Microbial *in vitro* model of root surface caries.

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Declaration

This thesis is the original work of the author.

List of abbreviations

Å	Ångström (10^{-10}m).
A.A.S.	atomic absorption spectroscopy.
A.B.B.	anaerobic blood broth.
arg.	arginine.
c	*circa*; about.
°C	degrees Celsius.
Ca	calcium.
Ca^{2+}	cationic calcium.
C.B.A.	Columbia Blood agar.
cf.	*confer*; compare.
c.f.u.	colony forming units.
Cl^-	anionic chloride.
cm	centimetre (10^{-2}m).
CO_2	carbon dioxide.
d	day.
D.F.S.	decayed or filled surfaces.
dl	decilitre (10^{-1}l).
D.M.F.	decayed missing or filled.
e.g.	*exempli gratia*; for example.
et al.	*et alia*; and others.
F.C.S.	foetal calf serum.
g	gram (10^0g).
HAp	hydroxyapatite powder.
HCl	hydrochloric acid.
H_2O	water.
hr	hour.
i.e.	*id est*; that is to say.
KCl	potassium chloride.
kg	kilogram (10^3g).
l	litre (10^0l).
L	Lower chamber of Ultrafree-CL ultrafiltration unit.
m	metre (10^0m).
M	molar (moles per litre).
m.f.p.	monofluorophosphate.
mg	milligram (10^{-3}g).
min	minute.
ml	millilitre (10^{-3}l).
mm	millimetre (10^{-3}m).
mM	millimolar (millimoles per litre).
mmol	millimole (10^{-3}mol).
mol	mole (10^0mol).
M.S.B.	Mitis Salivarius Bacitracin agar.
n	number of samples or replicates.
n.a.	not applicable.
n.c.p.	non-collagenous proteins.
NCTC	national collection of type cultures (strain designation).
n.d.	not done.
nm	nanometre (10^{-9}m).
n.s.	not supplied.

List of abbreviations (cont.)

P	phosphorus.
P.B.S.	phosphate buffered saline.
PO_4^{3-}	anionic phosphate.
pp.	pages.
p.p.m.	parts per million.
P.T.F.E	polytetrafluoroethylene.
P.V.D.F.	polyvinylidene difluoride.
R.C.I.	root caries index.
r.p.m.	revolutions per minute.
s	second.
S.D.	standard deviation of a normally distributed population.
spp.	species.
suc.	sucrose
t	time.
T.H.B.	Todd Hewitt broth.
U	Upper chamber of Ultrafree-CL ultrafiltration unit.
w/v	weight/volume.
µA	microamperes (10^{-6}A).
µg	microgram (10^{-6}g).
µl	microlitre (10^{-6}l).
µm	micrometre (10^{-6}m).
µmol	micromole (10^{-6}mol).
&	and.
[ ]	concentration.
>	greater than.
<	less than.
#	number.
±	plus or minus.

Summary

Root surface caries is an economically important disease in Western industrialised nations and appears likely to become more so in future due to demographic changes and improvements in oral hygiene. There are a variety of model systems which have been used to study root surface caries including; *in situ* studies, experimental laboratory animals, the use of pure acids *in vitro* and the use of bacterial cultures *in vitro*. However the published data fail to present a clear and definitive picture of the role that different bacterial species play or of the biochemical stages in the demineralisation of root surfaces by bacteria. Therefore it was decided to develop a novel *in vitro* model to study this problem.

The first system investigated was the Millicell-HA tissue culture insert (Millipore, U.K.), which consisted of a polystyrene cylinder sealed at one end with a 0.45μm pore-size membrane filter. These units are relatively cheap, easy to handle, provide a stable surface to support bacteria and also keep the bacteria separate from their broth supply to facilitate collection of samples for analysis. In chapter 3 a series of experiments is described in which three bacterial species - *Streptococcus mutans, Lactobacillus casei* and *Actinomyces viscosus* - were selected for study on the basis of previous scientific reports. Relatively reproducible films of the three test species were cultured in Millicell-HA inserts, with no significant differences between the retrievable viable counts of each species whether as pure or mixed cultures. Furthermore scanning electron micrographs confirmed that both *S. mutans* and *A. viscosus* could be cultured as healthy biofilms in the inserts, although technical difficulties precluded this conclusion for *L. casei*. Moreover there appeared to be a maximum attainable population density of approximately 10^8 c.f.u./cm^2, which was independent of the initial inoculum density.

However, when the incubation period was extended it was found that *S. mutans* passed from the Millicell-HA inserts into the nutrient broth phase by day 4 of incubation, although the population densities of both *L. casei* and *A. viscosus* remained stable for 14 days. It was therefore decided to discontinue work on the Millicell-HA model system.

The second system investigated was the Ultrafree-CL ultrafiltration unit (Millipore, U.K.), which is structurally similar to the Millicell-HA insert but available with smaller diameter pores. Chapter 4 characterised the properties of the model, which included the following; (i) reproducible films of the three test species could be grown as pure or mixed cultures with no significant differences between the final viable counts and (ii) *S. mutans* was retained by the filter membrane with a pore size of 0.22μm for around 7 - 8 days and for at least 21 days by a 0.1μm pore size. Since transverse sections of *S. mutans* films in Ultrafree-CL units with 0.22μm pores showed bacteria invading the membrane filter structure it is likely that this is the route of spread. Populations of *L. casei* remained stable within the Ultrafree-CL units for up to 21 days, whilst *A. viscosus* viable counts tended to decline between days 6 and 21 days. Furthermore there appeared to be a maximum population density of approximately 10^7 c.f.u./cm^2, which was independent of the viable counts in the initial inoculum. However, when the films were cultured with sucrose solution or distilled water instead of Todd Hewitt broth (T.H.B.) there were substantial decreases in viable counts, although these could be reversed by returning the films to T.H.B. This suggested that transfer from sucrose back to T.H.B. could allow one film to be used for a series of studies with few adverse effects on the overall viable counts.

In chapter 5, preliminary experiments are described which sought to determine whether the test bacterial species could cause demineralisation of root surfaces or hydroxyapatite powder within the Ultrafree-CL model system. The data indicated that they did indeed cause calcium release from mineral, with the rank order of *S. mutans* ≈ *L. casei* > *A. viscosus*, whilst lactate was usually the predominant anion. These results concur in general with the available literature. However, there was substantial variation in both the acid anion production and calcium release data between repeat experiments which indicated that modifications to the model system may be required before it can be successfully employed for demineralisation experiments. In chapter 6 a range of modifications to the Ultrafree-CL system are described which would help to reduce the variation to acceptable levels and allow the model to be employed to investigate root surface caries further.

Chapter 1 Studies in the bacteriology of root surface caries

1.1 Introduction - the importance of root surface caries

Root surface caries is a disease that often affects the elderly who have retained their natural dentition (e.g. Fejerskov *et al.*, 1991). In industrialised 'Western' countries population demographics are shifting towards an increasingly aged population whilst at the same time improvements in diet, oral hygiene and dental healthcare mean that ever greater numbers of elderly people are retaining their natural dentition for a longer time (Beighton, Decker & Homer, 1991). Progressive gingival recession occurs with age and can be accelerated by poor technique in oral hygiene, e.g. excessive pressure with too hard a tooth brush, (Konig, 1990) and once root surfaces are exposed they are then at risk of becoming carious (Syed *et al.*, 1974).

Thus, quite apart from the human suffering caused by root surface caries as a disease, it will also have a marked economic impact since an increasing number of people is likely to become affected. Therefore it is of value to study the disease; especially the microbiology to determine whether it is possible to identify 'at risk' individuals on the basis of plaque bacteriology so that they can be supplied with prophylactic treatment (Beighton, Decker & Homer, 1991). It is also of value to study the disease so that more effective treatment regimes may be designed for those who already suffer from it (Keltjens *et al.*, 1987).

1.2 A definition of root surface caries

Human dental root surface caries can be defined as a carious lesion which is initiated and wholly or predominantly located on the surface of a human tooth root (Beck, 1990). Root surface caries is akin to enamel caries in that the disease damages and eventually

destroys the root structure leading to cavitation. It is widely accepted that an exposed root surface is very important for the development of root surface caries (Katz, 1980).

1.3 Current knowledge concerning root surface caries

1.3.1 Introduction

As the awareness of the problem of root surface caries and its future significance is investigated so the volume of information available increases. The existing data is here presented under four major headings, i.e. epidemiology, bacteriology, biochemistry and histology of root surface caries. This approach will summarise how widespread the disease is, which micro-organisms are thought to be involved, what is happening to the root surface at a molecular level and what is happening to the root surface on a structural level.

1.3.2 Epidemiology

Root surface caries is a disease which has the potential to affect anyone with a natural dentition and a variety of studies have attempted to assess its prevalence and incidence. However the risk of developing root surface caries is not equally distributed throughout the population and so most studies have concentrated upon its spread within selected groups. Efforts have been made to determine the relationship of various factors with root surface caries within a target population, e.g. age, periodontal disease and water fluoridation. Tables 1.1 and 1.2 summarise the prevalence and incidence rates of root surface caries reported in a range of papers. Additionally these tables summarise any association found with the selected factors noted above.

Table 1.1 The prevalence of root surface caries

Author(s)	Year	n*	Age (years)	Prevalence	Related factor‡
Heinrich, Kunzel & Heinrich	1990	963	25 - 64	40.2% females, 42.7% males	None
Burt, Ismail & Eklund	1986	315	Mean 41.5	23.8% in optimal fluoridated area, 7.3% in high fluoride area	Water fluoridation
Stamm, Banting & Immrey	1990	967	>18	Mean 3 D.F.S. in 0.2p.p.m. fluoride area mean 2.49 D.F.S. in 1.6p.p.m. fluoride area	Water fluoridation
Wallace, Retief & Bradley	1988	603	>60	70%	Age
Locker, Slade & Leake	1989	183	>50	57% (37% had active lesions)	Age
Fure & Zickert	1990a	208	55 - 75	89%	Age
Hellyer *et al.*	1990	146	>55	88% (31.5% had active lesions)	Age
Fejerskov *et al.*	1991	90	60 - 80	79% (60% had active lesions)	Age
Banting, Ellen & Fillery	1980	59	Mean 68	75%	Age and chronic hospitalisation
Ravald & Birkhed	1991	147	30 - 78	80%	Periodontal disease and surgery

* = number of subjects within sample population.

‡ = factor which author(s) related to root surface caries prevalence

Table 1.2 The incidence of root surface caries

Author(s)	Year	n*	Age (years)	Incidence	Related factor‡
Banting, Ellen & Fillery	1985	45	Mean 68.6	1.9 new lesions per 100 person-months at risk	Prior root surface caries
Hand, Hunt & Beck	1988	338	> 65	Mean 1.1 lesions per subject, or 1.8 lesions per 100 exposed surfaces per year	Age
Gustavsen, Clive & Tveit	1988	2839	20 - 29 & > 70	20 - 29, mean 1.2 carious root surfaces > 70, mean 8.5 carious root surfaces	Age

* = number of subjects within sample population

‡ = factor which authors related to root surface caries incidence

Prevalence rates for root surface caries give an estimate of the spread of the disease throughout a given population at the time of sampling. As shown in Table 1.1 the reported prevalence rates are quite variable, ranging from approximately 7% (Burt, Ismail & Eklund, 1986) to almost 90% (Fure & Zickert, 1990a). However, the data presented in the papers listed in table 1.1 indicate that the prevalence of root surface caries is greater in elderly populations of subjects than in young ones. In addition root surface caries was more prevalent (80%) in subjects who had suffered periodontal disease (Ravald & Birkhed, 1991) than when they had not (approximately 40%) (Heinrich, Kunzel & Heinrich, 1990). In contrast, a protective environmental factor such as a fluoridated water supply would appear to reduce the prevalence rate of root surface caries - to 23.8% in an optimally fluoridated area and 7.3% in a super-optimally fluoridated area (Burt, Ismail and Eklund, 1986).

Prevalence rate data, as previously mentioned, give an estimate of how widespread root surface caries is in a population at a single point in time. However these data give no indication of the number of lesions per member of the population studied. In contrast, incidence data give an indication of the number of carious lesions present in each subject and also the number of new lesions developing over a period of time. Table 1.2 gives a summary of some studies which have reported upon the incidence of root surface caries.

Comparison between each of the papers mentioned is not straightforward, since each presents the data in a very different fashion. Banting, Ellen & Fillery (1985) examined 150 exposed caries free root surfaces for up to 34 months and found 1.9 new lesions per 100 person months at risk. In contrast Hand, Hunt & Beck (1988), who performed their study over a similar period of time as Banting, Ellen & Fillery (42 months), examined

338 subjects and presented their data both as a mean per subject and also as a mean per 100 susceptible surfaces - 1.1 lesions per subject or 1.8 lesions per 100 exposed root surfaces per year. Finally Gustavsen, Clive & Tveit found 1.2 carious root surfaces in the age group 20 - 29 compared with 8.5 in the over 70 age group

In 1980 Katz suggested the Root Caries Index (R.C.I.) as a means of standardising the presentation of data concerning root surface caries' spread within a population. The R.C.I. expresses the number of root caries lesions as a function of the number of exposed root surfaces per person and gives an estimate of the attack rate for root surface caries. This method of expressing the epidemiological data for root surface caries takes into account the variations in the number of exposed root surfaces between subjects; comparisons between subjects are then more meaningful, since fewer exposed root surfaces mean fewer potential sites for caries to develop.

Table 1.3 summarises a number of studies which presented their data in the form of a Root Caries Index. Three of the reports - Katz *et al.* (1982), Wallace, Retief & Bradley (1988) and Fure & Zickert (1990b) - correlated the age of the subjects with their experience of root surface caries. Both Katz *et al.* (1982) and Fure & Zickert (1990b) subdivided their subjects into decade age groups and the resulting data showed a steady increase in R.C.I. with increasing age; up to 22% in the oldest groups. However Wallace, Retief & Bradley (1988), who also examined an older group of subjects, found a much lower R.C.I. amongst them than the other two studies. In addition several reports that studied the intra-oral distribution of root surface caries found that, in general, mandibular molars were at the greatest risk of developing carious lesions upon their roots (Katz *et al.*, 1982, Gustavsen, Clive & Tveit, 1988 and Wallace, Retief & Bradley, 1988).

Table 1.3 Root caries indices

Authors	Year	n*	Age (years)	Root caries index	Related factor‡
Fure & Zickert	1990b	208	55, 65 & 75	14, 16 and 22% respectively	Age
Katz *et al.*	1982	473	20 - 64	1.1, 4.7, 13.0 and 22.0% for each decade respectively Mandibular molars = 25%, maxillary canines = 23%, whilst mandibular incisors = 2%	Age Root surface
Wallace, Retief & Bradley	1988	603	>60	8.1 $\pm$ 9.5% Mandibular molars = 28%, mandibular incisors = 2% Buccal = 14%, lingual = 3%	Age Root surface
Gustavsen, Clive & Tveit	1988	2839	>20	21% Mandibular molars = 32.9%, others = 15 - 25% interproximal and buccal = 20 - 25%, lingual = 10 - 17%	None Root surface
Keltjens *et al*	1988	83	22.4 - 71.5	6.3%	Periodontal disease

* = the number of subjects within the sample population

‡ = the factor which was associated with root surface caries by the authors of each paper

To summarise, root surface caries would appear to affect in excess of 40% of a general adult population with no especial risk factors (Heinrich, Kunzel & Heinrich, 1990). However, this prevalence rate can increase to greater than 60% when a more elderly population is studied (table 1.1) and to more than 75% if the subjects are chronically hospitalised (Banting, Ellen & Fillery, 1980) or if they have exposed roots due to periodontal disease/ surgery (Ravald & Birkhed, 1991). There is a steady attack rate of exposed root surfaces (table 1.2) and this attack rate increases as the subjects of the study increase in age (Katz *et al.*, 1982 and Fure & Zickert, 1990b).

1.3.3 Bacteriology

A complex microflora is known to inhabit the human oral cavity and bacteria are found in saliva and also adherent to soft and hard tissues as plaque. A wide range of bacterial species has been found in plaque from both healthy and carious human tooth root surfaces. Table 1.4 summarises the prevalence and proportions of the different bacterial species recorded by 15 different 'cross-sectional' studies. In contrast table 1.5 summarises the different bacterial species, their relative proportions and their prevalence rates in 4 'longitudinal' studies.

'Cross-sectional' and 'longitudinal' studies require different approaches and give different information concerning the bacterial populations present in root surface plaque and within the carious lesion itself. The 'cross-sectional' type of study takes a population at a single point in time and samples root surface plaque from carious (and non-carious) roots in order to quantify and characterise the bacterial species present at that point in time. In addition carious dentine from the root structure may also be sampled. Some 'cross-sectional' studies have chosen target organisms only (e.g. Sumney & Jordan,

Table 1.4 The bacterial species isolated from root surface caries in cross-sectional studies

Author(s)	Year	n*	Sample source	Organisms isolated	% proportion of total count	% prevalence in samples
Jordan & Hammond	1972	20	Carious dentine	*R. dentocariosa*	25	n.d.[†]
				A. viscosus	20	n.d.
				A. naeslundii	10	n.d.
				A. odontolyticus	5	n.d.
				A. eriksonii	5	n.d.
				Atypical *A. viscosus*	10	n.d.
				Uncharacterised	25	n.d.
Sumney & Jordan	1974	24	Carious dentine	*S. mutans*	18	n.d.
				S. sanguis	4	n.d.
				S. mitis	12	n.d.
				Enterococcus spp.[‡]	16	n.d.
				Micrococcus	23	n.d.
				Staphylococcus		
				Neisseria sicca	3	n.d.
				Actinomyces spp.	12	n.d.
				Arthrobacter spp.	10	n.d.
Syed *et al.*	1974	15	Root surface plaque	*S. mutans*	15	62
				S. sanguis	24.5	55
				(other) *Streptococcus* spp.	13.5	67
				A. viscosus	44	100
				Veillonella spp.	3	33

Table 1.4 (cont) **The bacterial species isolated from root surface caries in cross-sectional studies**

Author(s)	Year	n	Sample source	Organisms isolated	% proportion of total count	% prevalence in samples
Bryan, Reynolds & Zambon	1985	9	Root surface plaque and carious dentine	*A. viscosus*	13	78
				A. naeslundii	2	38
Brown, Billings & Kaster	1986	47	Non-carious root surface plaque	*S. mutans*	18	96
				S. sanguis	n.s.[+]	51
				S. salivarius	n.s.	21
				(total) *Streptococcus* spp.	33	85
				A. viscosus	15	72
				(total) *Actinomyces* spp.	24	98
				Lactobacillus spp.	3	51
				Candida spp.	n.s.	12
		50	Carious root surface plaque	*S. mutans*	24	100
				S. sanguis	n.s.	38
				S. salivarius	n.s.	24
				(total) *Streptococcus* spp.	38	76
				A. viscosus	8	71
				(total) *Actinomyces* spp.	12	94
				Lactobacillus spp.	2	54
				Candida spp.	n.s.	12
Fure *et al.*	1987	24	Non-carious root	*S. mutans*	3.1	83
				Lactobacillus spp.	0.03	25
				A. viscosus and *A. naeslundii*	21.7	91.5

Table 1.4 (cont) **The bacterial species isolated from root surface caries in cross-sectional studies**

Author(s)	Year	n	Sample source	Organisms isolated	% proportion of total count	% prevalence in samples
Fure *et al.* (cont.)		24	Carious root surface plaque	*S. mutans*	10.1	91.5
				Lactobacillus spp.	3.5	41.5
				A. viscosus and *A. naeslundii*	11.8	100
Keltjens *et al.*	1987	47	Non-carious root surface plaque	*S. sanguis*	10	96
				S. mutans	0.1	84
				A. viscosus/ naeslundii	1	90
				Lactobacillus spp.	n.s.	11
				Capnocytophaga spp.	n.s.	69
			Carious root surface plaque	*S. sanguis*	10	92
				S. mutans	1	92
				A. viscosus/ naeslundii	10	89
				Lactobacillus spp.	n.s.	18
				Capnocytophaga spp.	n.s.	52
Emilson, Klock & Sanford	1988	11	Non-carious root surface plaque	Mutans streptococci	0.01	n.d.
				S. sanguis	12.1	n.d.
				Actinomyces spp.	7.7	n.d.
		24	Carious root surface plaque	Mutans streptococci	1	n.d.
				S. sanguis	5.1	n.d.
				Actinomyces spp.	12.5	n.d.

Table 1.4 (cont) **The bacterial species isolated from root surface caries in cross-sectional studies**

Author(s)	Year	n	Sample source	Organisms isolated	% proportion of total count	% prevalence in lesions
Bowden *et al.*	1990	165	Non-carious root surface plaque	*S. mutans*	0.75	20.5
				S. oralis	0.45	45.5
				S. mitis 1	0.78	58
				S. sanguis	0.75	32.5
				Lactobacillus spp.	<0.05	<1
				A. viscosus (serovar 2)	2.20	75
				A. naeslundii (serovar 1)	3.55	45
			Carious root surface plaque	*S. mutans*	3.05	44
				S. oralis	0.45	29
				S. mitis 1	0.89	53.5
				S. sanguis	0.35	23.5
				Lactobacillus spp.	0.18	34
				A. viscosus (serovar 2)	3.90	67.5
				A. naeslundii (serovar 1)	1.65	33
Nyvad & Kilian	1990	6	Root surface plaque and dentine	*S. gordonii*	1.3	50
				S. mitis 1	8	100
				S. mitis 2	0.5	50
				S. anginosus	0.1	17
				S. mutans (serotype c)	5.7	100
				S. mutans (serotype f)	11	17
				S. sobrinus (serotype d)	4.7	33
				S. sobrinus (serotype g)	0.1	17
				Mutans streptococci	0.3	33

Table 1.4 (cont) **The bacterial species isolated from root surface caries in cross-sectional studies**

Author(s)	Year	n	Sample source	Organisms isolated	% proportion of total count	% prevalence in lesions
Nyvad & Kilian (cont.)				*Streptococcus* spp.	2.7	33
				A. viscosus	10	83
				A. naeslundii	8.4	83
				B. dentium	4	33
				Propionibacterium spp.	1.2	17
				Unidentified gram positive rods	0.2	17
				L. acidophilus	0.7	33
				L. salivarius	0.3	17
				L. casei subspecies *casei*	1.9	17
				L. casei rhamnosus	0.9	33
				Lactobacillus spp.	0.2	17
				V. parvula	8.7	83
				Gram negative rods	4	67
				Unclassified	3.4	83
van Houte *et al.*	1990	40	Non-carious root surface plaque	(total) *Streptococcus* spp.	54.3	100
				Mutans streptococci	1.1	47.4
				S. salivarius	1.3	22.9
				S. sanguis	17.7	100
				(other) *Streptococcus* spp.	31.9	99.7
				Enterococcus spp.	0.08	27.8
				(total) *Actinomyces* spp.	16.8	97.2
				A. viscosus	11.9	86.1
				A. naeslundii	6.4	61.1

Table 1.4 (cont) **The bacterial species isolated from root surface caries in cross-sectional studies**

Author(s)	Year	n	Sample source	Organisms isolated	% proportion of total count	% prevalence in lesions
van Houte *et al.* (cont.)				*Lactobacillus* spp.	0.18	10.5
			Carious root surface plaque	(total) *Streptococcus* spp.	47.6	97.5
				Mutans streptococci	1.8	63.2
				S. salivarius	0.59	20.0
				S. sanguis	16.5	97.4
				(other) *Streptococcus* spp.	27.6	97.4
				Enterococcus spp.	0.4	33.3
				(total) *Actinomyces* spp.	18.1	100
				A. viscosus	12.2	91.7
				A. naeslundii	3.9	52.8
				Lactobacillus spp.	3.5	21.1
Beighton, Lynch & Heath	1993	59	Soft carious dentine	Mutans streptococci	7.2	94.6
				Lactobacillus spp.	1.6	67.3
				Gram positive pleomorphic rods	36.0	98.2
			'Leathery' carious dentine	Mutans streptococci	5.2	54.0
				Lactobacillus spp.	1.4	21.4
				Gram positive pleomorphic rods	26.8	83.3
			Hard carious dentine	Mutans streptococci	0.1	5.8
				Lactobacillus spp.	n.d.	0
				Gram positive pleomorphic rods	37.6	52.5

Table 1.4 (cont) **The bacterial species isolated from root surface caries in cross-sectional studies**

Author(s)	Year	n	Sample source	Organisms isolated	% proportion of total count	% prevalence in lesions
Sansone *et al.*	1993	10	Non-carious dentine	Mutans streptococci	0.5	n.d.
				Total streptococci	28.3	n.d.
				Lactobacillus spp.	0 - 0.04	n.d.
			Carious dentine	Mutans streptococci	20.4	n.d.
				Total streptococci	42.8	n.d.
				Lactobacillus spp.	0.01 - 10.3	n.d.
Ozaki *et al.*	1994	50	Carious dentine	*S. mutans*	2.8	47
				S. sobrinus	0.6	13
				S. sanguis	2.8	39
				A. viscosus	10.2	81
				L. casei	0.5	14
				L. plantarum	1.3	24
				P. micros	0.8	17
				E. alactolyticum	0.9	27
				P. acnes	1.1	27
				S. aureus	0.1	9
				F. nucleatum	<0.1	4
Van Houte, Lopman & Kent	1994	25	Non-carious root surface plaque	*Gemella* spp.	3.6	50
				Mutans streptococci	1.4	25
				P. micros	0.3	12.5
				S. intermedius	0.6	12.5
				S. milleri II	3.1	37.5

Table 1.4 (cont) **The bacterial species isolated from root surface caries in cross-sectional studies**

Author(s)	Year	n	Sample source	Organisms isolated	% proportion of total count	% prevalence in lesions
Van Houte, Lopman & Kent (cont.)				*S. mitis*	1.1	37.5
				S. salivarius	0.3	12.5
				S. sanguis I	12.0	62.5
				S. sanguis II	24.3	87.5
				Veillonella spp.	17.6	87.5
				A. israelii	2.2	50
				A. naeslundii	6.4	37.5
				A. odontolyticus	3.4	62.5
				A. viscosus	10.3	75
				(other) *Actinomyces* spp.	0.8	12.5
				Clostridium spp.	6.4	50
				Eubacterium spp.	0.3	12.5
				Propionibacterium spp.	0.3	12.5
				Bacteroides spp.	5.0	25
				Capnocytophaga spp.	1.4	25
				Fusobacterium spp.	1.7	37.5
				A. viridans	0.5	11
			Carious root surface plaque	*Gemella* spp.	3.3	11
				Mutans streptococci	9.0	67
				S. constellatus	0.3	11
				S. intermedius	0.8	11
				S. milleri II	1.3	11
				S. mitis	1.0	11
				S. salivarius	0.3	11
				S. sanguis I	1.5	33

Table 1.4 (cont) **The bacterial species isolated from root surface caries in cross-sectional studies**

Author(s)	Year	n	Sample source	Organisms isolated	% proportion of total count	% prevalence in lesions
Van Houte, Lopman & Kent (cont.)				*S. sanguis* II	0.5	11
				S. uberis	0.3	11
				Veillonella spp.	11.8	100
				A. israelii	4.4	33
				A. naeslundii	20.8	78
				A. odontolyticus	1.8	67
				A. viscosus	4.4	44
				(other) *Actinomyces* spp.	4.6	56
				Bifidobacterium spp.	4.9	33
				Clostridium spp.	13.1	67
				Eubacterium spp.	1.0	44
				Lactobacillus spp.	1.8	22
				Propionibacterium spp.	5.1	44
				Actinobacillus spp.	0.3	11
				Bacteroides spp.	3.1	56
				Capnocytophaga spp.	0.3	11
				Fusobacterium spp.	1.5	22
				Leptotrichia spp.	0.3	11
				Wolinella spp.	0.5	22

* = number of samples † = not determined ‡ = species + = not supplied

1974), whilst others have sampled for chosen target organisms and expressed these as a ratio of the total viable count (e.g. Ozaki *et al.*, 1994). Other workers have identified the predominant micro-organisms present and expressed these as a percentage of the total viable count (e.g. Van Houte, Lopman & Kent, 1994). The 'longitudinal' type of study involves determining the bacterial flora in a chosen population of subjects at intervals over a period of time. As in the 'cross-sectional' study selected target organisms may be sought (e.g. Emilson, Ravald & Birkhed, 1993) or alternatively the predominant organisms of the total flora may be enumerated (e.g. Nyvad & Kilian, 1987). The difference in the numbers of studies of each type in the literature is probably indicative of the relatively low time and costs involved in cross-sectional compared with longitudinal studies.

The information provided by 'cross-sectional' and 'longitudinal' studies also differs. The 'cross-sectional' type of study provides an estimate of the relative proportions and prevalence of the bacterial species at a given point in time; it does not give any indication as to the population dynamics of the developing lesion. In contrast 'longitudinal' studies do provide more information on the dynamics of the developing bacterial population; an estimate of the predominant organisms is given as well as data about the relative increase or decrease in the proportions and prevalence of the various species as the lesion develops. This means that the data from longitudinal studies should show more clearly which organisms are of importance in root surface caries, since it can be seen which organisms increase in proportions as lesions develop. However, 'longitudinal' studies are more labour intensive and more expensive than 'cross-sectional' since repeated sampling sessions followed by isolation and identification of the bacteria are required.

Although many different species are known to comprise the bacterial population of root surface plaque, there are three bacterial groups which have been most regularly implicated with the development of root surface caries. In table 1.4 (and also table 1.5) it can be seen that these three groups are actinomyces, streptococci and lactobacilli. Within these three groups the most important species are *Actinomyces viscosus* (and to a lesser extent *A. naeslundii*), the mutans streptococci (especially *S. mutans*) and *Lactobacillus* species.

Upon studying the data summarised in tables 1.4 and 1.5 it becomes obvious that the relative importance of these three groups of organisms in root surface caries is unclear. *Actinomyces viscosus* is found in more than two thirds of carious plaque and carious dentine samples and constitutes from 3.9% (Bowden *et al.*, 1990) to 44% (Syed *et al.*, 1974) of the viable microflora. A number of the studies have compared the prevalence and proportions of this species in plaque from carious and non-carious root surfaces and found that both were greater in carious material (Keltjens *et al.*, 1987, Emilson, Klock & Sanford, 1988, Bowden *et al.*, 1990 and Van Houte *et al.*, 1990). However, in contrast, several other studies have reported that both the prevalence and proportions of *A. viscosus* were lower in carious compared with non-carious plaque (Brown, Billings & Kaster, 1986, Fure *et al.*, 1987 and Van Houte, Lopman & Kent, 1994). In addition Beighton, Lynch & Heath (1993) found a higher proportion of gram positive pleomorphic rods (which include the actinomyces) in hard (arrested) carious dentine than in soft or 'leathery' (active) carious dentine, although the prevalence of these organisms was less in hard than soft or 'leathery' dentine.

Mutans streptococci form a smaller proportion of the bacterial flora of carious root plaque and dentine; from about 1% (Keltjens *et al.*, 1987, Emilson, Klock & Sanford, 1988, Van Houte *et al.*, 1990, Beighton, Lynch & Heath, 1993 and Van Houte, Lopman & Kent, 1994) to around 24% (Brown, Billings & Kaster, 1986). The prevalence rates for the mutans streptococci range from a minimum of 44% (Bowden *et al.*, 1990) to a maximum of 100% (Brown, Billings & Kaster, 1986, Nyvad & Killian, 1990). The data from those studies which sampled plaque from non-carious as well as carious root surfaces indicate that mutans streptococci become more widespread and form a larger proportion of the bacterial flora as the root surface becomes carious. For instance van Houte, Lopman & Kent (1994) found that mutans streptococci comprised a higher percentage of the total count in carious (9.0%) than in non-carious (1.4%) sites and that prevalence rates were also higher (67% in carious and 25% in non-carious plaque). In addition Bowden *et al.* (1990) found the same increase in proportions (up from 0.75 to 3.05%) and prevalence (from 20.5 to 44%) of this organism when non-carious and carious root surface plaques were compared.

Most of the studies quoted in table 1.4 did not identify the lactobacilli to species level. These studies found that the proportions of lactobacilli in carious samples (plaque or dentine) ranged from 0.18% (Bowden *et al.*, 1990) to 3.5% (Fure *et al.*, 1987) whilst the prevalence rates ranged from 18% (Keltjens *et al.*, 1987) to 67.3% (Beighton, Lynch & Heath, 1993). A few studies did speciate the lactobacilli (Nyvad & Kilian, 1990 and Ozaki *et al.*, 1994). The former found that *L. casei* was the predominant *Lactobacillus* species present (1.9% of the total count compared with 0.7% for *L. acidophilus*) whilst the latter found that *L. plantarum* was the commonest species although *L. casei* was also present. Nyvad & Kilian (1990) found that *L. casei* was present in around 33% of

samples whilst Ozaki *et al.* (1994) found that it was present in 14% of samples. Neither of the two groups compared carious with non-carious samples and therefore it is not possible to state whether the prevalence and proportions of *L. casei* were greater in carious compared with non-carious material.

However, the studies which examined both carious and non-carious plaque or dentine and also presented data on lactobacilli as a group did not entirely concur on whether the prevalence and proportions were higher or lower in carious compared with non-carious. Bowden *et al.* (1990) found that both the prevalence and proportions of lactobacilli were greater in carious than non-carious plaques; proportions rose from < 0.05% to 0.18%, whilst prevalence rose from < 1% to 34%. Furthermore van Houte *et al.* (1990) and van Houte, Lopman & Kent (1994) recorded similar observations; the proportions rose from 0.18% to 3.5% (van Houte *et al.*, 1990) and from undetected to 1.8% (van Houte, Lopman & Kent, 1994), whilst the prevalence rose from 10.5% to 21.1% (van Houte *et al.*, 1990) and from undetected to 22% (van Houte, Lopman & Kent, 1994). In addition Keltjens *et al.* (1987) found a higher prevalence of *Lactobacillus* species in carious than non-carious root surface plaque (11% to 18%), although the numbers were too low to present as a proportion of the total count. In contrast Brown, Billings & Kaster (1986) found that the proportion of *Lactobacillus* species of the total count was slightly reduced in carious root surface plaque, although this study also found that the prevalence rate increased from 51 to 54% in carious compared with non-carious root surface plaque. Despite the relatively low proportions of the total count being comprised of *Lactobacillus* species, Ellen, Banting & Fillery (1985b) found that *Lactobacillus* species were closely correlated with *S. mutans* although in contrast Keltjens *et al.* (1987) found that only *S. mutans* was closely related to root surface caries.

Although species of *Actinomyces*, mutans streptococci and *Lactobacilli* have been explicitly correlated with root surface caries by many researchers (e.g. Beighton, Lynch & Heath, 1993, Ellen, Banting & Fillery, 1985a, Emilson, Klock & Sanford, 1988, Keltjens *et al.*, 1987 and Ozaki *et al.*, 1994), some have suggested that other organisms may have a role to play in less severe lesion development. For instance Nyvad & Kilian (1990) found that the microflora from plaque associated with root surfaces that were undergoing a large and rapid mineral loss was dominated by either *A. viscosus* or a combination of mutans streptococci and *Lactobacillus* species. However other plaque samples from root surfaces which demonstrated a lesser degree of demineralisation were found to have a far more complex microflora including mutans streptococci, *S. mitis* type 1, *Veillonella* species and *Lactobacillus* species. Syed *et al.* (1974) found that the plaque from root surface lesions could be divided into two groups (I and II) on the basis of the streptococcal species harboured. In group I plaques *S. mutans* formed 30% of the total cultivable flora whilst *S. sanguis* was present in only very low numbers. On the other hand, in group II plaques *S. mutans* was not detected whilst *S. sanguis* formed 48% of the total cultivable flora. In addition both Sansone *et al.* (1993) and Van Houte, Lopman & Kent (1994) suggested that organisms other than mutans streptococci and lactobacilli (e.g. non-mutans streptococci) may well play a part in the aetiology of root surface caries. The data discussed so far demonstrates the problem with attempting to determine the relative importance of the various bacterial species found in plaque, since the predominant *Actinomyces* species are found to decline in numbers as a root surface site becomes carious, whilst those organisms which do increase in number (i.e. the mutans streptococci and lactobacilli) tend to form only a small proportion of the total viable count.

Despite the efforts of researchers, the relative importance of the various bacterial species associated with root surface caries is still unclear. Are actinomyces the most important organisms since they generally predominate, but if so why do their proportions sometimes decrease in carious plaques? Alternatively, are the mutans streptococci and lactobacilli central to the development of root surface caries lesions since their numbers are greater in carious plaques? Additionally if mutans streptococci and lactobacilli are essential to development of carious lesions, why are there lesions from which they cannot be isolated? To some extent the answers to these questions may lie in laboratory experiments where conditions can be much more precisely controlled.

So far the variation in the bacterial populations of plaque between carious and non-carious surfaces has been discussed; however there are also changes in the bacterial population within a carious lesion with time. Table 1.5 summarises the data from a number of longitudinal studies which demonstrate clearly that changes occur in the proportions of different bacterial species which inhabit both the plaque overlying a carious root surface lesion and also the carious dentine itself, over a period of between 24hr and 34 months.

Nyvad & Kilian (1990) studied the microflora which developed on root surfaces which had been embedded in dental appliances and worn by volunteers for 24hr. The microflora was dominated by streptococci and Gram positive pleomorphic rods; *Streptococcus sanguis* contributed 6 - 18% of the early colonisers, whilst *S. mitis* and *S. oralis* contributed 24 - 42% and 1 - 27% respectively. The relative proportions of *S. oralis* increased significantly during the 24hr observation period. However *S. salivarius* and *S. mitis*, which were both also early colonisers of the exposed root surfaces, decreased in

Table 1.5 The bacterial species isolated from root surface caries in longitudinal studies

Author(s)	Year	n*	Sample source	Organisms isolated	% isolation frequency at (over a period of 32 months):- 1st	2nd	3rd	4th	5th	6th	7th sampling
Ellen, Banting & Fillery	1985a	45	Non-carious root surface plaque	*S. mutans*	58	62	50	54	48	64	58
				Lactobacillus spp.	46	50	52	40	48	48	40
			Carious root surface plaque	*S. mutans*	84	94	78	90	88	90	86
				Lactobacillus spp.	58	60	64	42	82	82	84

Author(s)	Year	n	Sample source	Organisms isolated	% proportion of total count at:- 0	12	20	24	28	32	34 months
Ellen, Banting & Fillery	1985b	44	Non-carious root surface plaque	*S. mutans*	0.07	0.27	0.02	0.69	0.30	0.01	0.01
				Lactobacillus spp.	0.29	0.96	0.86	0.81	0.24	2.53	5.69
				A. viscosus	7.94	7.21	7.48	5.55	3.83	7.68	6.61
				A. naeslundii	1.57	1.56	2.77	5.08	1.71	5.10	3.29
			Carious root surface plaque	*S. mutans*	0.006	0.13	0.003	0.61	0.13	0.003	0
				Lactobacillus spp.	0.42	0.47	0.61	0.22	0.25	1.46	11.24
				A. viscosus	9.19	11.42	3.14	10.99	4.25	2.97	12.01
				A. naeslundii	0.04	0	0.03	2.29	3.24	6.91	3.85

Author(s)	Year	n	Sample source	Organisms isolated	% proportion of total count at:- 4	8	12	24 hours
Nyvad & Kilian	1987	4	Root surface plaque	*S. sanguis*	15	12	13	18
				S. oralis	5	24	19	27

Table 1.5(cont.) **The bacterial species isolated from root surface caries in longitudinal studies**

Author(s)	Year	n	Sample source	Organisms isolated	% proportion of total count at:- 4	8	12	24 hours
Nyvad & Kilian (cont.)				*S. mitis* (arg^-)‡	16	17	31	23
				S. mitis (arg^+)†	17	7	9	5
				S. salivarius	19	12	7	2
				(other) Streptococci	5	7	9	4
				Gram positive rods	11	3	2	9
				Other cocci	11	14	9	8

Author(s)	Year	n	Sample source	Organisms	% proportion of total count at:- 0	12 months
Emilson, Ravald & Birkhed	1993	15	Non-carious root surface plaque	*S. mutans*	0.1	0.3
				S. oralis (group)	0.9	1.8
				A. naeslundii (group)	7.1	7.0
			Carious root surface plaque	*S. mutans*	0.7	7.3
				S. oralis (group)	0.2	0.7
				A. naeslundii (group)	2.3	1.4

* = number of samples

‡ = arginine negative

† = arginine positive

proportions as the experiment continued. *Actinomyces* species were also observed to colonise the root surfaces within the first 4hr, but their relative proportions did not increase until later in the experiment (8 - 12hr of exposure).

Ellen, Banting & Fillery (1985a and 1985b) determined the microflora present in carious and non-carious root surface plaque over a period of almost three years. In that time the percentage proportions of *Lactobacillus* species and *A. naeslundii* increased in both carious and non-carious plaque, whilst those of *S. mutans* and *A. viscosus* showed little change in either type of plaque. However, an important point to note is that none of the bacterial species isolated showed a steady increase in numbers over the course of the study, but instead increased and decreased with time in a relatively haphazard fashion that was not apparently related to disease onset. When the prevalence rates for *S. mutans* and *Lactobacillus* species were examined it was found that again there was a large amount of variation between the data for each time-point. *Streptococcus mutans* did not show any significant increase in prevalence over the course of the study in either carious or non-carious plaque. However the prevalence of the *Lactobacillus* species did increase in carious plaques (from 58% to 84%) but not in non-carious plaques.

Emilson, Ravald & Birkhed (1993) followed the developing bacterial populations in subjects with root surface caries who were given professional tooth cleaning and treatment with fluoride varnish over a period of year. The proportions of *S. mutans* were higher (but not significantly) in carious than compared with non-carious plaques over the twelve month period and in addition there were no significant differences in the proportions of *S. oralis* group or *A. naeslundii* group in the plaques from the different types of surfaces sampled (sound, inactive caries and active caries). The viable counts for

the three groups of organisms were found to vary widely, which complicates the assessment of the data by statistical tests.

In summary, the majority of the data presented suggest that actinomyces (especially *A. viscosus*), mutans streptococci (especially *S. mutans* and *sobrinus*) and lactobacilli are correlated with the development of root surface caries lesions. However, the proportions and prevalence of *Actinomyces* species show little if any change (at most a slight decline) as the disease develops, whilst those of the mutans streptococci and the lactobacilli tend to increase. In addition there are combinations of bacterial species in root surface plaque which may still be cariogenic but contain far fewer mutans streptococci or lactobacilli along with a wide range of other organisms including *S. mitis* biovar 1, and *Veillonella parvula* (Nyvad & Kilian, 1990).

1.3.3 Biochemistry

The data available on the biochemical development of root surface caries in human tooth roots are relatively limited compared with the epidemiology and bacteriology of the disease. Some studies have employed bovine dental root tissue as their model system (e.g. Boonstra, ten Bosch & Arends, 1990), whilst others have tested powdered hydroxyapatite (as an enamel analogue)(e.g. Chestnutt, MacFarlane & Stephen, 1994) or human tooth root material (e.g. Hoppenbrouwers, Driessens & Borggreven, 1987). From the available information it would appear that human tooth root surface caries does not usually develop in a smooth progressive manner but in alternating episodes of demineralisation and remineralisation (Furseth & Johansen, 1968). This suggests that the many factors which interact to produce root surface caries exist in a dynamic equilibrium with the potential to cause demineralisation which if unchecked leads to lesion formation,

or arrestment of the carious lesion with remineralisation. The factors which could combine to determine whether a root surface caries lesion will develop or not include the presence and composition of dental plaque, the susceptibility of the root mineral structure to demineralisation, the ability of the micro-organisms inhabiting the plaque to degrade the root's collagenous matrix, composition of saliva, the dietary habits of the individual and the level of fluoride intake.

Bacterial acidogenicity A number of studies have attempted to determine the acidogenicity of plaque micro-organisms when challenged with dietary carbohydrates. Wijeyeweera & Kleinberg (1989) determined the ability of a range of micro-organisms to produce acid, both from endogenous metabolites and also exogenous glucose. Of the bacterial species tested *S. mutans*, *S. milleri*, *L. casei*, *A. viscosus*, *A. odontolyticus*, *A. naeslundii* and *Neisseria subflava* generated the greatest decrease in pH from neutrality from endogenous metabolites. When supplied with exogenous glucose (4.2mM) *S. mutans*, *S. sanguis*, *L. fermentum*, *L. casei* and *A. odontolyticus* produced the greatest decrease in pH. However, the ability of *S. mutans* to generate a pH drop from either endogenous metabolites or an exogenous supply of glucose differed between the serotypes tested. *Streptococcus mutans* E49 (serotype 'a') caused the greatest reduction in pH whilst utilising endogenous metabolites, whilst strain OMZ 175 (serotype 'f') gave the least change. In contrast, strain OMZ 175 caused the greatest reduction in pH from 4.2mM exogenous glucose and was closely followed by strain E49, whilst strain GS-5 (serotype 'c') gave the least reduction with exogenous glucose.

Chestnutt, MacFarlane & Stephen, (1994) found that when a range of *Streptococcus* species were incubated with hydroxyapatite powder and 146mM sucrose, all generated a

rapid drop in the pH of the medium. Mutans streptococci (*S. mutans* and *S. sobrinus*) were responsible for the greatest pH drop under the experimental conditions; from approximately pH 6.75 at time zero to little more than pH 4 after 5hr of incubation. Other species of streptococci (*S. gordonii*, *S. sanguis*, *S. vestibularis* and *E. faecalis*) were also capable of acidogenesis but not to the same extent as the mutans streptococci. De Soet, Toors & de Graaff (1989) also explored the acidogenesis of oral streptococci when cultured with glucose in a pH-stat at pH 7.0, 6.0, 5.5 and 5.0. *Streptococcus sobrinus* was the most acidogenic species whilst *S. sanguis* was the least active at each of the pH values tested; indeed *S. sanguis* was almost incapable of acidogenesis below pH 5.5. Sansone *et al.* (1993) found that the proportions of both mutans and non-mutans streptococci were higher in plaque from carious than non-carious root surfaces. Furthermore, non-mutans streptococci capable of acidogenesis at low pH generally outnumbered mutans streptococci in all of the plaques sampled. When streptococcal cells are adherent to a surface rather than being in suspension, the amount of acid produced from glucose can be greater; e.g. some strains of *S. mutans* produced 14% more lactate when adsorbed to hydroxyapatite crystals than when unadsorbed (Berry & Henry, 1977).

However, simply to examine the ability of oral micro-organisms to reduce the pH of their immediate environment does not give a complete picture of the 'cariogenicity' of dental plaque. It has been noted that some oral bacteria are capable of generating a pH rise when supplied with amino acids or urea. Levy & Eisenberg (1992) found that the addition of arginine along with glucose to the culture medium of *S. rattus* and *S. milleri* led to a higher terminal pH than when the organisms were supplied with glucose alone. In addition Sissons & Cutress (1988) found that bacteria sedimented from saliva metabolised urea to give a pH rise, whereas when supplied with glucose they produced a

drop in pH. When a mixture of urea and glucose was supplied to bacteria, an initial pH drop followed by a pH rise resulted, which reached a final value intermediate between those obtained with either pure glucose or urea.

Thus the pH of root surface plaque is likely to be in a state of continual flux depending on a variety of factors. The bacterial composition of the plaque is important in its ability to modify the pH; if it contains a high proportion of acidogenic bacteria then the pH is more likely to be reduced further than if particularly proteolytic or ureolytic bacteria are present in high proportions. The carbohydrate and amino-acid content of an individual's diet could then interact with the bacteria present in the plaque; a high carbohydrate diet combined with a preponderance of acidogenic bacteria might lead to a more pronounced pH drop, whilst a high protein diet in conjunction with a greater proportion of proteolytic bacteria in the plaque might be less likely to lead to a pH drop and may even lead to a pH rise within the plaque fluid (e.g. Wijeyweera & Kleinberg, 1989).

Tooth root biochemistry Chemically the human tooth root is constructed of inorganic and organic components, with the former comprising about 80% of the total by weight (Katz, Park and Palenik, 1987). Selvig & Selvig (1962) analysed human cementum and dentine for their mineral content and found that calcium and magnesium ions together comprised between 26% and 27% of the dry weight of both tissues. Phosphorous accounted for approximately 12% of cementum dry weight and about 13% of dentine dry weight, giving a calcium (and magnesium) to phosphate ratio of around 2.1:1. Hoppenbrouwers, Driessens & Borggreven (1986 and 1987), using human tooth roots that had not been exposed in the mouth found that the critical pH for mineral dissolution was about pH 6.7, which is close to neutrality. It appears that the further the pH is

reduced from 7.0, the greater the rate of mineral dissolution. Boonstra, ten Bosch & Arends (1990), employing bovine tooth roots, found that demineralisation rates were on average 1.5 times faster at pH 4.5 than at pH 5.0.

Little is known about the precise bacterial products released onto human tooth roots from overlying plaque or the amounts and types of mineral and organic components released from root surfaces during demineralisation *in vivo*. The studies which enumerated the inorganic composition of human tooth roots and the investigations which determined demineralisation chemically, did so using organic acids which did not mimic the acids produced by plaque bacteria either qualitatively or quantitatively (e.g. Herkströter, Witjes & Arends, 1991, Hoppenbrouwers, Driessens & Borggreven, 1986 and 1987, Nakata, Stepkin & Zipnik, 1972, Phankosol *et al.*, 1985 and Selvig & Selvig, 1962). Studies that have employed bacterial cultures to generate artificial root surface caries lesions have all determined the extent of demineralisation of root sections by microradiography rather than by biochemical means (e.g. Clarkson *et al.*, 1987 and Firestone *et al.*, 1993). Chestnutt, MacFarlane & Stephen (1994) examined the rate of demineralisation caused by selected oral bacteria using biochemical means, but employed hydroxyapatite powder as the mineral source.

The organic component of human tooth roots comprises approximately 20% of the total by weight (Katz, Park & Palenik, 1987) and is largely collagen which is an insoluble fibrous protein (Gage, Francis & Triffitt, 1989). Collagen forms long fibrils which provide a cross-linked matrix around which the mineral component is deposited (Klont, 1990) thus producing a structure with the combined physical characteristics of both and is analogous to steel reinforced concrete. The collagen proteins found so far in human

tooth roots are largely type I with a small amount of type III (Klont, 1990). There is also a range of non-collagenous proteins (n.c.p.) present, which include proteoglycans and glycoproteins in cementum, whilst in dentine there is a more complex mixture of citrate, lipids, proteoglycans, phosphoproteins, acidic glycoproteins and serum proteins (Klont, 1990). The non-collagenous protein fraction of the organic component of dentine would not appear to be extensively acid-soluble. Boonstra, ten Bosch & Arends (1990) found that although between 6 and 11mg of protein were released per gram of solubilised hydroxyapatite from bovine dentine when exposed to demineralising solutions, protein release and pH were not directly related. In addition "a large fraction of the n.c.p." were not released from dentine during demineralisation.

Native collagen is a very resilient molecule with a high resistance to acidic degradation. Boonstra, ten Bosch & Arends (1990) found that when bovine dentine was exposed to demineralising solutions at pH 5.0 or 4.5 for one week which was sufficient to dissolve the mineral structure, no collagen could be detected in the demineralisation buffer. In contrast Klont, Damen & ten Cate (1991) and Klont & ten Cate (1991) found that a small proportion of the collagen matrix could be degraded by acids alone. However, when human tooth roots become carious, cavitation may result if the lesion is not treated, which indicates that the collagen structure has become severely disrupted. This may be due to either physical processes (such as abrasion), microbial enzyme activity, or a combination of both.

Studies have attempted to determine the effect of proteolytic enzymes upon demineralised and undemineralised tooth roots. Klont & ten Cate (1991) found that the amount of collagen degradable by collagenase in bovine tooth roots was directly

proportional to the amount of calcium released in a prior demineralisation step. A small amount of collagen was degraded during the demineralisation prior to collagenase treatment, but this amounted to a maximum of 40μg/cm^2 after 4 weeks compared with 3,000μg/cm^2 after 1 week's treatment with collagenase. Collagen was found to be almost four times more collagenase-sensitive in erosive lesions (where the whole of the lesion is demineralised) than in sub-surface lesions (where a hypermineralised surface layer overlies the demineralised body of the lesion). Klont, Damen & ten Cate (1991) showed that if collagen was exposed to trypsin, a similar pattern to that obtained with collagenase was found; the amount of trypsin degradable collagen increased in direct proportion to the degree of demineralisation. However, the total amount of collagen which could be degraded by trypsin was about 2% of that which could be hydrolysed by collagenase, indicating that only a small proportion of the collagen had been denatured during demineralisation. Van Strijp *et al.* (1994) examined the ability of various bacterial species to colonise and degrade decalcified bovine dentine mounted in dental appliances and worn by volunteers. Collagen loss varied from between 0 and 67% and the range of bacterial species that colonised the dentine was diverse, although no significant correlation between known proteolytic organisms and collagen break-down was found.

The action of fluoride A number of studies have attempted to elucidate the effect of fluoride upon root surface caries development. Mellberg & Sanchez (1986) found that when root surfaces with artificial caries lesions were treated with a fluoride dentifrice (2 x 5min per day), and then bathed in a Ca^{2+}-remineralisation buffer *in vitro*, remineralisation occurred to a significantly greater extent than when a placebo dentifrice was employed. Al-Joburi & Koulourides (1984) found that the treatment of root surfaces with three different fluoride formulations prior to and during exposure to a

demineralising buffer reduced the severity of mineral loss as compared with untreated controls. However, Retief, Wallace & Bradley (1988) reported no significant correlation between the fluoride concentration of cementum and the root surface caries experience of 475 elderly subjects with gingival recession. This result was attributed to the fact that subjects may not have had a steady long term exposure to fluoride so that caries could have developed in some individuals prior to exposure to fluoride. It is interesting to note that Schaeken, Keltjens & van der Hoeven (1991) found no significant changes over 9 months in the viable counts of mutans streptococci in the plaque microflora of subjects treated with fluoride varnish which was applied to exposed roots at 3 month intervals. The total and the actinomyces viable counts both increased slightly. In contrast, the number of decayed, missing or filled surfaces (the D.M.F. value) was significantly lower than in the non-fluoride-treated controls, which tends to suggest that fluoride is exerting an effect more at the chemical rather than the bacteriological level.

In summary, it would seem that a significant proportion of the bacterial species present in root surface plaque are capable of generating a pH drop, from endogenous metabolites or exogenous carbohydrates, that could be low enough to lead to loss of mineral from the root structure. Although many bacterial species are capable of generating a pH drop, certain streptococcal, lactobacillus and actinomyces species (i.e. mutans streptococci, *L. casei* and *L. plantarum* and also *A. viscosus* and *A. naeslundii*) would appear to be the most effective. However, the pH reducing capacity of a plaque can be offset to some extent by the ability of some bacterial species to raise plaque pH. Dissolution of the mineral component, which can begin at relatively high pH, can be slowed or even reversed by the application of fluoride, whilst degradation of the organic matrix would appear to be dependent upon proteolytic activity occurring after demineralisation.

1.3.5 Histology

The outermost layer of the human tooth root consists of cementum although this can undergo changes, such as hypermineralisation (Furseth & Johansen, 1970), when it is exposed to the oral environment. Embedded in the cementum are the fibres of the periodontal ligament which anchor each tooth to the surrounding periodontal bone. The layer directly beneath the cementum, which makes up the major part of the tooth bulk, is the dentine. Structurally the dentine is composed of bundles of collagen filaments surrounded by mineral crystals. Each healthy mineral crystal is usually 296Å wide, 31.6Å thick and needle shaped (Daculsi *et al.*, 1979). At the centre of the tooth root is the pulp which carries the tooth blood supply and also the nerves.

There are a number of steps involved in the development of a root surface caries lesion; (i) colonisation of the root surface by cariogenic bacteria (or the invasion of a 'benign' plaque by more 'virulent' organisms)(Donoghue & Perrons, 1991), (ii) mineral loss begins to take place (Nyvad, Ten Cate & Fejerskov, 1989), (iii) some of the organisms present upon the lesion surface begin to invade the root structure and (iv) degradation of the collagen matrix of the dentine occurs leading to destruction of the root structure in that area (Frank, Steuer & Hemmerle, 1989).

Cementum caries begins as decalcification of the surface layer of the root which can proceed inwards to a depth of 0.33mm without any visible defects occurring (Hals & Selvig, 1977). Decalcification results in a loss of mineral from hydroxyapatite crystals which compose the inorganic structure of the root; these crystals are initially irregularly shaped (varying from 'needle' to 'tablet' and circular to triangular) but become larger and more regular in shape (Furseth & Johansen, 1970). Mineral loss is believed to result

from dissolution of the crystal lattice by inorganic acids liberated by plaque bacteria in response to dietary carbohydrate intake (Clarkson *et al.*, 1987) and develops as an increasing inward gradient (Nyvad & Fejerskov, 1990). In contrast Frank, Steuer & Hemmerle (1989) found no gradient in the surface layer, but identified one in the deeper layers of advanced caries lesions.

Carious lesions tend to have a more densely mineralised surface layer which is also much thicker than that present in normal cementum (Furseth & Johansen, 1968, Hals & Selvig, 1977, Phankosol *et al.*, 1985 and Nyvad & Fejerskov, 1990). Carious cementum viewed in transverse section also has alternating bands of mineralised and demineralised tissue (Furseth & Johansen, 1968) which is due to alternating periods of de- and remineralisation (Furseth and Johansen, 1968 and 1970 and Phankosol *et al.*, 1985). The collagen fibres which comprise much of the organic matrix of the tooth root structure would appear to be unaffected (or, at worst, only minimally affected) at this stage in the development of a root surface caries lesion (Furseth, 1971 and Nyvad & Fejerskov, 1990). Along with the process of demineralisation members of the bacterial plaque invade the lesion, initially along the calcified bundles of collagen fibre in the cementum (Frank, Steuer & Hemmerle, 1989 and Nyvad & Fejerskov, 1987b and 1990).

Initially a root surface caries lesion appears as an area of demineralisation of the cemental layer, but without treatment it soon progresses into the dentinal structure to form an advanced lesion (Brown, Billings & Kaster, 1986). The mineral content of the hydroxyapatite crystals in dentine continues to be lost as advanced caries lesions develop. The crystals appear to be granular and irregularly scattered in later lesions (Ohgushi & Fusayama, 1975). Moreover the dentinal tubular matrix is destroyed and the collagenous

component degraded, although there is a zone of hypermineralisation at the advancing front of the lesion (Frank, Steuer & Hemmerle, 1989, Levine, 1974 and Westbrook *et al.*, 1974). Under experimental conditions advanced lesions have been observed to extend for up to 240μm into the dentine after one month of *in situ* exposure and up to 630μm after three months (Nyvad, ten Cate & Fejerskov, 1989). Advanced caries lesions can involve cavitation of the root surface; to the naked eye they appear to be discoloured (brown or black) and have a distinctive 'leathery' texture that can be felt upon examination with a dental probe (Lynch and Beighton, 1994). Bacteria have been observed invading the dentinal tubules surrounding lesions and spreading towards the dental pulp cavity (Schüpbach, Guggenheim & Lutz, 1990).

If a root surface caries lesion is treated before cavitation occurs, it is possible to arrest its progress (Nyvad & Fejerskov, 1986) and perhaps even encourage remineralisation (Schüpbach, Lutz & Guggenheim, 1992). Schüpbach, Lutz & Guggenheim (1992) postulated that the arrestment of root surface caries lesions occurred via a number of steps; firstly an inner barrier forms which prevents diffusion of substrates from the pulp to invading bacteria; secondly a hypermineralised outer barrier forms which prevents bacterial products from diffusing into the dentine and thirdly a layer of remineralisation develops progressively from the outer barrier towards the pulp. This report noted that intertubular dentine was fully mineralised and that the dentinal tubules had become sclerosed, with some still containing the outlines of mineralised bacterial cells. The crystals which form the inorganic matrix of arrested caries lesions were noted to be very similar in shape and size to those of non-carious dentine (Daculsi *et al.*, 1979). However for remineralisation of a root surface lesion to occur it would seem that the collagen matrix must be largely intact to provide points for crystal growth (Klont, 1990).

1.4 Current and potential model systems available for the study of root surface caries

1.4.1 Introduction

There are a variety of different model systems available which can be used to study human root surface caries. These include *in vivo* studies in which the epidemiology, bacteriology and histology of root surface caries lesions are examined, *in situ* models, experimental animal systems and *in vitro* experiments that investigate the effect of organic acids or bacterial acids and enzyme products on the root structure. Finally *in vitro* bacterial model systems have been developed which attempt to simulate the oral environment to some degree i.e. 'artificial mouths'. Each of these model systems has advantages and disadvantages related to ease of use and expense together with its degree of 'relatedness' to naturally occurring root surface caries.

1.4.2 *In vivo* studies

In vivo studies, which encompass the epidemiology, bacteriology and histology of root surface caries development were reviewed in section 1.3. Since these studies investigate naturally occurring carious lesions they are by definition closely 'related' to the disease in humans. The results of these studies have shown how widespread root surface caries is in selected populations (Fejerskov *et al.*, 1991) and have identified the individual groups most likely to be affected (Fure & Zickert, 1990b). In addition such data can give an indication of the bacterial species most commonly associated with root surface caries (Bowden *et al.*, 1990) and can also reveal the structural changes that occur in the carious root lesion (Schüpbach, Guggenheim & Lutz, 1990 and Schüpbach, Lutz & Guggenheim, 1992). However, although these studies give closely 'related' data, they have a number of potential drawbacks. Firstly, for statistically robust data to be

generated it is preferable to have the largest sample population possible. Secondly, long-term longitudinal studies provide more detailed information than cross-sectional ones but require substantial resources due to repeated sample collection. Finally, it is difficult to control the processes which occur intra-orally over a period of time; for instance the oral microflora is known to differ between individuals and over time within the same individual (Nyvad & Kilian, 1990 and 1987), dietary intake can vary from day-to-day and salivary flow rate can also fluctuate. Therefore a variety of approaches to tackle these problems have been made and these are discussed below.

1.4.3 *In situ* model systems

Several studies have looked at *in situ* root surface caries development by embedding either native human tooth root sections (Nyvad & Kilian, 1987 and 1990) or demineralised bovine dentine sections (van Strijp *et al.*, 1994) in dental appliances. The dental appliances were then worn intra-orally by volunteers for varying periods of time and the microflora characterised and enumerated. In addition the degree of structural degradation of the dentine sections was determined for the longer term experiments.

In one study the number of bacteria colonising root surfaces exposed to the oral environment for 24hr was 10^7 per $0.024cm^2$ (Nyvad & Kilian, 1987), with *S. mitis* tending to be the predominant gram positive coccus present (a mean of 24 - 42% of the total). However the proportions of *S. mitis* declined over 24hr, whereas *S. oralis*, which was found in lower proportions (up to 27% of the total), increased significantly during the same period. Actinomyces were found to adsorb to the root surfaces within the first 4hr of the experiment but did not increase in proportions until about 12hr into the experiment, when they comprised 1% of the total. In 1990 Nyvad & Kilian reported on

the microflora present on bacterial roots mounted in dental appliances which had been exposed to the oral environment for 3 months and found a high degree of mineral loss with the overlying plaque being dominated by either *A. viscosus* (up to 82% of the total cultivable flora) or a combination of mutans streptococci and lactobacilli (up to about 76% of the total cultivable flora). Root surfaces which exhibited a less pronounced demineralisation were colonised with a wider range of micro-organisms.

Rather than examine the effect of oral bacteria upon whole dentine, van Strijp *et al.* (1994) examined the ability of oral micro-organisms to colonise and degrade the demineralised matrix of bovine dentine mounted in an intra-oral appliance. This study found that a range of streptococci, lactobacilli, actinomyces, veillonellae and gram negative rods colonised the demineralised dentine over a period of 7 weeks. However, the plaques which had the greatest degradative capability tended to be dominated by *S. mitis*, *Peptostreptococcus productus* and *Veillonella parvula*, although the proportions of these varied.

Thus *in situ* model systems can provide information upon the bacterial colonisation of root (or other tooth) surfaces and also about the ability of the colonising plaque bacteria to degrade the tooth structure. However, such studies do not provide comparative data concerning the ability of individual bacterial species to cause degradation, nor do they give any data concerning the biochemical changes that take place; i.e. the identity, quantity and rate of ions released and the enzymes involved in protein degradation. *In situ* model systems also suffer to some extent from the drawbacks mentioned for *in vivo* studies (e.g. difficulties in controlling the intra-oral experimental conditions).

1.4.4 Experimental animal model systems

Model systems which employ laboratory animals have been used to investigate both the ability of selected oral organisms to colonise tooth surfaces and to induce caries and periodontal disease. It is possible that a model system using gnotobiotic laboratory animals could be developed to study root surface caries in the absence of periodontal disease to compare the ability of human oral bacterial species to induce disease. However a number of technical problems would require to be solved; such as a method of reproducibly exposing root surfaces without stressing the experimental animals to such an extent that their habits were to change significantly and their dietary intake affected. Nevertheless, the data provided by the current experimental laboratory animal models do give some useful insights into the relative cariogenic activities of different oral bacterial species, even if the data do relate more to coronal/ fissure caries than root surface caries.

In the oral environment it is essential for a potential pathogen to invade and colonise the existing plaque successfully before it can produce caries. Beckers & van der Hoeven (1982) examined the ability of *Actinomyces viscosus* and *Streptococcus mutans* to colonise the dental plaque of rats and found that the *S. mutans* viable counts decreased slightly for the first 6 - 24hr and then steadily increased over the remaining 14 days. However, two strains of *S. mutans* were employed and one (T2) had no apparent effect upon the indigenous oral flora whilst the other (C67-1) appeared to compete actively and reduce the numbers of indigenous bacteria. The doubling time for both of the strains during the first 24 hours were approximately 5 hours. *Actinomyces viscosus* also showed an initial drop in viable counts over the first 6 hours followed by a rapid increase, although its doubling time at approximately 7.3 hours was slower than the *S. mutans* strains. No competitive effect between the test strain of *A. viscosus* and the indigenous

bacterial flora was noted. In 1984 Beckers & van der Hoeven examined the effects of *S. mutans* and *A. viscosus* upon one another in gnotobiotic rats and found no competitive effect between the two.

A number of papers have investigated the ability of various bacterial strains to induce (coronal) caries when inoculated into laboratory rodents. De Soet *et al.* (1991) infected gnotobiotic laboratory rats, that received a diet containing 20% sucrose and 5% glucose, with *S. mutans* and *S. sobrinus* freshly isolated from the human mouth. No significant difference was found between the colonisation activity of the two species, but *S. sobrinus* caused a significantly higher number of advanced dentinal lesions than *S. mutans*, although there was no difference between the two species for less severe forms of coronal caries. The ability of various oral streptococcal species both to colonise a glass surface and to induce dental caries in rats have been the subject of an investigation by Willcox, Drucker & Hillier, (1988). Mutans streptococci (*S. mutans*, *S. sobrinus*, *S. rattus* and *S. cricetus*) were found to have the greatest ability to induce fissure caries, whilst *S. lactis* had the least. *Streptococcus salivarius* had the next greatest relationship with fissure caries after mutans streptococci, followed by *S. milleri*, then *S. sanguis*, *S. faecalis* (now renamed *Enterococcus faecalis*) and finally *S. mitis*. In 1991 Willcox *et al.* compared the characteristics of *S. vestibularis* with *S. salivarius* using the same techniques as described in Willcox, Drucker & Hillier (1988). All *S. vestibularis* strains were capable of producing limited caries in fissures whilst *S. salivarius* produced many more caries lesions. The abilities of either species to adhere to sucrose coated hydroxyapatite or to buccal epithelial cells were approximately equal; neither sucrose nor saliva particularly increased aggregation of the two test species although *S. salivarius* was much more hydrophobic.

The lactobacilli adhere poorly to oral surfaces (Marsh & Martin, 1992) and this may affect determination of their *in vivo* cariogenic ability. However, Fitzgerald *et al.* (1980) inoculated hamsters with fifty different strains of lactobacilli (isolated from school children) and found that 3 strains (2 provisionally identified as *L. salivarius* and one as *L. fermentum*) had significant cariogenic activity. In addition it was found that sucrose but not glucose or starch was a prerequisite for cariogenicity, although the 3 cariogenic lactobacillus strains failed to form adherent plaques in the presence of sucrose *in vitro*. *Actinomyces* species have been inoculated into both hamsters and rats with the intention of studying periodontal disease (Jordan & Keyes, 1964). Human isolates of actinomyces (including *A. viscosus*) inoculated into gnotobiotic rats were found to induce the symptoms of gingivitis and periodontitis and in addition they were found to produce root surface caries lesions (Jordan, Keyes & Bellack, 1972).

Thus model systems which employ experimental animals have been used to provide information on the cariogenic properties of different bacterial species. However, the experiments generally aimed to investigate coronal caries and only recorded root caries when the bacteria succeeded in inducing sufficient destruction of the gingivae to expose the tooth roots. The use of experimental animals can overcome some of the problems associated with controlling dietary intake and the effect of a competitive plaque microflora if gnotobiotic animals are used. However, it is still difficult to investigate and quantify the biochemical processes taking place because the complex composition of saliva and the variability of salivary flow rate can compromise biochemical investigations of the products released from the roots during the development of disease. Moreover, the oral environment of laboratory animals and their tooth structure tends to be different from humans which can make it difficult to extrapolate any data gathered using animals.

Due to the problems associated with *in vivo* and *in situ* model systems, involving either humans or laboratory animals, some researchers have turned to using a variety of *in vitro* model systems.

1.4.5 *In vitro* chemical model systems

Rather than employing bacterial populations, some *in vitro* studies have used bacteria-free chemicals i.e. organic acids and/ or purified proteolytic enzymes. Some of those investigations have attempted to elucidate the rate at which root mineral dissolves under defined conditions, whilst others have tried to explore the degradation of the organic components. The effect of various compounds (mostly containing fluoride) upon the demineralisation and remineralisation of root surfaces has also been explored in chemical-based model systems. The events that occur in a chemical-based *in vitro* system can be analysed by x-ray microradiography or by chemical analysis. In other words the structure or degree of mineralisation of the tooth root can be examined before and after the experiment and the fluid phase of the system can be sampled before and after the experiment to determine changes in chemical composition.

Studies which measured caries progress by microradiography Hoppenbrouwers, Driessens & Borggreven (1986 and 1987) examined the rate at which mineral was lost from roots when they were exposed to solutions containing varying calcium and phosphate ion concentrations at pH 5.0 (1986) or pH 5.0, 5.5, 6.0 and 6.5 (1987). The results of both studies indicated that the threshold pH for dissolution of the mineral structure of human tooth roots was in the region of pH 6.7. Another study which employed a relatively large volume of demineralising buffer was that of Herkströter, Witjes & Arends (1991) who found that during cycled exposure to de- and re-

mineralising solutions, the total exposure time to the demineralising solution had a direct correlation with mineral loss from root structure. However, the presence or absence of an intact surface layer on the root dentine was found to have a significant effect upon overall demineralisation; root dentine which had been abraded and lost its surface layer was found to lose about twice as much mineral compared with that which had simply been polished (approximately 0.12kg/m^2 versus 0.06kg/m^2 respectively).

Rather than using a relatively large volume of demineralising buffer, as described above, some workers have opted to use an acidified gel to generate the root surface lesions. Phankosol *et al.* (1985) found that when exposed to a gelatin gel containing 1M acetic acid at pH 4.5, none of the human tooth root sections developed cavitated lesions although all lesions had a 'saucer shaped' appearance. Also almost half of the lesions were found to have a radiopaque surface layer (i.e. a surface layer which was hypermineralised with respect to the body of the lesion) which was thought to be reprecipitation of mineral solubilised from the deeper areas of the lesion; the collagen structure appeared to be unaffected. Feagin *et al.* (1987) also employed an acidified gelatin gel system which contained 75mM lactic acid and 25mM acetic acid, although in contrast to Phankosol *et al.* (1985) the buffer also contained calcium, phosphate and sodium fluoride at concentrations of 1, 0.6 and 0.05mM respectively. This study found that root surface caries lesions had progressed to a depth of 20μm at 1hr and 245μm at 528hr.

The effect of fluoride upon root surface caries lesion progression has also been investigated. Al-Joburi & Koulourides (1984), when they reacted human tooth root surfaces with demineralising buffer containing 0.01M lactic acid, 3mM calcium, 1.8mM

phosphate and 1% carboxymethylcellulose, found that when the surface layer was removed by planing with a curette the lesion depth was significantly greater than when the surface was largely intact. Herkströter, Witjes & Arends (1991) reported similar results. Treatment of the planed root surfaces with various topical fluoride preparations (for 3min at t = 0, 3, 6 and 9 days) led to the formation of a thick densely mineralised layer overlying the body of the lesion. When demineralised root sections were treated with a toothpaste containing monofluorophosphate (m.f.p.) or a placebo paste for five minutes twice a day and then immersed in a remineralising solution between treatments, a significant degree of remineralisation was found to occur (Mellberg & Sanchez, 1986). However, the pattern of mineral deposition appeared to vary between the two treatments, with the placebo treated lesions showing even remineralisation whilst the fluoride treated lesions showed remineralisation of the body of the lesion with a more densely mineralised outer surface layer. Moreover it was found, by Almqvist and Lagerlöf (1993), that mineral loss from root blocks was slowed by fluoride concentrations of between 0.02 and 0.2p.p.m. but not by 2p.p.m. fluoride.

Studies which measured caries progress by chemical analysis Instead of employing microradiography, root surface caries can be analysed by examining the demineralisation/ remineralisation buffer solution for specific chemicals liberated during the experiment. Some studies have determined the release of calcium and phosphate ions from root mineral, whilst others have sought the breakdown products from the organic matrix e.g. collagen, collagen subunits and non-collagenous proteins. As is the case with microradiographic analyses, the effect of fluoride ion upon the dissolution of mineral from root has usually been part of the experimental protocol.

Boonstra, ten Bosch & Arends (1988) and (1990) reported the results of studies into the rate of demineralisation of bovine dentine when exposed to a demineralising buffer of acetic acid at either pH 4.5 or pH 5.0. The rate of demineralisation at pH 4.5 and 5.0 was 0.5 and 0.32mmol Ca^{2+}/cm^2 dentine/week respectively and 0.31 compared with 0.1832mmol PO_4^{3-}/cm^2 dentine/week (Boonstra, ten Bosch & Arends, 1988), which yielded a molar Ca:P ratio of 1.74 ($\pm$0.07) at both of the pH values employed. Similar molar Ca:P ratios were also reported in a further series of experiments described by Boonstra, ten Bosch & Arends (1990) ranging from 1.60 ($\pm$0.04) at pH 4.5 to 1.83 ($\pm$0.08) at pH 5.0. A maximum of 614µmol calcium ions per cm^2 and 383µmol phosphate ions per cm^2 was dissolved from dentine at pH 4.5. Demineralising buffer at pH 4.5 caused greater demineralisation than buffer at pH 5.0.

Feagin *et al.* (1987) applied an acidified gel of pH 4.4, containing 25mM acetic acid and 75mM lactic acid, to tooth root surfaces. After 1hr the calcium ion concentration increased from 1.0 to 1.04mM and the phosphate from 0.6 to 1.02mM, whilst at 22d the concentrations had increased to 5.8mM and 3.6mM respectively. Taking into account the buffer volume (0.1ml) and the exposed root surface area (0.15 - 0.22mm^2), the data indicate that a total of 2.4µmol calcium/cm^2 exposed root and 1.5µmol phosphate/cm^2 was released in 22 days. The molar Ca:P ratio was 1.6 - very close to that determined by Boonstra, ten Bosch & Arends (1988) and (1990). The good agreement between the Ca:P ratios determined by these studies suggests that the differences in total calcium and phosphate ion concentrations are likely to be a result of the experimental design, since Boonstra, ten Bosch & Arends (1988) and (1990) used a buffer volume of 100ml per cm^2 exposed dentine whereas Feagin *et al.* (1987) made use of approximately 0.5ml of buffer per cm^2 exposed root surface.

Protein degradation of the root matrix can be divided into breakdown/ loss of (i) the collagenous matrix and (ii) the non-collagenous proteins (n.c.p.). Klont, Damen & ten Cate (1991) reported that when demineralised root powder was exposed to either an acidic or neutral buffer, collagen was released from the root matrix into both of the buffers although at a greater rate and to a greater final concentration in the acidic buffer. In addition, greater amounts of collagen were released when the ionic strength of the demineralising buffers was increased. In 1991 Klont & ten Cate found that the amount of degradable collagen in bovine tooth root sections exposed to demineralising buffer increased proportionally to the amount of calcium released, up to a ceiling of between 80 and 100μmol calcium released/cm^2 exposed root surface. The results of the study suggested that the degree of demineralisation of the root would have an impact upon the ease with which collagen could be degraded; i.e. collagen in completely demineralised regions would be more easily degraded than that in partially demineralised areas. However Boonstra, ten Bosch & Arends (1990) could not detect collagen released from dentine exposed to demineralising conditions, although 50% or more of the non-collagenous proteins were found to have been removed from the dentine.

In vitro model systems which employ chemicals rather than bacteria supply invaluable data about the rate of mineral and protein loss from the tooth root structure. These model systems can be controlled to some extent since it is possible to introduce pure, specific chemicals into the system and to determine their effect without fear of interference from other factors, such as saliva, salivary flow rate and diet. In addition the analyses of samples from these systems are relatively straightforward since there will be little contamination by human secretions or bacterial metabolites. However, although control of external environmental factors is possible with these *in vitro* model systems,

natural variation in the tooth root structure could confound the results of experiments using this type of model system just as with *in situ* or experimental animal models. Moreover, a major disadvantage of these *in vitro* model systems is that the experimental conditions tend to be relatively static and the complex interactions that occur *in vivo* cannot be simulated reducing their 'relatedness' to the *in situ* environment.

1.4.6 *In vitro* biological model systems

Various attempts have been made to elucidate the cariogenicity of micro-organisms inhabiting dental plaque. Some studies have explored the ability of micro-organisms to produce acids, alter pH or to produce proteases under defined conditions. The cariogenicity of some oral bacteria has also been ascertained by determining the rate at which they cause demineralisation - estimated either by chemical means or by microradiography.

The determination of bacterial cariogenicity by the measurement of acid and/ or protease production Studies have attempted to determine the cariogenicity of oral plaque micro-organisms by examining the amount of acid or pH change that they produce under specified conditions. De Soet, Toors & de Graaff (1989) compared the abilities of a range of oral streptococci to liberate acids from glucose at predetermined pH values. *Streptococcus sobrinus* was found to be capable of producing much more acid than other streptococcal species at low pH (as low as pH 5.5), suggesting that it may have an important role in cariogenesis. However, in contrast to this, Homer, Patel & Beighton (1993) compared the ability of strains of *S. mutans* and *S. sobrinus* to ferment glucose in the presence of N-acetylglucosamine, which is naturally present in human saliva, and found that the ability of *S. sobrinus* to ferment glucose was inhibited to a marked degree

by this compound. Furthermore, Sansone *et al.* (1993) found that the non-mutans streptococci present in plaque capable of acidogenesis at low pH also had a weak positive correlation with root surface caries. Wijeyeweera & Kleinberg (1989) also reported that non-mutans streptococci (e.g. *S. sanguis* and *E. faecalis*) were capable of producing a pH reduction to match that of *S. mutans*, although the picture was somewhat confused by the fact that different strains of the same species (both of *S. mutans* and of *S. sanguis*) had different pH lowering capacities.

The acidogenicity of various *Actinomyces* species has also been explored in an attempt to determine their cariogenic potential. Wijeyweera & Kleinberg (1989) reported that *A. viscosus* was responsible for much less of a pH reduction than *A. odontolyticus*, *A. israelii* or *A. naeslundii*. In contrast Komiyama, Khandelwal & Duncan (1986), Komiyama, Khandelwal & Heinrich (1988) and Komiyama & Khandelwal (1992) in a number of studies examined the ability of freshly isolated *A. viscosus* and *A. naeslundii* to synthesise and degrade glycogen - an intracellular carbohydrate source. *Actinomyces viscosus* isolated from carious root surface plaque had a greater ability to convert radiolabelled glucose to glycogen than either *A. viscosus* from non-carious plaque or *A. naeslundii* from carious and non-carious plaque. Furthermore, when starved, strains isolated from carious plaque continued to degrade stored intra-cellular glycogen for a longer period than those isolated from non-carious plaque even when the pH was as low as pH 4.5. This indicates that these organisms will be able to continue to metabolise and to excrete acids for an extended period even though the plaque pH is very low. In addition bicarbonate, which is present in saliva (Shellis, 1978), was shown to increase the acid production of *Actinomyces viscosus* (Takahashi & Yamada, 1992).

Few investigations have studied the ability of *Lactobacillus* species to lower pH, although those which did (e.g. Wijeyeweera & Kleinberg, 1989) showed that lactobacilli were generally very effective at reducing the pH in response to glucose exposure. *Lactobacillus salivarius, L. casei* and *L. acidophilus* were all found to be able to reduce the pH of the culture medium to a greater extent than any of the *S. mutans* strains tested. However, Sansone *et al.* (1993) found that the proportions of lactobacilli in carious plaque tended to be low.

In the case of root surface caries, it would seem likely that the ability to degrade collagen is also an important factor in the cariogenic potential of a plaque, since cavitation involves the eventual loss of both organic and inorganic matrix. In addition the micro-organisms present within plaque may be able to gain an extra energy source when food is scarce by hydrolysing proteins present in saliva and gingival crevicular fluid (Homer & Beighton, 1992). Dental plaque is known to have proteolytic activity in general (Söder & Frostell, 1966) and also specific collagenolytic activity (Loesche *et al.*, 1974). Of the micro-organisms which inhabit plaque, *Streptococcus* species (including *S. mutans*) are capable of proteolysis, although both *S. oralis* and *S. sanguis* have greater activity than mutans streptococci (Homer, Whiley & Beighton, 1990). Knuuttila & Mäkinen (1981) reported that after 18 months' exposure of *S. mutans* to xylitol (a carbohydrate source which it is incapable of metabolising), the ability of this species to hydrolyse various proteins was significantly increased. It was presumed that the organism was utilising proteins as both a carbon and nitrogen source. Other organisms which inhabit the oral cavity and exhibit proteolytic activity include *Candida albicans* (Hagihara *et al.*, 1988), *Bacteroides (Prevotella) melaninogenicus* and *Clostridium histolyticum* (Loesche *et al.*, 1974) and *Porphyromonas gingivalis* (Homer & Beighton, 1992).

The determination of bacterial cariogenicity by the chemical measurement of mineral dissolution It is also possible to measure the amount of mineral released in response to bacterial activity as either a supplement or an alternative to measuring the amount of metabolic products excreted by bacteria. Both Chestnutt, MacFarlane & Stephen (1994) and Reynolds & Riley (1981) have used this approach to examine the cariogenicity of streptococcal species. Reynolds & Riley exposed hydroxyapatite powder to *S. mutans* in the presence of sucrose at varying concentrations and found that the saturating concentration of sucrose was 2mM. Chestnutt, MacFarlane & Stephen (1994) employed a similar technique, but widened their study to incorporate other strains of streptococci. Mutans streptococci (*S. mutans* and *S. sobrinus*) had the greatest cariogenic potential, in terms of calcium released from hydroxyapatite powder.

The determination of bacterial cariogenicity by microradiographic measurement of mineral dissolution A third alternative method for exploring the cariogenicity of plaque micro-organisms is to expose root sections to cultures of bacteria for a period of time and then to measure the change in mineralisation in any lesions produced using microradiography. A number of studies have adopted this technique to examine the root surface caries-inducing potential of selected human plaque bacteria that have been strongly implicated in caries. Clarkson, Wefel & Miller (1984) coated human tooth root molars with varnish such that only a small window of root surface was exposed to *S. mutans* in a gel matrix containing dextrose. In later experiments *S. mutans* or *A. viscosus* in broth containing sucrose alone, sucrose + starch, sucrose + starch + amylase or sucrose + amylase were tested (Clarkson *et al.*, 1987). At the end of the exposure period the root sections were examined by microradiography. The lesions produced by both *S. mutans* and *A. viscosus* were similar to natural dentine lesions,

although there were differences in lesion depth depending upon growth substrate. *Streptococcus mutans* caused deeper lesions than *A. viscosus* when the substrate was sucrose. (In contrast when the substrate was sucrose + starch, *A. viscosus* caused the deeper lesions. Furthermore, in the gel based system (Clarkson, Wefel & Miller, 1984) the lesions were found to be of the 'sub-surface' type which is analogous to the type of root caries lesion most commonly found *in vivo* (Wefel, Clarkson & Heilman, 1985). In the broth-based system (Clarkson *et al.*, 1987) *A. viscosus* reduced the pH further than *S. mutans* did and also produced deeper lesions when the carbohydrate source was mixed. Similarly, Firestone *et al.* (1993) found root surface caries to progress more rapidly with *A. viscosus* than with *S. sobrinus* when either glucose or sucrose was present as the carbohydrate source. In addition *A. viscosus* cells were observed to invade the carious root structure whilst *S. sobrinus* did not. However Kaufman, Pollock & Gwinnett (1988) found that *S. mutans* caused greater demineralisation of exposed root sections than *L. casei* whilst *A. viscosus* was responsible for least.

In summary, *in vitro* biological model systems have provided useful information concerning the relative cariogenic properties of a range of bacterial species which would have been exceedingly difficult to collect by means of *in situ* studies. For example *Streptococcus* species, *Lactobacillus* species and *Actinomyces* species when examined in monoculture all possessed the ability to generate sufficient acid from carbohydrate to dissolve hydroxyapatite powder, but a number of external factors affects their relative cariogenicity; e.g. *Actinomyces* species were capable of storing carbohydrate as intracellular glycogen and also of utilising starch to a much greater extent than mutans streptococci, which might be an advantage in producing dental caries in the oral environment. Moreover, the relative abilities of various micro-organisms to degrade

collagen have also been reported and whilst the majority of oral species examined would appear to have proteolytic capabilities, a much more restricted range of micro-organisms possess specific collagenolytic activity. However, although *in vitro* model systems of this sort have an advantage over *in situ* studies in the degree of control over external factors, it is possible that unexpected or as yet unknown synergistic or competitive interactions between organisms may have an impact upon their cariogenic properties. Thus some researchers have attempted to mimic the oral environment to some degree *in vitro* by building a 'model mouth', which is described in the following section.

1.4.7 In vitro 'model mouth' systems

An *in vitro* model system which mimics the oral environment - a 'model mouth' or 'artificial mouth' - has been developed by a number of researchers. In this model a bacterial plaque is grown upon a hard surface in a laboratory environment under controlled conditions. An *in vitro* 'model mouth' has been used to develop carious lesions in dental enamel (Sorvari, Spets-Happonen & Luoma, 1994) but as yet no reports appear to be available of a 'model mouth' being employed in the study of root surface caries.

Much work on the development and applications of the *in vitro* 'model mouth' has been performed by Donoghue & Perrons (1988) and (1991), Donoghue, Perrons & Hudson (1985), Hudson, Donoghue & Perrons (1986) and Perrons & Donoghue (1988). The 'model mouth' system employed by these workers consisted of a sterile tooth assembly suspended beneath a nutrient inlet port within an incubation chamber which could be maintained at a preselected temperature. Bacterial cultures were inoculated to the tooth surface and then a nutrient supply was dripped onto the tooth assembly from the inlet

port (Hudson, Donoighue & Perrons, 1986). The resultant bacterial plaque which developed upon the tooth surface was analysed in various ways depending upon the aims of the experiment. An earlier but comparable *in vitro* 'model mouth' system was described by Dibdin, Shellis & Wilson (1976).

The model mouth system described above has been used to study the effect of environment upon selected oral bacteria and *vice versa*. Donoghue, Perrons & Hudson (1985) studied the effect of salivary components upon the interaction between two species of streptococcus; *S. rattus* and '*S. mitior*'. It was found that, supplementing the nutrient supply with catalase or lactoperoxidase altered the balance of the two organisms in mixed plaque; both enzymes increased the relative proportions of *S. rattus*, although lactoperoxidase caused a reduction in the total viable count. In addition Donoghue & Perrons (1991) explored the ability of pre-formed plaques to resist colonisation by an invading organism when supplied with different nutrients. Colonisation of the tooth surface by *S. mutans* was reported to be 50-fold enhanced by glucose supplementation and 200-fold by sucrose supplementation of the growth medium. Furthermore, Hudson, Donoghue & Perrons (1986) reported that *S. rattus*, when supplied with glucose for 1hr at 5hr intervals, caused a progressive decrease in plaque pH; from pH 7 at baseline to pH 5.95 at 48hr, whilst '*S. mitior*', under identical conditions caused a decrease from pH 6.40 to pH 5.35.

An *in vitro* 'model mouth' system which used the same principle as the studies described above, but which was constructed differently, was developed by Noorda *et al.* (1985). In this system up to 12 tooth sections were attached to the upper surface of a disc-shaped sample holder platform. The sample holder platform was rotated intermittently by a

motor which brought each tooth section to rest for a short period of time beneath a media inlet port to allow medium to be supplied to the growing bacterial plaque. Pumping of growth medium to the inlet port was synchronised with stopping of the sample holder. This 'model mouth' system was found to be capable of growing bacterial plaques and also producing demineralisation of human enamel slabs. Zampatti, Roques & Michel (1994) used a modification of this model system (the tooth sections were stationary and each had its own medium inlet port) to study the effect of antiplaque agents upon the formation of dental plaque.

A more sophisticated *in vitro* 'model mouth' system was developed by Sorvari, Spets-Happonen & Luoma (1994). This study literally attempted to recreate a mouth by embedding sections of bovine teeth in the teeth of a plastic denture and then submerging the denture in artificial saliva. The system was inoculated with *S. mutans* and supplied with sucrose to a final concentration of 3%. The physical movement of the human mouth and tongue were also mimicked in this model system and the main purpose of this study was to determine the effect of fluoride and chlorhexidine varnishes upon carious lesion formation. The results suggested that each alone could reduce the amount of demineralisation of the enamel by the plaque and that the two substances could also act synergistically.

Each of the *in vitro* 'model mouth' systems described above has been employed for the production of bacterial plaques upon tooth surfaces, for the development of artificial caries lesions and for the determination of the effect of antibacterial and fluoride upon plaque and lesions development. However, all of the studies made use of human or bovine enamel as their sample tooth surfaces; none employed human root surfaces.

Therefore, although this type of model system has potential for use in the study of root surface caries, as yet no experiments appear to have been reported in the scientific literature.

In vitro 'model mouth' systems have been developed in which plaques of oral organisms can be produced reproducibly and to generate carious lesions in exposed tooth enamel samples. The 'model mouth' system is attractive because, in general, it offers some of the advantages of *in vitro* systems such as the ability to control external variables and also collect samples for biochemical analysis to an extent not possible in *in situ* studies. However, 'model mouth' systems are technically more demanding than simple test-tube based *in vitro* models because of their increased sophistication; many parts require to be assembled and maintained in sterile conditions. These models are also more difficult to control and since all of the models described involved medium being dripped onto the sample there may be differential rates of bacterial removal from the sample due to differences in the adhesive abilities of different species (Fitzgerald *et al.*, 1980).

1.5 Summary of literature review

Root surface caries can be defined as a carious lesion which is wholly or largely confined to the tooth root surface and root surfaces exposed to the oral environment are commonly a prerequisite for its development (Katz, 1980). Although the data can be variable depending upon the population sampled, the general consensus is that at least some members of any population with a natural dentition will have experienced root surface caries and that it is more prevalent in the elderly than the young (Fure & Zickert, 1990b). Increased retention of a natural dentition by elderly people means that root surface caries is likely to become more prevalent (Thomson, 1990).

A wide range of bacterial species has been found to be associated with non-carious and carious plaques and also with carious dentine. Although various species found in carious plaque and dentine have been associated with the development of root surface caries, three groups of bacteria have been more commonly implicated in the development of the disease than the others; *Actinomyces* species, the mutans streptococci and *Lactobacillus* species (Bowden, 1990). However, the relative cariogenicity of each of the three groups is uncertain, and although *Actinomyces* species (particularly *A. viscosus*) are generally the predominant organisms in plaques their proportions can be slightly lower in plaques associated with carious lesions (Fure *et al.*, 1987). Mutans streptococci generally form a smaller proportion of the total viable bacterial count than *A. viscosus*, whilst *Lactobacillus* species often amount to barely 1% of the total viable count in carious plaque. However mutans streptococci and lactobacillus species have been found to be significantly associated with the development of root surface caries. All three groups of bacteria are capable of producing coronal caries in laboratory animals (section 1.4.4) and of generating a sufficient pH drop when supplied with sucrose *in vitro* to cause dissolution of a mineral source (section 1.4.6).

Human tooth root mineral is known to be soluble at pHs close to neutrality (Hoppenbrouwers, Driessens & Borggreven, 1986 and 1987). Furthermore, dental caries lesions can be arrested by fluoride and by antibacterial therapy (Sorvari, Spets-Happonen & Luoma, 1994). However, little is known about the dissolution rates of the human tooth root structure in response to acids produced by the bacterial species associated with the disease or about the order in which the structural components are lost. Natural tooth root caries lesions develop either as subsurface lesions where a region of demineralised dentine is overlaid by a layer of hypermineralisation (Nyvad & Fejerskov,

1990), or as an erosive lesion in which the root structure is degraded and cavitation occurs. In addition bacterial cells have been observed to progressively colonise the dentinal tubules of root dentine and to advance along them as the lesions develop (Schüpbach, Guggenheim & Lutz, 1990) . However, little is known about human root surface caries lesion development in response to challenge by different oral bacteria or about the abilities of the different bacteria to invade and degrade the root structure.

In an attempt to elucidate the relative cariogenic properties of the bacterial species found in plaque and also to determine their effect upon degradation of the root structure a variety of model systems have been devised. Potential model systems include *in situ* models and a range of *in vitro* models (section 1.4) and each has its advantages and its drawbacks, although all of them provide valuable information upon differing aspects of the development of human root surface caries. However, it was felt that there could be advantages to the development of a model system that might allow a range of bacterial species to be screened at relatively low cost and relatively rapidly.

1.6 The aims of the project

The primary aim of the current study was, therefore, to develop a novel *in vitro* model system for the growth of bacteria, which would be: simple, reproducible, relatively easy to handle and permit the growth of a number of different species of oral bacteria both in pure and in mixed culture. Furthermore the model system would allow for simple and rapid collection of 'fluid phase' samples, free from bacterial cells. The secondary aim was to determine whether the new model system could be used to study the demineralisation of human tooth root surfaces.

Chapter 2 Preparation of bacterial cultures

2.1 Introduction

The identity of the bacterial species employed was confirmed by the same means throughout the experiments described in the following chapters (3 - 5). Furthermore the bacterial suspensions inoculated into the model systems were prepared in the same way each time. Therefore it is simpler to describe here the identification and preparation protocols employed and then to refer back to this chapter; this has the added merit of reducing repetition. Aseptic technique was employed throughout the experiments described in this thesis.

2.2 Materials and methods

2.2.1 The organisms employed and verification of their identity

The bacterial strains employed in the experiments which follow were *Streptococcus mutans* NCTC 10449 (human isolate), *Lactobacillus casei* NCTC 6375 (source not given; Snell & Strong, 1939) and *Actinomyces viscosus* NCTC 10951 (hamster). These were supplied as freeze dried cultures in glass ampoules. The organisms were resuspended by pipetting 0.1ml of fastidious anaerobe broth (F.A.B.)(LabM, Amersham, U.K.)(appendix 1) into one ampoule containing each organism. Once resuspended the contents of each ampoule were transferred to separate Columbia blood agar (C.B.A.) plates (Gibco B.R.L., U.K.)(appendix 2) and smeared over the surface of the agar with a preflamed nichrome inoculating loop. The blood agar plates were incubated at 37°C for 48hr in an anaerobic incubator (Anaerobic workstation Mk. 3, Don Whitely Scientific, U.K.) with an atmosphere comprising 80% oxygen free nitrogen, 15% carbon dioxide and 5% hydrogen (B.O.C. gases, U.K.). Figure 2.1 shows the anaerobic incubator. In all of the experiments in the following chapters 'anaerobic incubation' indicates incubation in this anaerobic incubator containing the atmospheric

gases stated. One typical colony from each of the three cultures was then subcultured to a blood agar plate to verify purity (i.e. homogeneous colony morphology). In addition a colony from the *S. mutans* culture was inoculated onto Mitis Salivarius Bacitracin agar (M.S.B.)(Difco Labs., U.S.A.)(appendix 3) and a colony from the *L. casei* culture was transferred to Rogosa agar (Difco Labs., U.S.A.)(appendix 4). At the end of the incubation period all of the plates were examined for purity and colonial morphology.

The growth obtained on the blood agar plates was used to confirm the identity of the Type cultures as follows:-

(a) A colony was picked from the 24hr blood agar culture of each species and used to prepare a Gram film (appendix 5), which was examined microscopically under oil at 1,000x magnification using an Olympus BH2 Microscope (Olympus, Japan) and Gurr Microil immersion oil (BDH Chemicals Ltd., U.K.).

(b) Proprietary biochemical identification systems were used:- api 20 STREP (BioMérieux SA, France)(appendix 6) for *S. mutans*, and Minitek Anaerobe II (Becton Dickinson, U.S.A.)(appendix 7) for *L. casei* and *A. viscosus*. The techniques employed were as follows:-

(i) *Streptococcus mutans*. The blood agar bearing the 24hr *S. mutans* culture was swabbed with a sterile cotton wool swab. The harvested bacteria were then transferred to 2ml of sterile distilled water in a sterile glass bijoux by rotating the swab against the inside wall. Equal volumes (20µl) of this suspension were inoculated to each cupule of an api 20 STREP identification strip. Each well contained a different metabolite (appendix 6) and an indicator in powdered form (figure 2.2 shows an api 20 STREP identification strip). The identification strip was incubated aerobically for 4hr at 37°C and then the appropriate reagents were added to the cupules as specified in the

manufacturer's instructions. The result of each test was noted and the resulting code interpreted with the aid of the manufacturer's manual.

(ii) *Lactobacillus casei* and *A. viscosus*. Blood agar cultures (24hr) were swabbed with sterile cotton wool swabs and the bacterial cells transferred to 1.5ml of sterile broth (supplied by the manufacturer) by rotating the swab against the inner wall of the tube using gentle pressure. Standard volumes of each species (50µl) were inoculated to all the wells of a Minitek Anaerobe II identification gallery, each of which contained a paper disc impregnated with an individual metabolite and an indicator (appendix 7). The galleries were incubated anaerobically for 48hr at 37°C and then the appropriate disclosing reagents were added to the wells specified by the manufacturer. A positive or negative result for each biochemical test was recorded on a result sheet and the resultant code interpreted with the aid of the manufacturer's manual to identify the organisms.

2.2.2 Criteria for the identification of bacterial species

(a) *Streptococcus mutans*:- Gram positive cocci that produced medium-sized (c. 3mm diameter), white, rough and matt colonies which appeared to be embedded in the blood agar and small, dark-blue, rough and matt 'embedded' colonies on M.S.B. agar. In addition an acceptable pattern of biochemical reactions according to the manufacturer's database was necessary.

(b) *Lactobacillus casei*:- Gram positive bacilli that produced large-sized (c. 5mm), white, smooth, glossy and domed colonies on blood agar and large creamy-white, smooth, glossy and domed colonies on Rogosa agar. In addition an acceptable pattern of biochemical reactions according to the manufacturer's database was necessary.

Figure 2.1 **The anaerobic incubator**

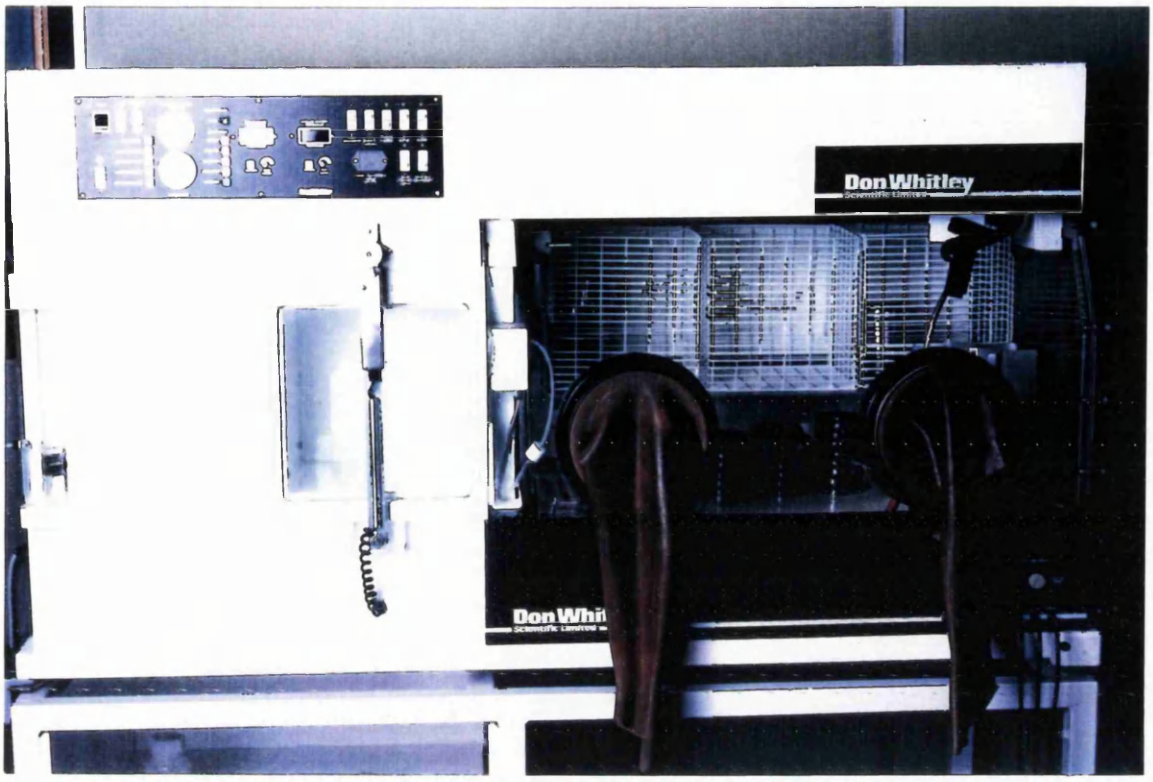


Figure 2.2 **Photograph of an api 20 STREP identification strip**

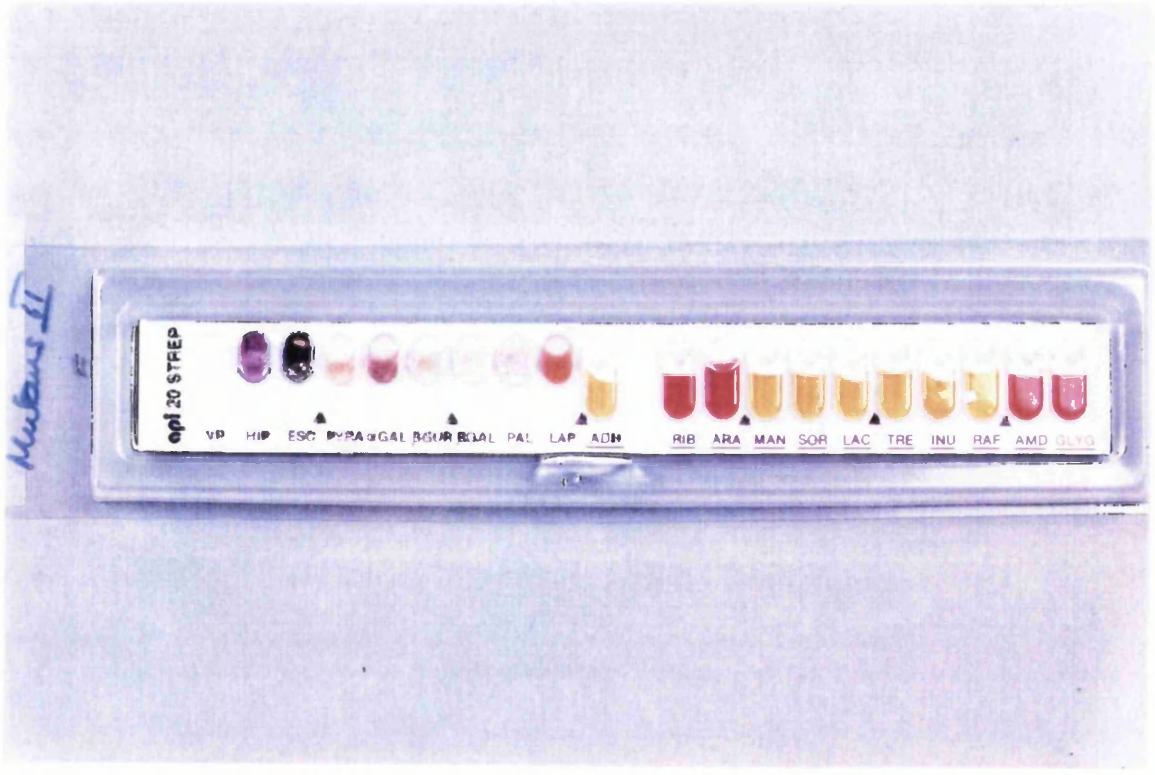
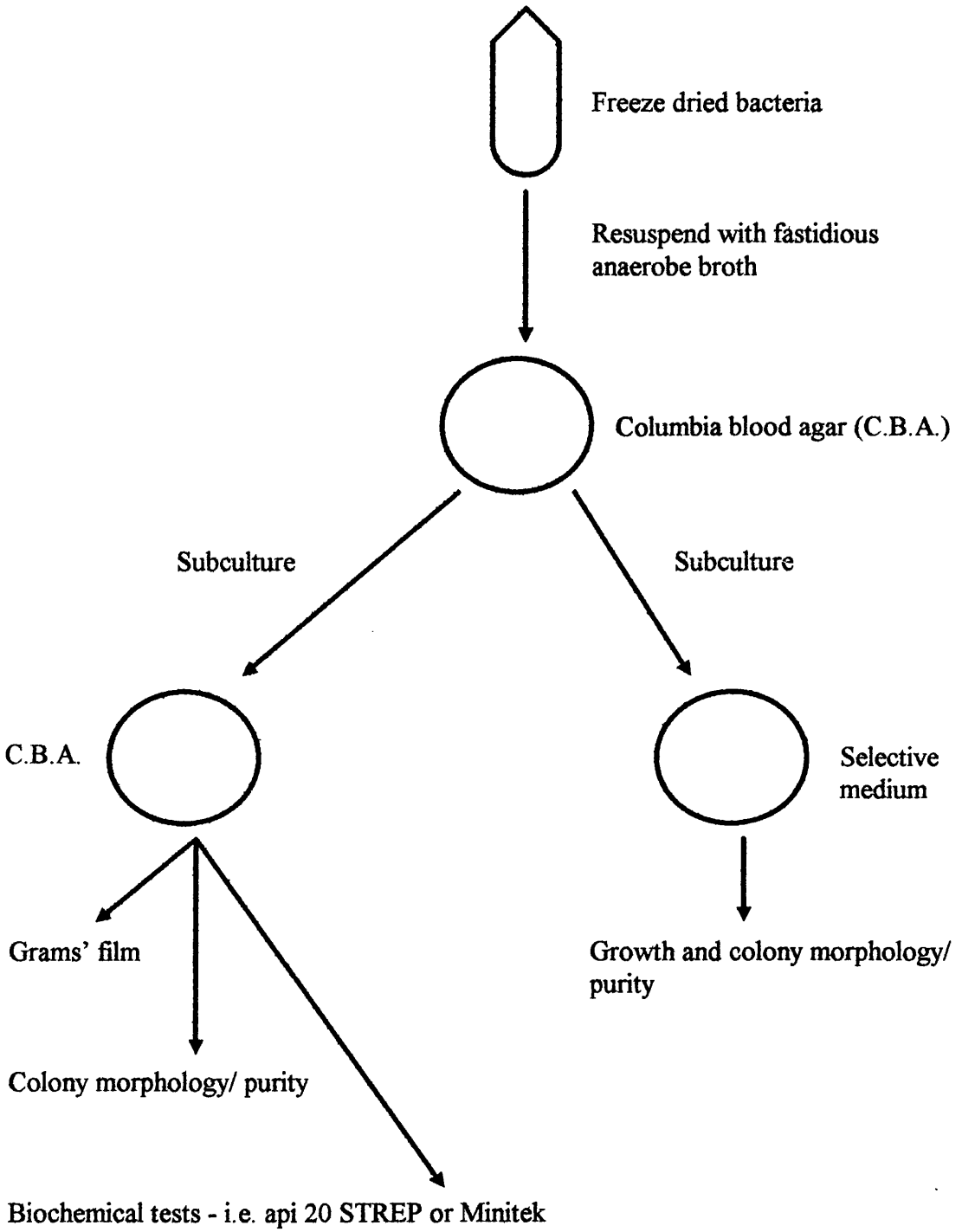


Figure 2.3 **Flow diagram for confirmation of bacterial identity**



(c) *Actinomyces viscosus*:- Gram positive rods that produced small-sized (c. 2mm), beige/white, smooth, glossy and domed colonies on blood agar and an acceptable pattern of biochemical reactions according to the manufacturer's database.

The identity of the three Type cultures was confirmed by these criteria.

Figure 2.3 shows the standard protocol for confirmation of bacterial identity. In general a combination of Gram stain and colonial morphology on blood agar and on semi-selective medium were employed to verify the identity of the three species during the experiments detailed in the following chapters. Commercially available biochemical identification systems were used only initially and thereafter at infrequent random intervals during the experimental period due to the expense involved.

2.3 Storage of bacteria

Once the identity of the three type cultures had been confirmed, they were subcultured to quadruplicate blood agar plates and incubated for 48hr at 37°C in an anaerobic environment. After incubation, the Type cultures were stored using both 'Protect' Bacterial Preserver beads (Technical Service Consultants Ltd., U.K.) and by freeze drying:-

2.3.1 Protect beads

After incubating each species for 48hr, growth on 3 of the 4 blood agar plates was harvested with sterile cotton wool swabs and transferred to the cryopreservative broth supplied by the manufacturer by rotating the swab against the inside wall of the tube whilst immersed in the cryopreservative broth. One blood agar culture from each species

was inoculated to one tube of 'Protect' beads. The tubes were capped and inverted several times to coat the beads with the bacterial cells. After 30s the excess broth was removed and discarded. Two tubes of 'Protect' beads inoculated with each species were stored at -30°C and these acted as the day-to-day 'working' stock of bacteria. The third was stored at -70°C to act as a 'reserve' stock.

2.3.2 Freeze drying

At the end of the incubation period the fourth blood agar culture of each species was swabbed with a sterile cotton wool swab. The harvested organisms were suspended in separate 2ml volumes of anaerobic blood broth (A.B.B.)(Gibco B.R.L., U.K.)(appendix 8) supplemented with 33% foetal calf serum (F.C.S.) (Life Technologies, Gibco, U.K.). Fifty microlitre volumes of each bacterial suspension were added to sterile glass ampoules, which were labelled with the name and identification code of the organism inoculated. The ampoules were freeze dried at - 30°C in a Modulyo Freeze Dryer (Edwards, U.K.) according to the manufacturer's instructions. Finally the evacuated ampoules containing the freeze dried bacterial cultures were sealed and stored at room temperature. The freeze dried cultures acted as an emergency reserve stock for use in the event of a power failure that could lead to the loss of the frozen 'Protect' bead stocks. This situation, however, did not occur.

2.4 Resuscitation of bacteria and preparation of initial inocula

2.4.1 Resuscitation of bacteria from 'Protect' beads

At the beginning of each experiment described in the following chapters, each of the bacterial species used was resuscitated from the 'Protect' beads stored at -30°C. One of the bacteria-coated beads was removed from the tube with a sterile nichrome inoculating

loop and then gently smeared across the surface of a sterile blood agar plate to transfer the bacterial inoculum. The culture was then incubated anaerobically at 37°C for 48hr.

2.4.2 Growth of bacterial cultures

Each of the three species, once resuscitated from 'Protect', was subcultured to four blood agar plates and incubated anaerobically at 37°C for 48hr. Three of the blood agar plates were used to prepare the bacterial inoculum for each experiment, whilst the fourth blood agar culture was used for quality control (section 2.2.2). After 48hr of incubation, each of the three blood agar cultures was harvested with a sterile cotton wool swab, and the bacteria transferred to 20ml of Todd-Hewitt Broth (T.H.B.)(Oxoid, Unipath Ltd., U.K.)(appendix 9) in a sterile plastic container. The broth cultures were incubated anaerobically at 37°C for 24hr. Three separate 20ml volumes of T.H.B. were used to prepare the bacterial inocula for ease of handling during the subsequent preparation stages described below. A Gram's film was prepared from the fourth blood agar plate for quality control purposes and in addition colonial morphology was noted (section 2.2.2).

2.4.3 Harvesting of bacterial cultures

Each of the broth cultures (3 x 20ml total) was centrifuged at room temperature for 10min at 3,000r.p.m. in an M.S.E. Centaur 2 bench centrifuge (M.S.E., U.K.). The supernatant was discarded and each pellet resuspended by vortexing for 30s at the highest power setting in 1ml of T.H.B. The three 1ml suspensions of each strain were combined to give a single 3ml suspension which was then transferred to a preweighed sterile plastic bijoux bottle (L.I.P. Equipment and Services Ltd., U.K.) and centrifuged at 4,500r.p.m. for 20min at room temperature. The supernatants were discarded and the weight of the bijoux plus the wet bacterial pellet noted using an Ohaus Galaxy 160

electronic balance (Ohaus, Germany). The wet weight of each bacterial pellet was then calculated and the pellets resuspended with T.H.B. to give 100mg/ml (wet w/v). Fifty microlitre volumes of each of the 100mg/ml (wet w/v) bacterial suspensions were then 100-fold serially diluted in T.H.B. to give the working suspension (as noted in the relevant section of each chapter).

This procedure for the preparation of the initial bacterial inoculum was adopted to standardise the density of the initial inoculum, and ensure that the cells were predominantly in the stationary phase of growth.

2.5 Determination of the viable count of initial inocula

2.5.1 Preparation and inoculation of the initial inoculum

The viable count of each initial inoculum was determined by first making 100-fold serial dilutions - 50µl into 4.95ml - using 135mM Potassium Chloride (KCl)(Analar grade, BDH Chemicals Ltd., U.K.) as the diluent (KCl was employed for preparing the dilutions since it was the diluent of choice in the fermentation experiments in later chapters). Fifty microlitre volumes of the serial dilutions of each bacterial suspension were then inoculated (in ascending order of concentration) to blood agar plates using a spiral plater (Spiral Systems Inc., U.S.A.)(figure 2.4 shows the spiral plater). The inoculated blood agar plates were incubated anaerobically for 48hr at 37°C.

The spiral plater inoculates a predetermined volume (50µl) of bacterial suspension onto an agar plate in an archimedean spiral pattern (Chestnutt, 1992). The stylus of the spiral plater which deposits the bacterial suspension on the agar was sterilised with a solution of Titan Chlor-Tabs disinfectant (Lever Industrial Ltd., U.K.) at standard concentration

Figure 2.4 **The spiral plater**

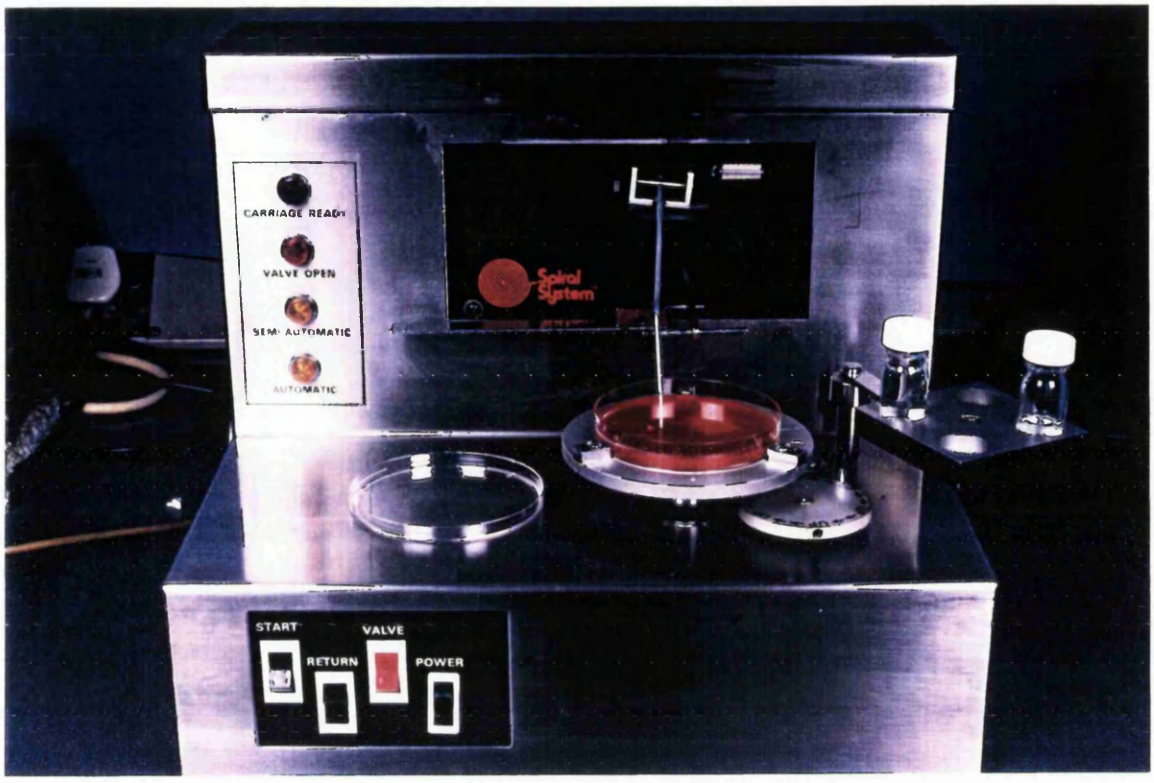
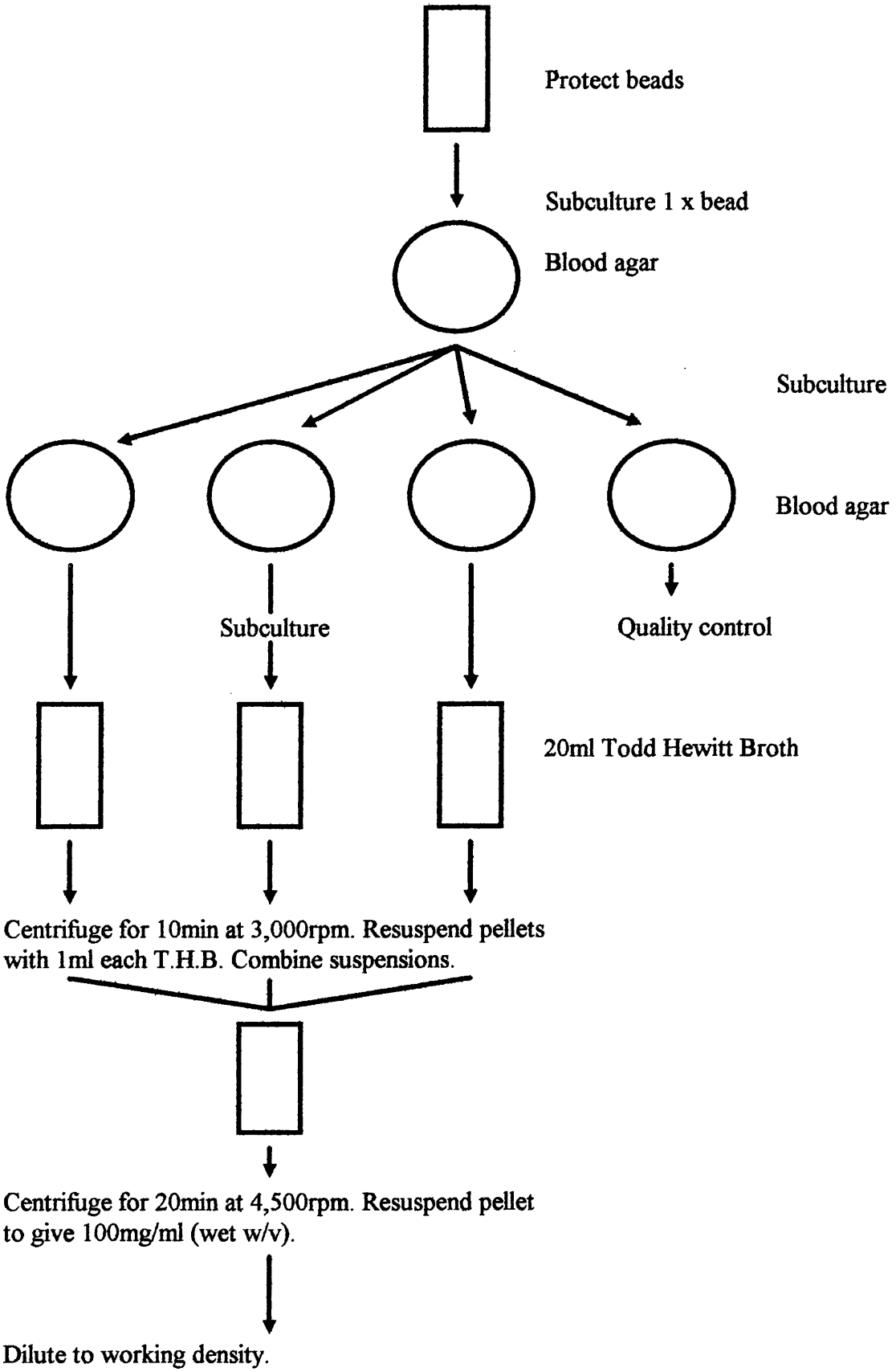


Figure 2.5 **Flow diagram for preparation of initial bacterial inoculum**



(2 tablets per litre giving 1,000p.p.m. chlorine) - initially and between inoculating each series of bacterial suspensions - followed by a thorough sterile distilled water wash.

Concurrently with the determination of the viable counts of each initial inoculum, a loopful of each culture was inoculated to blood agar and also to M.S.B. for *S. mutans* and Rogosa agar for *L. casei*, to verify the purity and identity of the cultures as described in section 2.2. Figure 2.5 shows the standard protocol for preparation of the initial inoculum.

2.5.2 Calculation of the number of viable counts

After incubation the number of bacterial colony forming units (c.f.u.) on each plate was counted using a colony counter (model CC50, Don Whitely Scientific, U.K.). Plates were selected for counting that had more than 10 but less than 300 colonies present.

The viable counts present on each plate in the original bacterial suspension were calculated as follows:-

(i) Multiply the number of c.f.u. on a plate by the inverse of the dilution inoculated to that plate (i.e. if a dilution of 10^{-4} was inoculated to the plate then the c.f.u. would be multiplied by 1×10^{4}) to give an estimate of the viable counts per 50µl.

(ii) Multiply the product of step (i) by 2 to convert from c.f.u. per 50µl to c.f.u. per 100µl, since this was the volume of the initial inoculum.

(iii) If more than one dilution of a particular bacterial suspension (e.g. 10^{-3} and 10^{-4}) both yielded counts of between 10 and 300, then steps (i) and (ii) were followed and then the mean calculated to give an estimate of c.f.u. per 100µl.

Chapter 3 The Millicell-HA model system for growing bacterial films

3.1 Introduction

3.1.1 The model systems currently available for the demineralisation of tooth roots

A variety of model systems are available which could be used for the study of root surface caries. Possible model systems include gnotobiotic animals, the incorporation of root sections into intra-oral appliances, the exposure of root sections to pure organic acids or bacteria *in vitro* and the culture of bacterial films upon root sections in a 'model' mouth (as described in section 1.4). However, each of the model systems has its disadvantages as well as its advantages, as has already been discussed in chapter 1.

Due to the perceived problems in the use of the past and current model systems for the study of demineralisation of human tooth root sections, it was decided to develop a new model system. It was felt that the experimental model should permit bacteria to be grown as surface films and allow close control over environmental factors. In addition it should be easier to handle and more flexible to use, compared with the model systems currently available. The Millicell-HA tissue culture insert manufactured by Millipore Ltd., U.K., was deemed to be suitable as a potential growth vessel for a novel *in vitro* model, since bacteria could be grown as a film on the surface of a membrane filter which also separated the intact cells from the liquid nutrient reservoir. The tissue culture inserts also appeared to be relatively easy to handle and therefore it was decided to test the Millicell-HA tissue culture inserts for the growth of films containing *S. mutans, L. casei* and *A. viscosus* in both pure and mixed culture since these three species appear to be related to root surface caries development and have also been employed by other workers; e.g.

Nagaoka *et al.* (1995), Clarkson *et al.* (1987), Kaufman, Pollock & Gwinnett (1988) and Switalski & Butcher (1994).

3.1.2 A description of the Millicell-HA model system

The Millicell-HA tissue culture insert is composed of an impact polystyrene cylinder, with an internal diameter of 10mm and a height of 10mm. One end of the cylinder is sealed with a surfactant-free membrane filter (Millipore HATF) with a pore size of 0.45μm and is constructed from mixed esters of cellulose. The working area of the membrane filter is $0.6cm^2$. The insert has three legs of 1mm height which raise it to allow fluid to circulate beneath it (see figures 3.1 and 3.2). Millicell-HA tissue culture inserts are supplied pre-sterilised by the manufacturer in individual blister packs.

To provide a nutrient source for cells growing within the Millicell-HA inserts, each was placed in a 60mm polystyrene petri dish (Nunc, Denmark) which contained sufficient Todd Hewitt Broth to provide a layer 1mm deep; i.e. a layer which made contact with the underside of the membrane filter, but which did not rise above it. A volume of broth giving a depth greater than 1mm would have risen above the level of the membrane filter and given a column of liquid within the insert. This would have been undesirable since the intention was to culture the bacteria as a surface film at the gas-liquid interface, not as free-growing organisms in fluid culture. Figures 3.3 and 3.4 show a Millicell-HA unit *in situ* with its broth reservoir.

Figure 3.1 **Diagram of a Millicell-HA tissue culture insert** (not to scale)

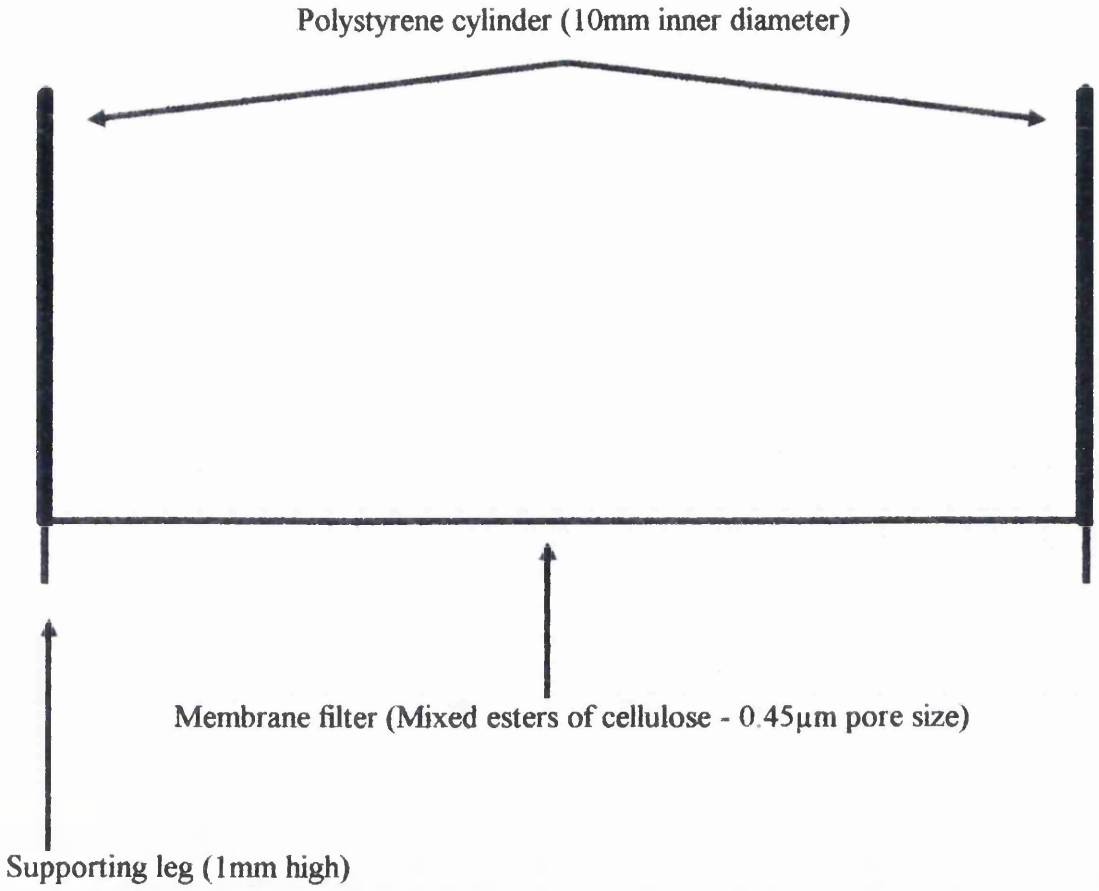


Figure 3.2 **Photograph of a Millicell-HA tissue culture insert and petri dish**

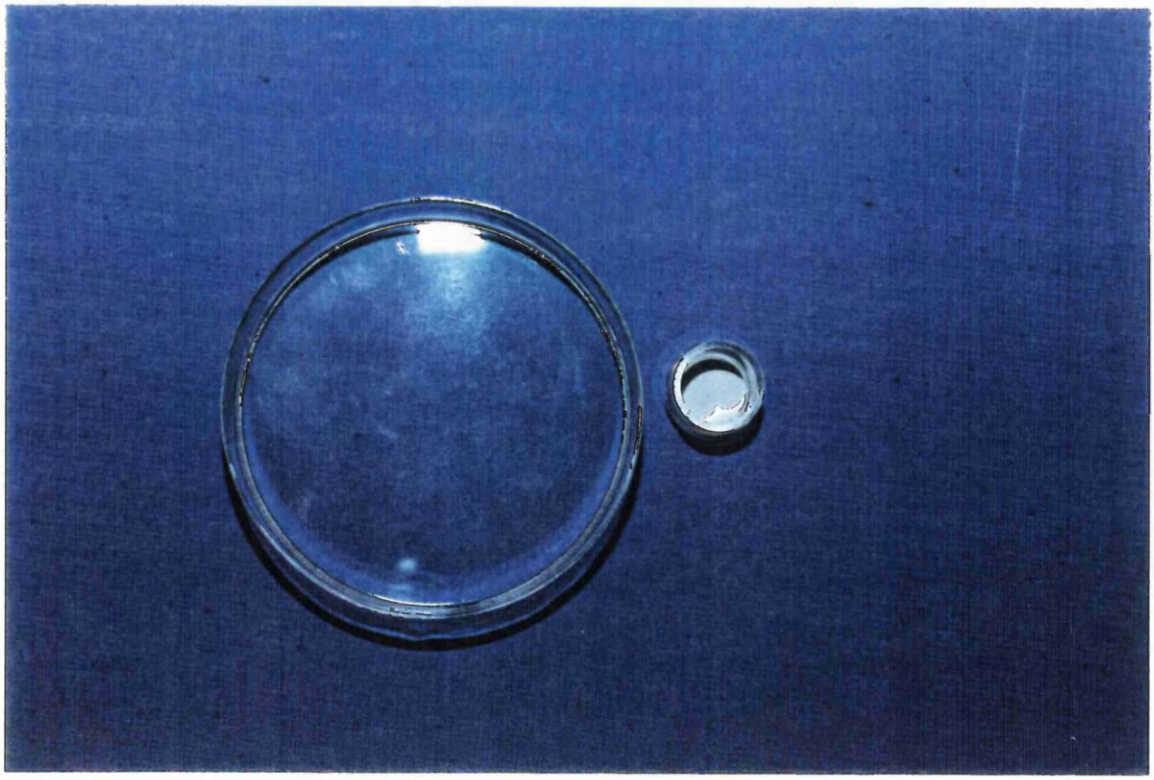


Figure 3.3 **Diagram of Millicell-HA unit in use** (not to scale)

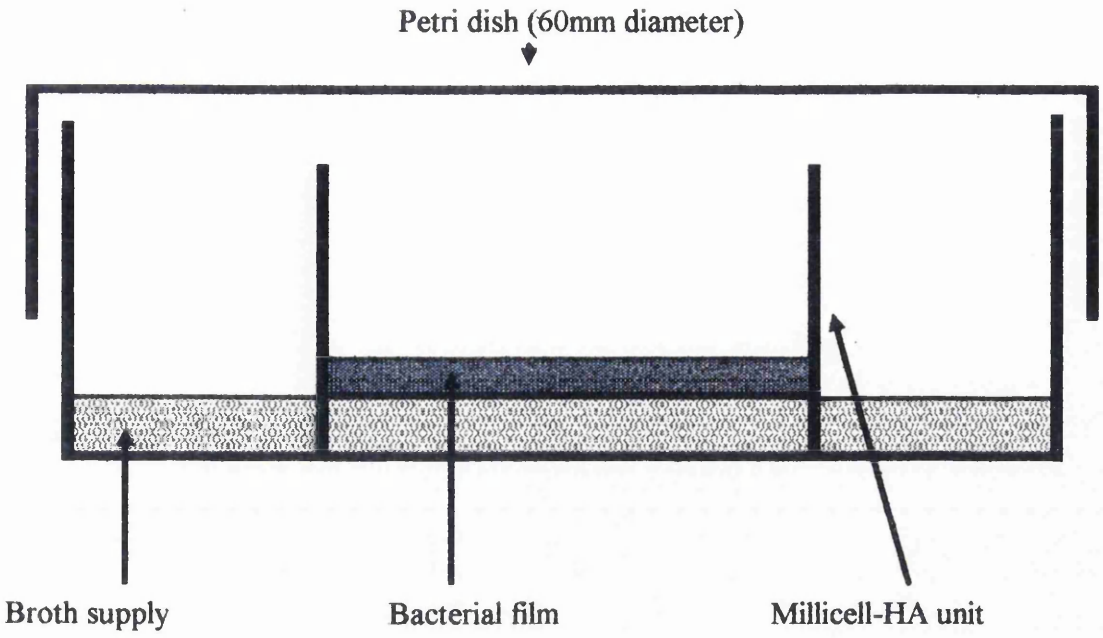
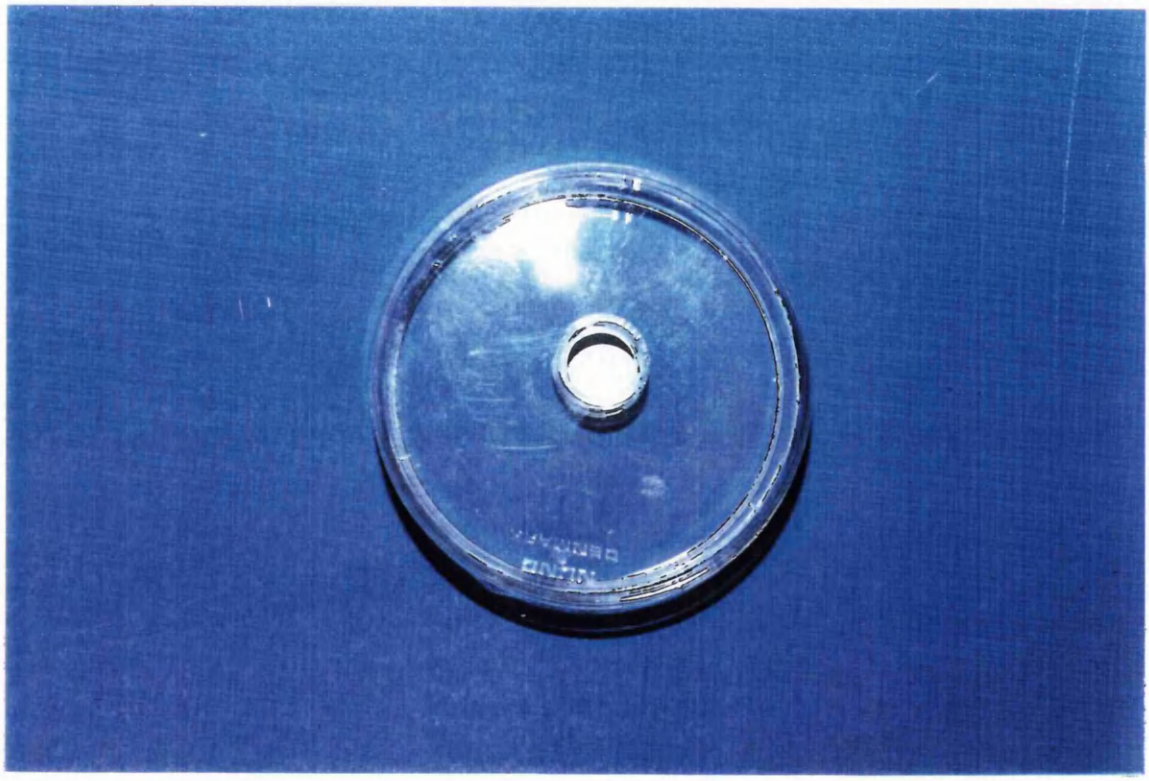


Figure 3.4 **Photograph of a Millicell-HA unit *in situ***



3.2 Exploration and evaluation of the potential of the Millicell-HA tissue culture insert as an *in vitro* model system

3.2.1 Preliminary experiments to determine the viability of bacterial cultures in Millicell-HA tissue culture inserts and determination of the optimal conditions for counting the number of bacteria present on the Millicell-HA membranes

(a) Introduction

Having selected the Millicell-HA tissue culture insert as a potential culture vessel for an *in vitro* system to model root surface caries, it was decided to perform a simple experiment which would demonstrate whether a selection of human oral bacteria thought to be involved in the disease process - i.e. *Streptococcus mutans, Lactobacillus casei* and *Actinomyces viscosus* (see chapter 1) - would grow as surface films.

A bacterial plaque is usually regarded as a layer of micro-organisms adherent to a surface which will require some mechanical force to detach it from that surface. Since an estimate of the number of viable bacteria present upon the Millicell-HA membrane surface is required to demonstrate viability and to allow a quantitative comparison between the populations of the bacterial species used, it was decided to investigate the optimum period of washing required to retrieve the maximum number of bacteria from the filter membrane surface. This information would then be incorporated into the design of future experiments to ensure optimal harvesting of bacteria. The aims of these experiments, therefore, were to examine whether 3 species of bacteria inoculated into the inserts would remain viable and multiply during a short period of incubation and also to explore the optimum period of washing with a vortex mixer to retrieve the maximum number of bacteria from the membrane and cylinder surfaces.

(b) Materials and methods

Organisms and growth conditions employed The bacterial cultures employed in this experiment were *S. mutans* NCTC 10449, *L. casei* NCTC 6375 and *A. viscosus* NCTC 10951. A 1mg/ml (wet w/v) pure suspension of each species was prepared as described in section 2.5.1. A quality control experiment was also performed, following the procedure detailed in section 2.5.2.

The culture vessels used in this experiment were Millicell-HA tissue culture inserts, each of which had been aseptically transferred to 3ml of T.H.B. in a 60mm polystyrene petri dish using pre-flamed forceps whilst working in a sterile-air cabinet (MDH Intermed, U.K.). Subsequently, working on the open bench, a 100µl volume of the *S. mutans* suspension (1mg/ml wet w/v as described in section 2.4) was inoculated into a Millicell-HA unit. This procedure was repeated for both *L. casei* and *A. viscosus* using a fresh sterile insert in each case.

The Millicell-HA units which had been inoculated with bacterial suspensions were incubated anaerobically for 48hr at 37°C. The broth in each petri dish was replaced with fresh medium (3ml) after 24hr. The Millicell-HA units were removed from the anaerobic incubator to perform the transfer of broth (this was performed aseptically on the open bench).

Determination of bacterial viable counts The number of c.f.u. present in the initial inocula was determined as described in section 2.5.1. At the end of the incubation period the number of bacterial colony forming units present in each Millicell-HA was determined as follows. The membrane filter was cut from the base of the Millicell-HA insert using a

sterile scalpel blade (number 11, Swann-Morton, U.K.). Each filter was then transferred to a 1ml volume of sterile 135mM KCl, using pre-flamed forceps. The polystyrene cylinder portion which remained of each Millicell-HA was transferred to 5ml of sterile 135mM KCl, also using pre-flamed forceps.

A 50µl sample of the KCl was taken at 0s to give a baseline count and then each filter was vortexed at the highest power setting for 60, 120, and 180s, whilst each cylinder component was washed for only 90s since fewer bacterial cells were expected to be adherent. Fifty microlitre volumes of the resulting bacterial suspensions were aspirated at the end of each wash period and then serially diluted 100-fold with sterile 135mM KCl. A 50µl volume of each serial dilution was inoculated onto a blood agar plate with a spiral plater. The inoculated plates were incubated anaerobically for 48hr at 37°C and then the number of c.f.u. at each dilution was calculated. The identity and cultural purity of the organisms washed from every filter and cylinder was also determined for quality control purposes (section 2.2). This experiment was performed on three separate occasions.

(c) Results

Figure 3.5 shows the mean number of colony forming units of *S. mutans* retrieved from the filter membranes after 0, 60, 120 and 180s of washing. The number of retrievable c.f.u. increased for 2min after which time further washing did not increase the retrievable c.f.u. significantly.

Figure 3.6 shows the mean number of colony forming units of *L. casei* retrieved from the filter membranes after 0, 60, 120 and 180s of washing. The number of retrievable c.f.u.

Figure 3.5 *S. mutans* NCTC 10449 colony forming units retrieved from a Millicell-HA inserts after 0, 60, 120 and 180s washing of filter membranes

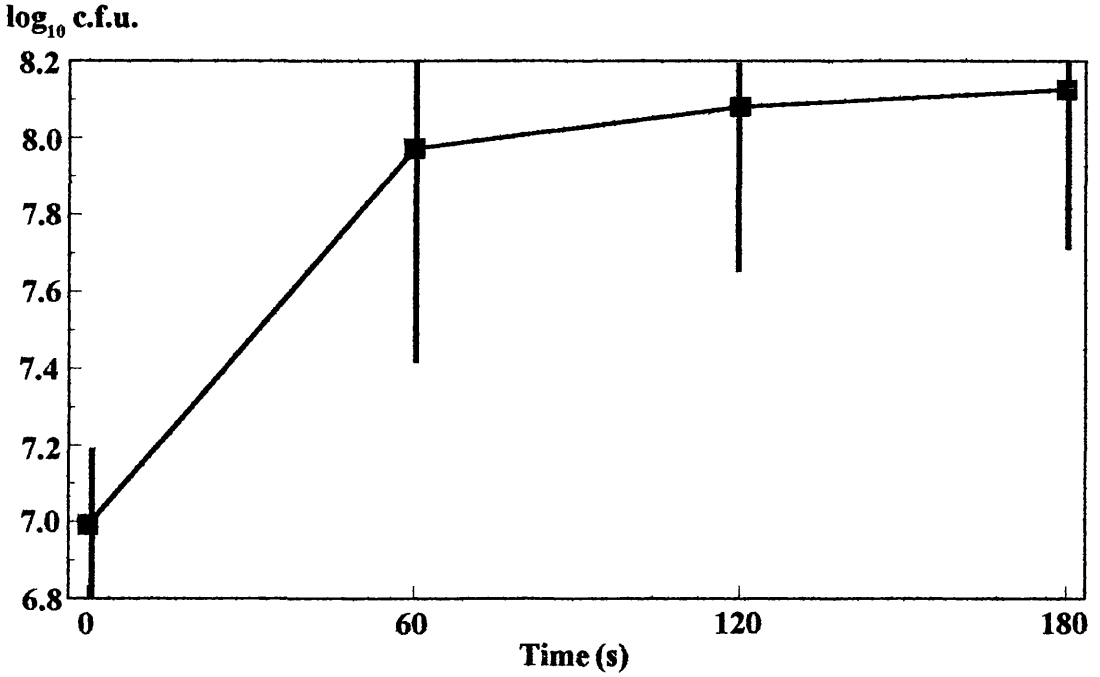
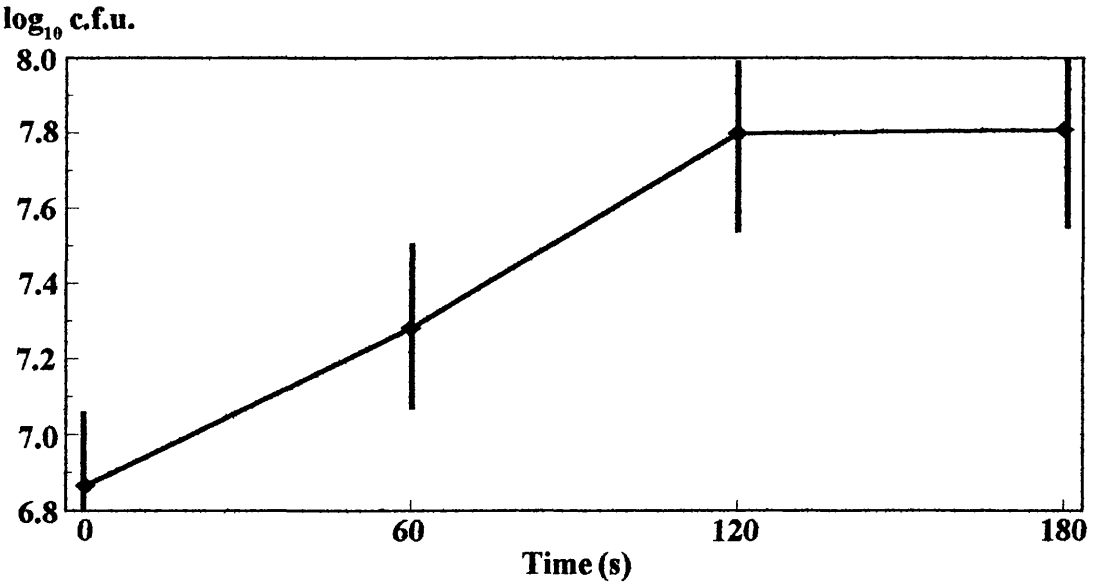
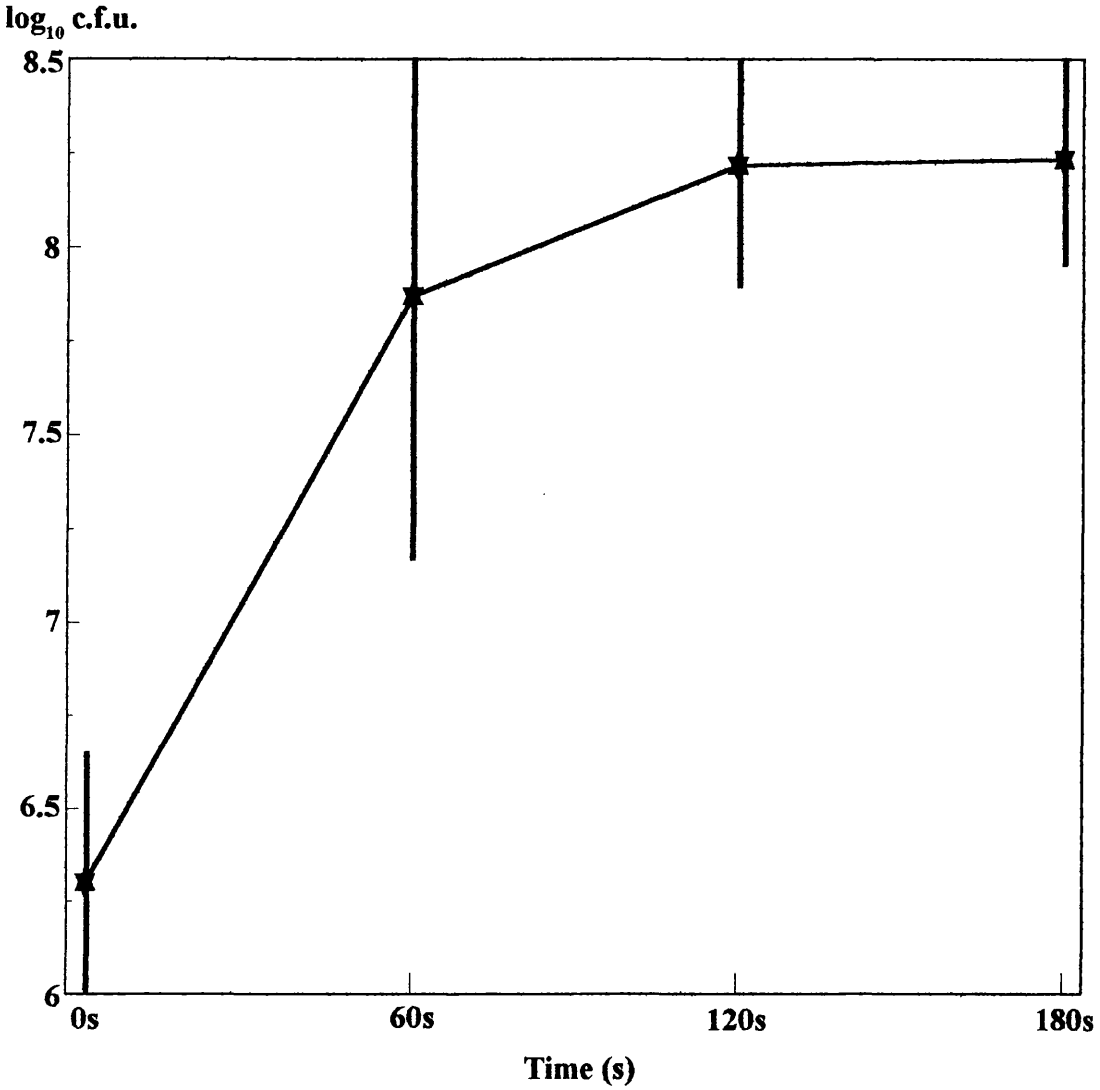


Figure 3.6 *L. casei* NCTC 6375 colony forming units retrieved from a Millicell-HA inserts after 0, 60, 120 and 180s washing of filter membranes



Error bars represent mean ± 1 S.D. n = 3

Figure 3.7 ***A. viscosus*** **NCTC 10951 colony forming units retrieved from a Millicell-HA inserts after 0, 60, 120 and 180s washing of filter membranes**



Error bars represent mean ± 1 S.D. **n = 3**

Table 3.1 **Colony forming units retrieved from Millicell-HA membrane filter and cylinder components**

Organism	Mean log_{10} c.f.u. inoculated (± 1 S.D.)	Mean log_{10} c.f.u. retrieved after washing of:- Filter (± 1 S.D.) (120 seconds)	Cylinder (± 1 S.D.) (90 seconds)	Snm of mean log_{10} c.f.u. from filter + cylinder (± 1 S.D.)
S. mutans NCTC 100449	7.956 (0.664)	8.081 (0.428)	7.691 (0.485)	8.230 (0.445)
L. casei NCTC 6375	7.792 (0.778)	7.798 (0.247)	7.742 (0.070)	8.079 (0.156)
A. viscosus NCTC 10951	7.138 (0.653)	8.217 (0.328)	6.755 (0.281)	8.227 (0.328)

n = 3

increased for 2min after which time further washing did not increase the retrievable c.f.u. significantly.

Figure 3.7 shows the mean number of colony forming units of *A. viscosus* retrieved from the filter membranes after 0, 60, 120 and 180s of washing. The number of retrievable c.f.u. increased for 2min after which time further washing did not increase the retrievable c.f.u. significantly.

Significantly more bacteria of each species were retrieved from the membranes after 180s of washing than after 0s ($p < 0.001$, one-way analysis of variance).

Figures 3.5 - 3.7 also show that the number of bacteria washed from the membranes increased during the first 2 minutes of vortexing but there was little increase after this. The number of c.f.u. of each species of organism recovered after 2min of vortex washing was greater than 89% of that recovered after 3min of washing. These results indicate that a minimum period of 2 minutes wash is required to retrieve a reproducible and significant proportion of the recoverable bacteria present upon the membrane surface.

Table 3.1 shows the mean total viable count of each organism washed from the Millicell-HA membrane filter, the corresponding cylinder component and the sum of the c.f.u. from the filter and cylinder combined. Although all of the viable counts at the end of the experiment were higher than those of the initial inoculum, the increase in bacterial c.f.u. over 2d of incubation was not statistically significant.

3.2.2 Determination of the optimum density of the initial bacterial inoculum for biofilm formation

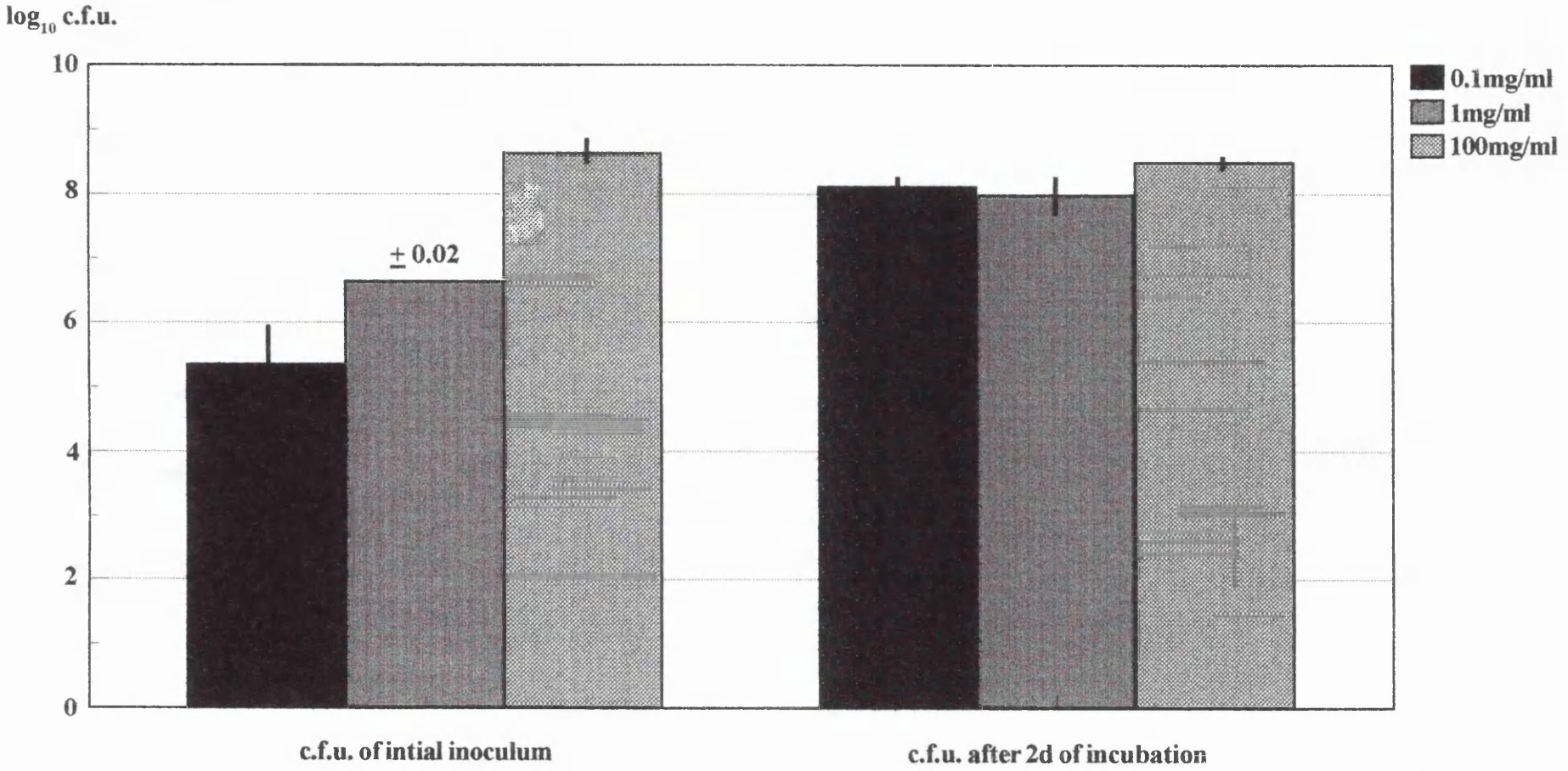
(a) Introduction

The results recorded in section 3.2.1 did not indicate any significant changes between the initial and final counts, although the reason for this was unclear. One possibility was that there is a maximum population density which Millicell-HA units can harbour and that the initial inoculum in section 3.2.1 was too close to this. For instance Donoghue & Perrons (1988) found no significant changes in plaque viable counts after 45hr (up to 90hr) of incubation *in vitro* in a model mouth. In addition Macpherson, MacFarlane & Stephen (1991) recorded a bacterial density of around 2.51 x 10^8 c.f.u./cm^2 on enamel surfaces exposed *in situ* for 2d in intra-oral appliances, which approximately equals the population density achieved in Millicell-HAs over the same time period. Therefore the current experiments were designed to determine whether bacteria would replicate in Millicell-HA units or whether the numbers would remain static whatever the initial inoculum. In addition the experiments would also demonstrate whether there was indeed a maximum density.

(b) Materials and methods

Organisms and growth conditions employed Cultures of *S. mutans* NCTC 10449, *L. casei* NCTC 6375 and *A. viscosus* NCTC 10951 were investigated and each was prepared to a density of 0.1, 1 and of 100mg/ml (wet w/v). A 100µl volume of each density of bacterial suspension was inoculated to a separate sterile Millicell-HA unit in T.H.B., which was incubated anaerobically for 48hr at 37°C. The T.H.B. was replaced with fresh medium after 24hr of incubation.

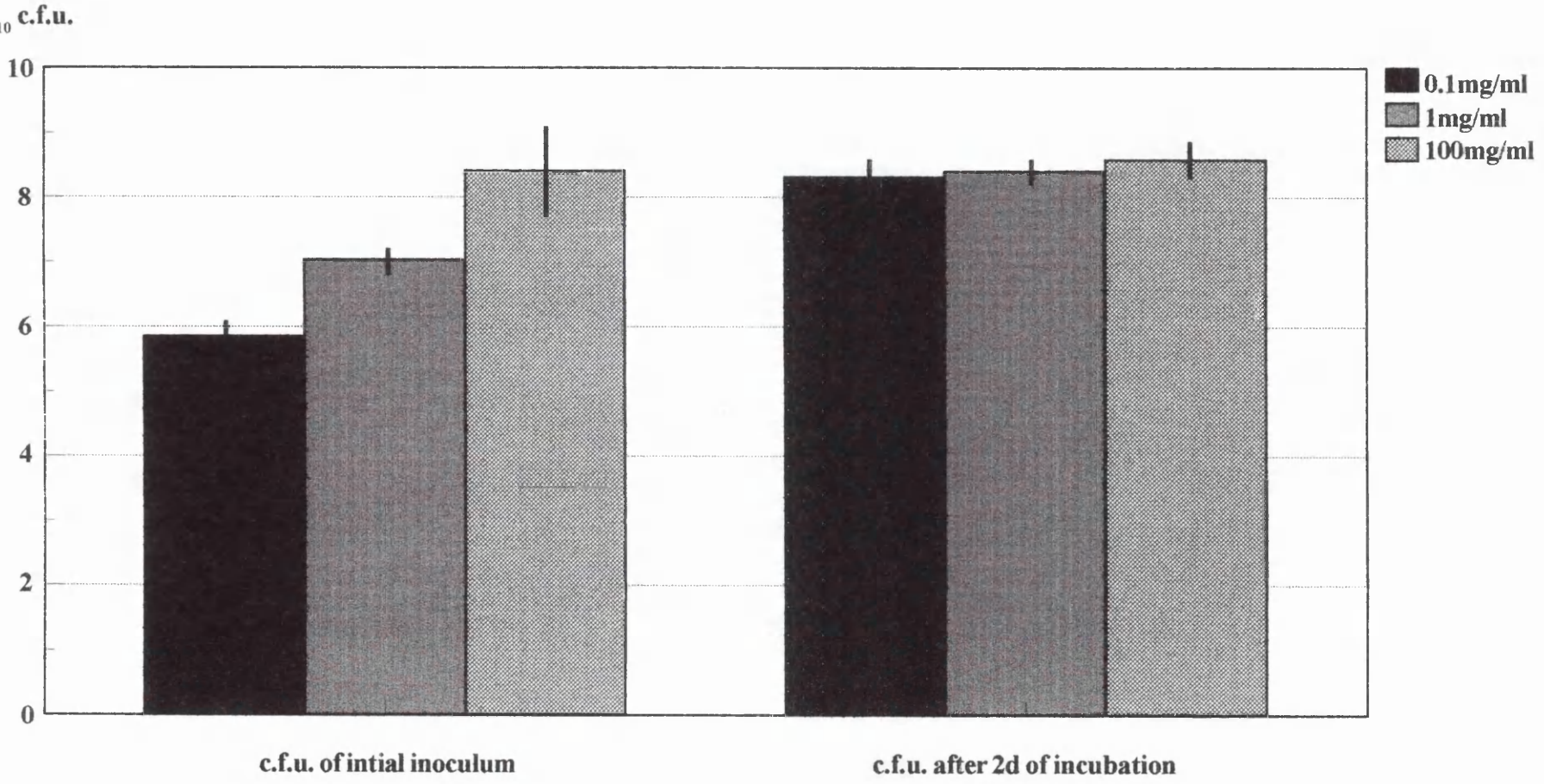
Figure 3.8 **The effect of different densities of initial inoculum on the final numbers of viable *S. mutans* NCTC 10449 after incubation for 2 days**



Error bars represent mean ± 1 S.D.

n = 3

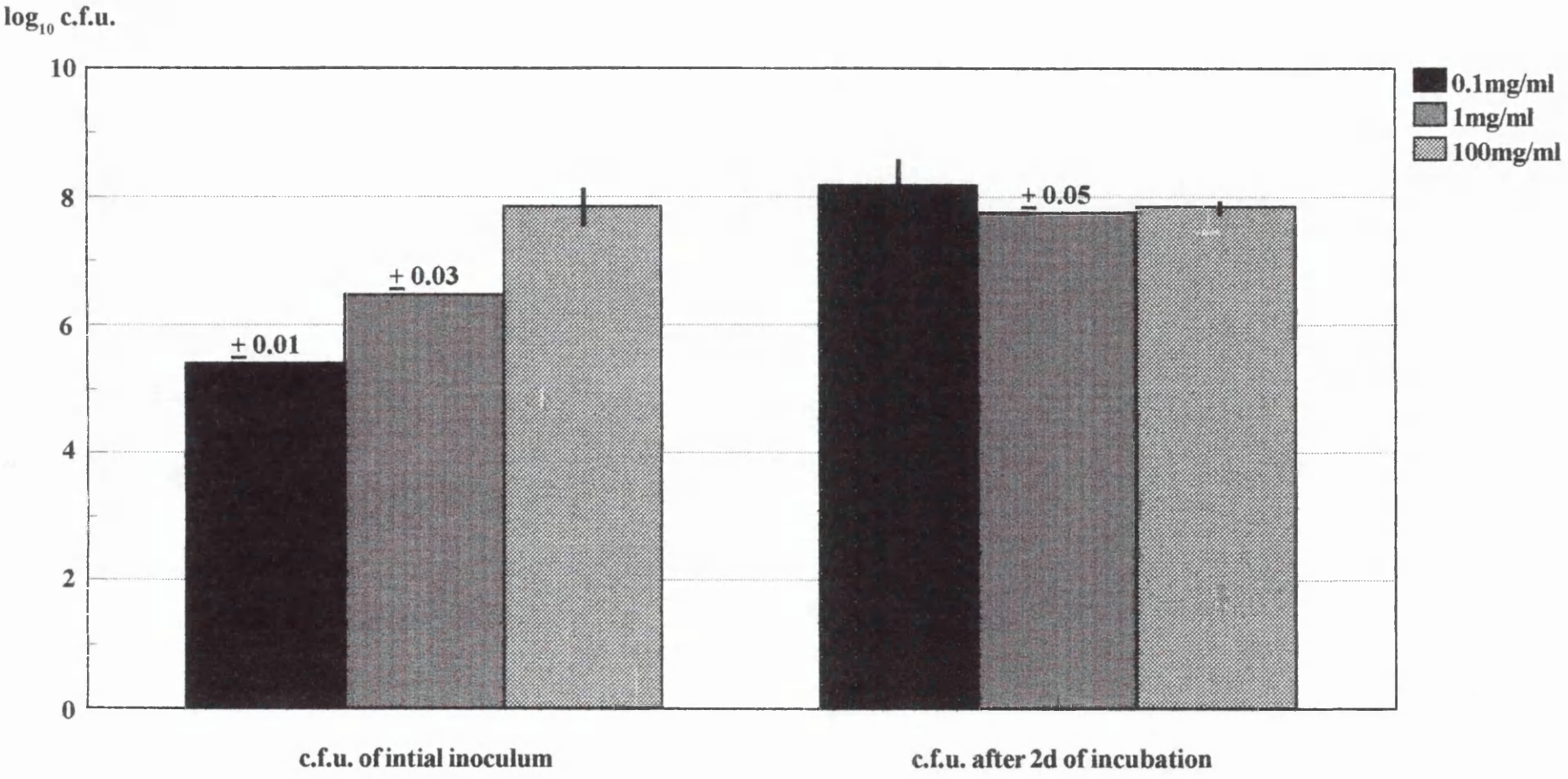
Figure 3.9 **The effect of different densities of initial inoculum on the final numbers of viable *L. casei* NCTC 6375 after incubation for 2 days**



Error bars represent mean ± 1 S.D.

n = 3

Figure 3.10 **The effect of different densities of initial inoculum on the final numbers of viable *A. viscosus* NCTC 10951 after incubation for 2 days**



Error bars represent mean ± 1 S.D.

n = 3

Determination of bacterial viable counts The viable counts and purity of the initial inocula and final biomass in each Millicell-HA were determined as indicated in section 3.2.1 (i.e. 2min vortexing). The experiment was performed on three separate occasions.

(c) Results

Figures 3.8, 3.9 and 3.10 show the mean number of c.f.u. (per 100µl) of *S. mutans*, *L. casei* and *A. viscosus* respectively for each of the three densities of initial inoculum and also the mean viable counts present in each Millicell-HA unit after 2 days' incubation. There was a significant increase in the number of c.f.u. retrieved from Millicell-HA units inoculated with bacteria at densities of both 0.1 and 1mg/ml ($p = 0.001$, one-way analysis of variance). However there were no significant differences in the number of bacteria present over time when the initial inoculum density was 100mg/ml ($p > 0.1$ for each species, Student's T-test). Furthermore there were no significant differences between the final viable counts that developed from each density of initial inoculum when the three species were compared ($p = 0.05$, one-way analysis of variance). The maximum density of recoverable bacteria developing from the initial inocula was approximately 1×10^8 per filter membrane (or about 1.7×10^8 c.f.u./cm^2).

3.2.3 The ability of mixtures of bacteria to grow on the Millicell-HA membrane surface

(a) Introduction

Previous reports have indicated that plaque associated with root surface caries lesions frequently contains *Actinomyces* species, *Streptococcus mutans* and lactobacilli, together with a range of other micro-organisms (especially streptococci)(Bowden, 1990). In light of the fact that root surface caries would appear to be associated with a complex mixture

of bacterial species, it was decided to discover whether mixtures of the test organisms could be grown together in the Millicell-HA tissue culture insert system or not. The ability of a mixture of bacteria to grow in this system is obviously of importance to its application to the modelling of the carious process which involves complex bacterial communities. In addition it was decided to extend the incubation time to determine whether the organisms could be sustained in Millicell-HAs for a further 2 days.

(b) Materials and methods

Organisms and growth conditions employed *S. mutans* NCTC 10449, *L. casei* NCTC 6375 and *A. viscosus* NCTC 10951 were examined and the initial inocula prepared to a density of 1mg/ml (wet w/v). Two-way mixtures of these suspensions were also prepared to a total cell density of 1mg/ml (wet w/v) by mixing equal volumes (0.5ml) to give suspensions of *S. mutans* + *L. casei*, *S. mutans* + *A. viscosus* and *L. casei* + *A. viscosus*. A 100µl volume of each species in pure culture and of the two-way mixtures was inoculated into a separate Millicell-HA insert resting in 3ml of T.H.B. Growth conditions were as employed previously except that the incubation period was extended to 4d.

Determination of bacterial viable counts The initial and final c.f.u. of the pure bacterial cultures were enumerated as before, as was their purity. The c.f.u. of the two-way mixtures present initially and at the end of the experiment was determined by washing the organisms from the filter membrane and cylinder components of each Millicell-HA and then inoculating 50µl volumes of dilutions prepared with the washes onto C.B.A. plates, which were then incubated as before. The colony count of each species present in the mixtures was calculated on the basis of colonial morphology on C.B.A., which was sufficiently distinctive. Initially the colony counts for *S. mutans* and *L. casei* were also

determined on M.S.B. and Rogosa agar, however since maximal retrieval was recorded with C.B.A.(see results below) it was decided that semi-selective media provided little extra useful data and so their use was discontinued. The experiments were performed on three separate occasions.

(c) Results

Table 3.2 shows a comparison of the mean number of bacterial c.f.u. present in the initial inocula as counted on C.B.A. (using colony morphology to differentiate between the species) with that obtained with semi-selective media (M.S.B. and Rogosa agar).There were no significant differences between the viable counts of *S. mutans* on M.S.B. compared with C.B.A. in mixed culture ($p > 0.1$, Student's T-test). However the counts of *L. casei* on Rogosa agar were significantly lower than on C.B.A. ($p < 0.05$ in both cases, Student's T-test).

Figure 3.11 shows the mean number of colony forming units that developed from each bacterial species when grown alone as a pure culture in Millicell-HA inserts over 4 days of incubation. The initial number of colony forming units of *A. viscosus* inoculated to Millicell-HAs was much lower than those of either *S. mutans* or *L. casei* but there were no significant differences between the final counts of the 3 species ($p = 0.636$, one-way analysis of variance). However the final number of c.f.u. of each organism was approximately 1×10^8 c.f.u. per unit (1.7×10^8 c.f.u./cm^2) and was significantly greater than the initial inoculum in each case ($p < 0.001$, one-way analysis of variance).

Figures 3.12, 3.13 and 3.14 show the mean number of each bacterial species present in 100µl of initial inoculum and in the Millicell-HA units after 4 days incubation, when the

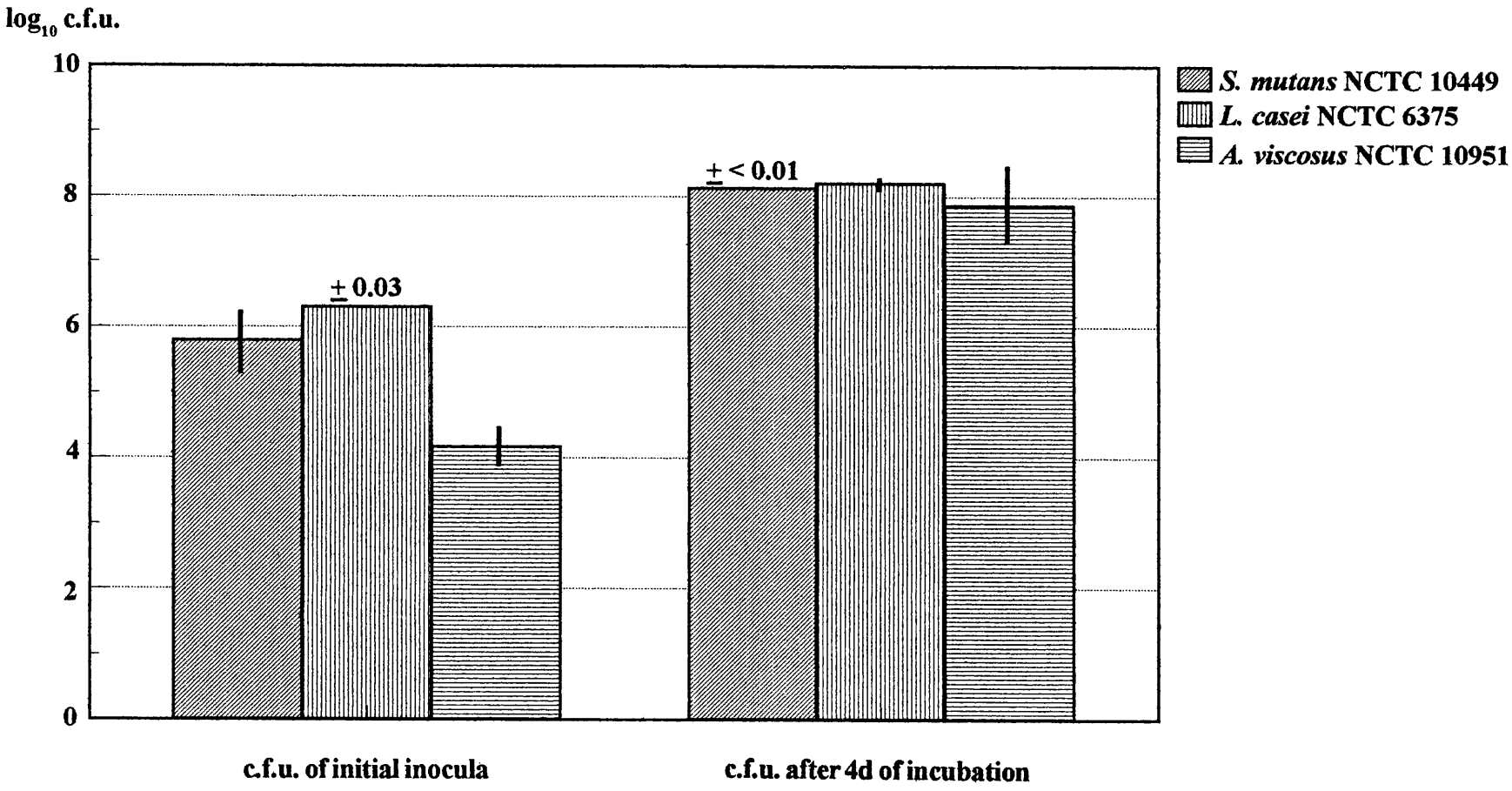
Table 3.2 **A comparison of the mean viable counts of *S. mutans*, *L. casei* and *A. viscosus* inoculated to Millicell-HA and then enumerated using Columbia blood agar and semi-selective media**

Organism	Mean $\log_{10}$ c.f.u. (± 1 S.D.) of initial inoculum counted using; Pure	Mixed C.B.A.	Rogosa	M.S.B.
S. mutans NCTC 10449	5.483 (0.112)	5.094 (0.252)	n.a.*	5.053 (0.131)
+				
L. casei NCTC 6375	6.296 (0.034)	6.030 (0.070)	5.738 (0.119)	n.a.
S. mutans NCTC 10449	5.483 (0.112)	4.902 (0.465)	n.a.	5.047 (0.063)
+				
A. viscosus NCTC 10951	4.168 (0.259)	3.745 (0.319)	n.a.	n.a.
L. casei NCTC 6375	6.296 (0.034)	5.919 (0.110)	5.497 (0.033)	n.a.
+				
A. viscosus NCTC 10951	4.168 (0.259)	3.834 (0.761)	n.a.	n.a.

* = not applicable

n = 3

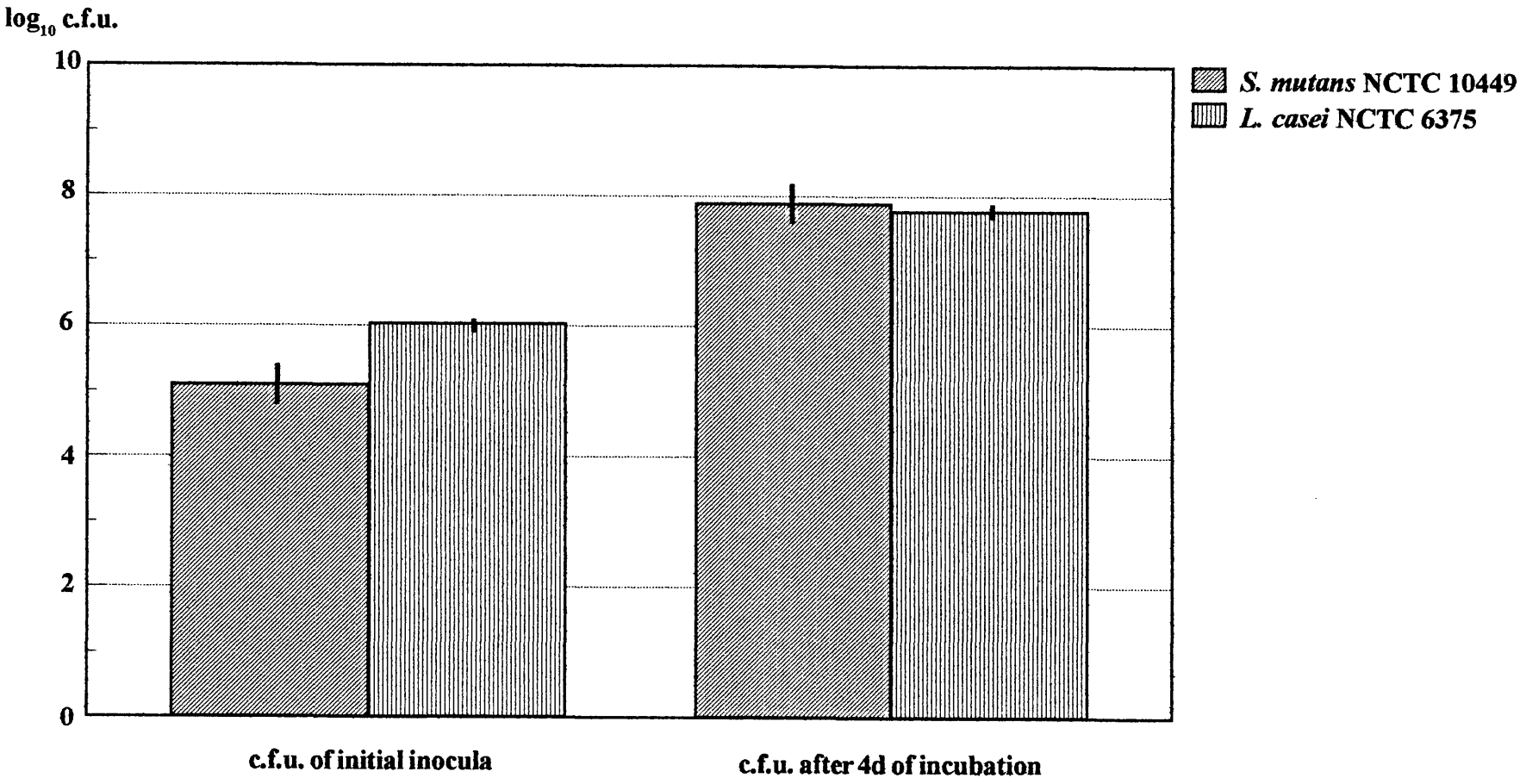
Figure 3.11 **The change in the numbers of *S. mutans* NCTC 10449, *L. casei* NCTC 6375 and *A. viscosus* NCTC 10951 when cultured separately for 4 days**



Error bars represent mean ± 1 S.D.

n = 3

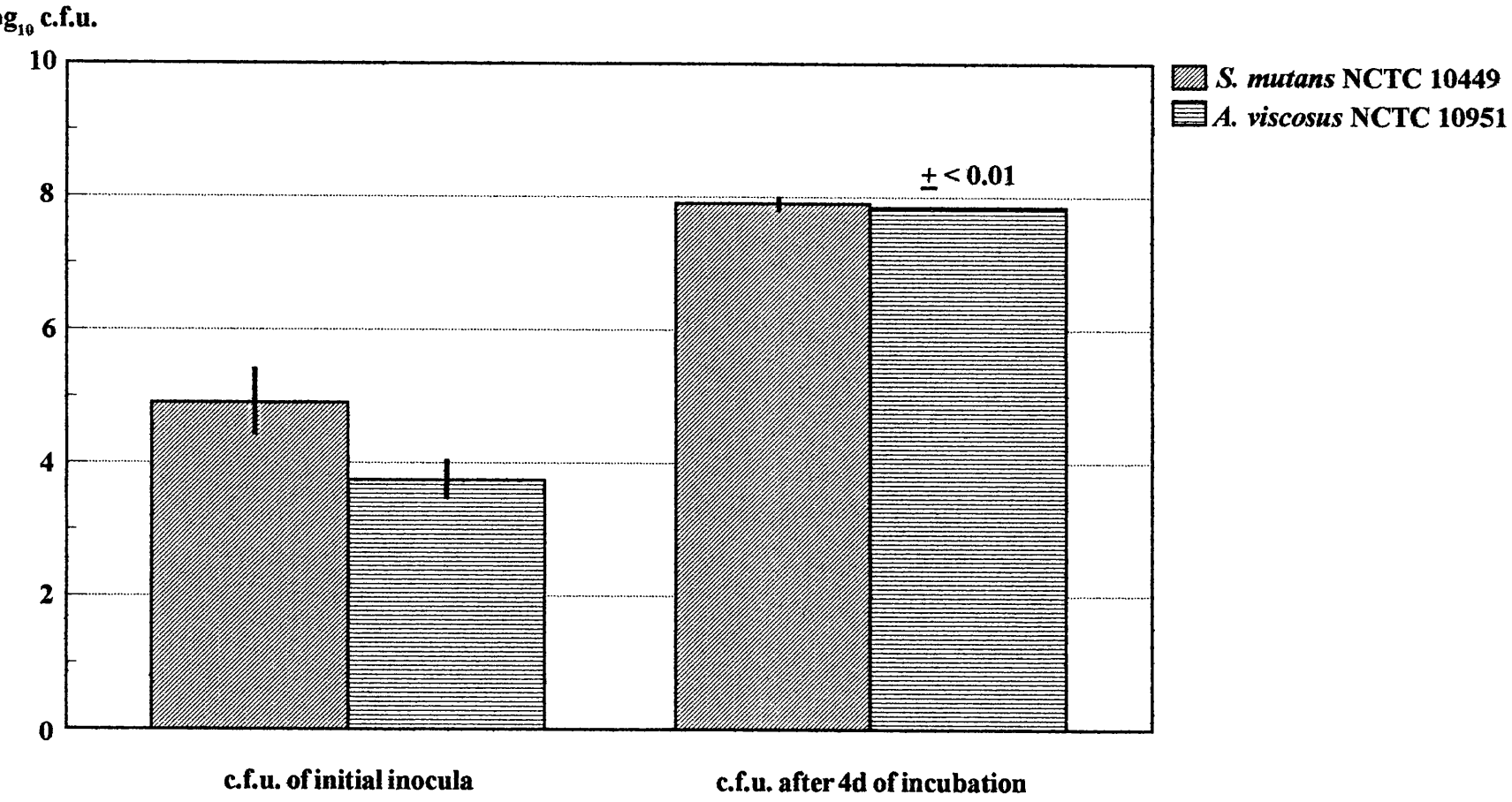
Figure 3.12 **The change in the numbers of *S. mutans* NCTC 10449 and *L. casei* NCTC 6375 when cultured together for 4 days**



Error bars represent mean ± 1 S.D.

n = 3

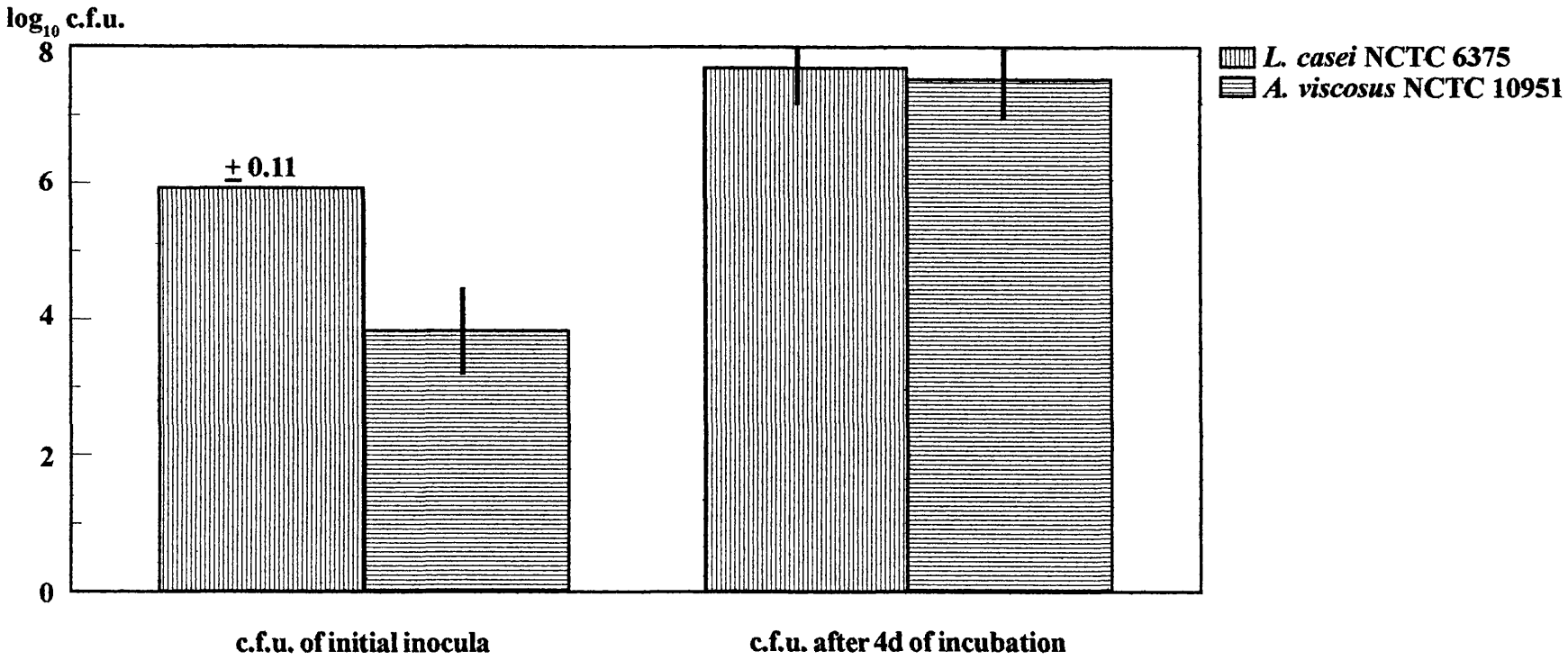
Figure 3.13 **The change in the numbers of *S. mutans* NCTC 10449 and *A. viscosus* NCTC 10951when cultured together for 4 days**



Error bars represent mean ± 1 S.D.

n = 3

Figure 3.14 **The change in the numbers of *L. casei* NCTC 6375 and *A. viscosus* NCTC 10951 when cultured together for 4 days**



Error bars represent mean ± 1 S.D.

n = 3

Millicell-HAs were inoculated with mixtures of *S. mutans* + *L. casei*, *S. mutans* + *A. viscosus* and *L. casei* + *A. viscosus* respectively. The number of viable bacteria increased significantly during the 4 days of incubation ($p < 0.001$, one-way analysis of variance) to a plateau of approximately 1×10^8 (1.7×10^8 c.f.u./cm^2) in each case. However there were no statistically significant differences between the final c.f.u. of each bacterial species when inoculated as pure cultures compared with two-way mixtures (figure 3.11 cf. figures 3.12 - 3.14)($p = 0.794$, one-way analysis of variance). Furthermore, the total viable counts (i.e. biomass) present in Millicell-HAs inoculated with mixtures of bacteria were no different from those inoculated with pure cultures ($p = 0.899$, one-way analysis of variance).

3.2.4 Examination of pure and mixed bacterial films in Millicell-HA tissue culture inserts by scanning electron microscopy

(a) Introduction

Bacteria initially colonise root surfaces as discrete micro-colonies which then proliferate to form confluent plaques (Nyvad & Fejerskov, 1987a). It was decided to study the growth of the experimental bacterial species on the surface of the Millicell-HA membrane filters to determine whether they are present, at the end of the incubation, as micro-colonies or as a plaque which covers the whole surface of the filter. In addition some information about the morphology of bacteria growing in this system would be obtained.

(b) Materials and methods

Organisms and growth conditions employed Pure cultures of the *S. mutans* NCTC 10449, *L, casei* NCTC 6375 and *A. viscosus* NCTC 10951 were prepared, inoculated into duplicate Millicell-HA units and incubated as described in section 3.2.2. In addition

a 100µl volume of a three-way mixture, which was prepared by mixing equal volumes (500µl) of each of the organisms, was also inoculated to duplicate Millicell-HAs and incubated as described previously.

Preparation of Millicell-HAs for microscopy At the end of the incubation period one of each pair of units was prepared for scanning electron microscopy in the following manner. The broth supply was aspirated from the petri dish in which each unit was sitting and discarded. Each Millicell-HA was then rinsed for 3 consecutive periods of 1hr by pipetting 3ml of sterile phosphate buffered saline (P.B.S.)(appendix 11) into the petri dish and incubating at 37°C in an orbital incubator. This wash step was performed to remove polypeptides and carbohydrates from the broth, which would have covered the surface of the bacterial film and masked its appearance. The final rinse was aspirated from each petri dish and discarded. The bacterial film was fixed by soaking (without agitation) for 1hr at 37°C in 3ml of 2.5% sterile filtered glutaraldehyde (Agar Scientific, U.K.) in P.B.S. which was introduced into the petri dish. The units were rinsed (without agitation) overnight at room temperature with a minimum of three volumes (3ml) of fresh P.B.S. The membrane filters were next separated form the cylinder components and dehydrated using a graded series of concentrations of ethanol (BDH, U.K.) - 50, 70, 90, 95 and 100%, ½hr per step. This was followed by controlled sublimation drying using Peldri II (Agar Scientific, U.K.) according to the manufacturer's instructions. The membrane filters were then affixed to individual scanning electron microscopy stubs using carbon adhesive discs and coated with a layer of gold using an Emscope SC500 gold coater (Emscope 2,000, U.K.). Finally the prepared samples were examined using a Cambridge 360 Scanning Electron Microscope (Cambridge Leica, U.K.). The second

Millicell-HA of each pair was employed for the determination of bacterial viable counts and cultural purity. This experiment was performed only once.

(c) Results

Table 3.3 shows the number of bacterial c.f.u. present in the initial inoculum and in the washings from the Millicell-HA inserts after 2 days of incubation. There was an increase in the viable counts over the 2d incubation period, with the final counts retrieved being in the range of 2.1×10^8 - 8.4×10^8 c.f.u./cm^2, except for the *A. viscosus* in mixed culture which only achieved a density of 2.1×10^7 c.f.u./cm^2.

Figure 3.15 shows a scanning electron micrograph of the surface of a Millicell-HA tissue culture insert membrane filter. The Millicell-HA membrane filter structure appears to be of globules linked by fibrils to form a dense interwoven mat. There appears to be no regular shape or size to the pores.

Figure 3.16 shows a representative scanning electron micrograph of the surface of a *Streptococcus mutans* plaque grown upon a Millicell-HA membrane filter. The bacterial cells appeared to have formed long chains in a relatively dense mat and the average cell diameter was 0.5µm.

Figure 3.17 shows a scanning electron micrograph of *Lactobacillus casei* grown upon a Millicell-HA membrane filter. The bacterial layer appears to be very sparse with only a few cells interwoven with the structure of the membrane filter. The cell length was variable but averaged 2.3µm, whilst the diameter averaged 0.5µm.

Table 3.3 **The number of bacterial cells inoculated to and recovered from Millicell-HA units during 2 days of incubation**

Organism	Initial inoculum (log_{10} c.f.u.)	Washings from Millicell-HA after 2 days (log_{10} c.f.u.)
S. mutans NCTC 10449	6.611	8.316
L. casei NCTC 6375	6.997	7.919
A. viscosus NCTC 10951	6.318	8.512
S. mutans NCTC 10449	6.182	7.874
+		
L. casei NCTC 6375	6.723	7.939
+		
A. viscosus NCTC 10951	5.342	6.904

n = 1

Figure 3.15 Scanning electron micrograph of the surface of a Millicell-HA tissue culture insert membrane filter (Magnification = 5,000x)

——————— 5μm

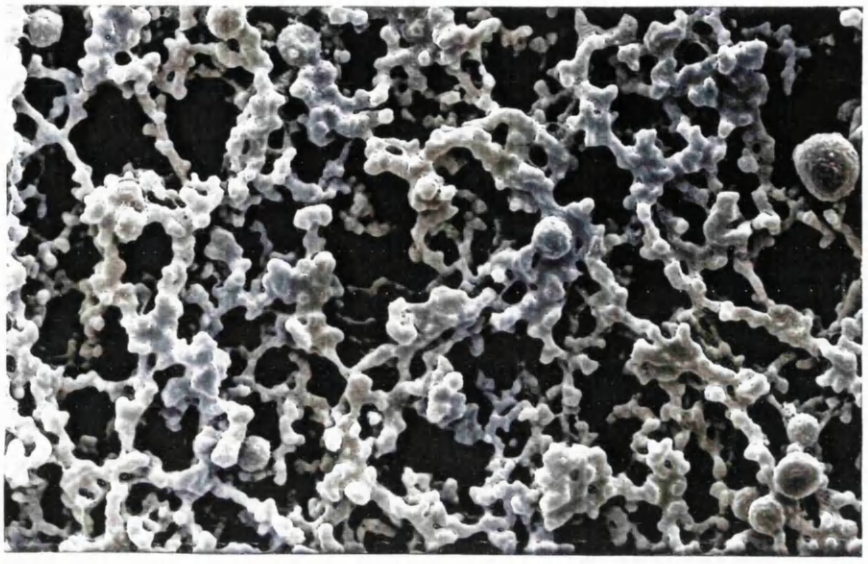


Figure 3.16 Scanning electron micrograph of a *Streptococcus mutans* plaque on the surface of a Millicell-HA filter (Magnification = 5,000x)

——————— 5μm

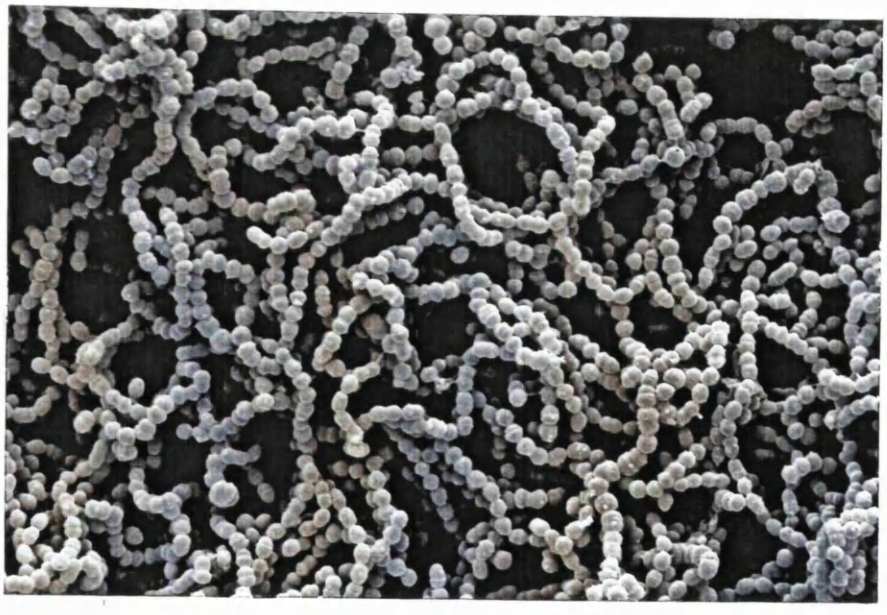


Figure 3.17 Scanning electron micrograph of a *Lactobacillus casei* plaque on the surface of a Millicell-HA filter (Magnification = 5,000x)

5μm

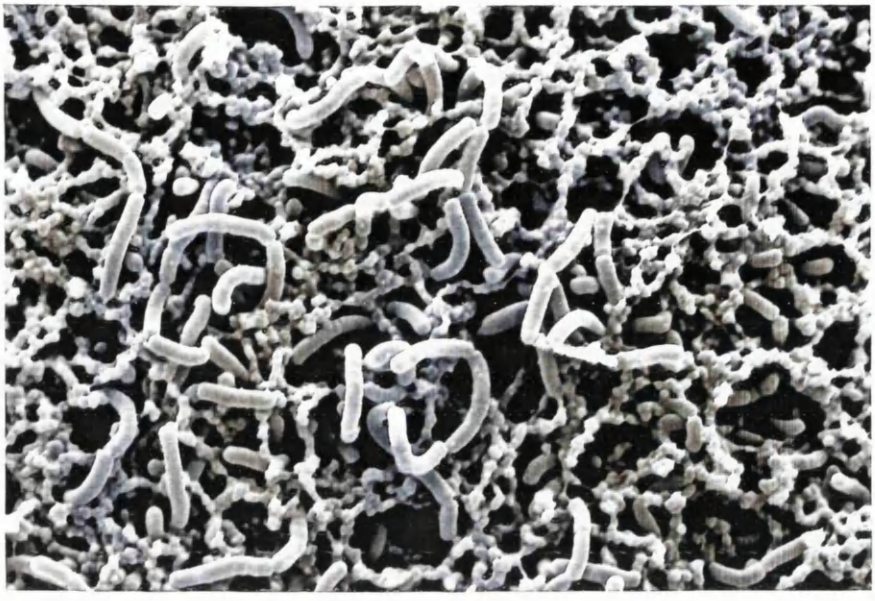


Figure 3.18 Scanning electron micrograph of an *Actinomyces viscosus* plaque on the surface of a Millicell-HA filter (Magnification = 5,000x)

5μm

Figure 3.19 Scanning electron micrograph of a mixed plaque containing *S. mutans*, *L. casei* and *A. viscosus* grown on the surface of a Millicell-HA filter

(Magnification = 5,000x) ——— 5μm

Figure 3.18 shows a typical scanning electron micrograph of the surface of an *Actinomyces viscosus* plaque grown upon a Millicell-HA membrane filter. The plaque appears to be composed of a mat of branching filaments and rods and whilst the cell length was too variable to make an average figure meaningful, the average cell diameter was 0.4μm.

Figure 3.19 shows a typical scanning electron micrograph of the surface of a mixed plaque of *S. mutans* + *L. casei* + *A. viscosus* grown upon a Millicell-HA membrane filter. The plaque appears to be composed of a mixture of bacterial cells (cocci, rods and filaments) with no segregation into micro-colonies, although chains of cocci predominate. Cell size and morphology are similar to that of the corresponding pure cultures.

3.2.5 Determination of bacterial viability in Millicell-HA tissue culture inserts with time

(a) Introduction

The development of root surface caries lesions is a slow process which takes place over a period of several months and is thought to involve bouts of demineralisation of tooth root structure interspersed with periods of remineralisation (Furseth & Johansen 1968). Klont & ten Cate (1991), who used pure organic acids at high concentrations *in vitro*, have reported that calcium was still being released from root surfaces after 28d. Thus dental caries, which is a chronic disease, will likely require a period of prolonged incubation with bacteria to achieve adequate demineralisation of human tooth root surfaces in an *in vitro* model system. Therefore it was decided to investigate bacterial viability over prolonged time in the Millicell-HA model.

(b) Materials and methods

Organisms and growth conditions employed *Streptococcus mutans* NCTC 10449, *L. casei* NCTC 6375 and *A. viscosus* NCTC 10951 were investigated and suspensions (100µl) of each species at a density of 1mg/ml (wet w/v) were inoculated into five separate Millicell-HA tissue culture inserts. The Millicell-HAs were incubated anaerobically at 37°C in 3ml each of T.H.B. which was replaced with fresh every day and the number of bacteria present in the Millicell-HAs was determined by culture at 1, 2, 6, 10 and 14d of incubation. One of the five Millicell-HAs was utilised at each time point to estimate the number of c.f.u. present. In addition samples of the wash from the filters and cylinders were tested for quality control purposes. The experiment was performed on three separate occasions.

(c) Results

Figure 3.20 shows the mean number of *S. mutans* viable counts inoculated to Millicell-HA units and then retrieved after 1, 2 and 6 days of growth. After a substantial initial increase of 100-fold to a plateau of approximately 2.5×10^8 per filter membrane (4.2×10^8 c.f.u./cm^2) there were no statistically significant differences between the viable counts retrieved on day 1 and thereafter ($p = 0.269$, one-way analysis of variance). It was found that the broth surrounding each Millicell-HA unit inoculated with *S. mutans* had become contaminated (turbid) between the third and fourth days of incubation. Subsequent analysis of the contaminated broth using the quality control protocol described in section 2.2 showed that the contaminating organism was *S. mutans*. Therefore, this part of the experiment was abandoned after 6d of incubation.

Figure 3.20 The number of bacteria retrieved at different time points from Millicell-HAs inoculated with *S. mutans* NCTC 10449 and cultured for a period of 6 days

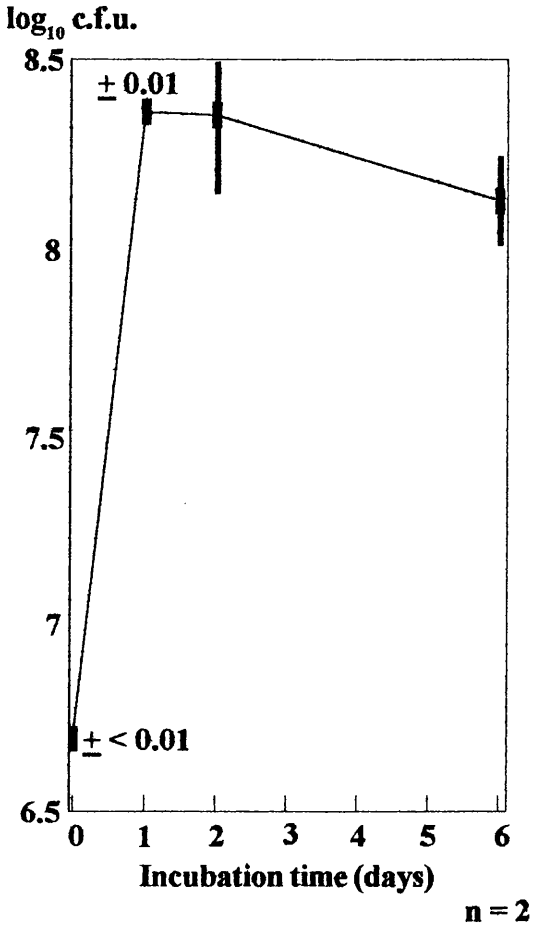
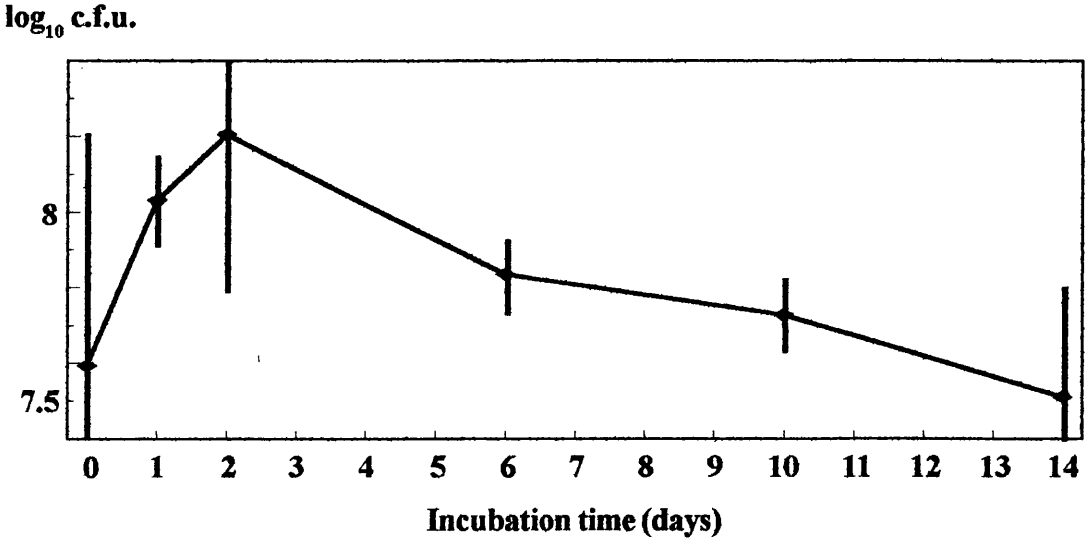
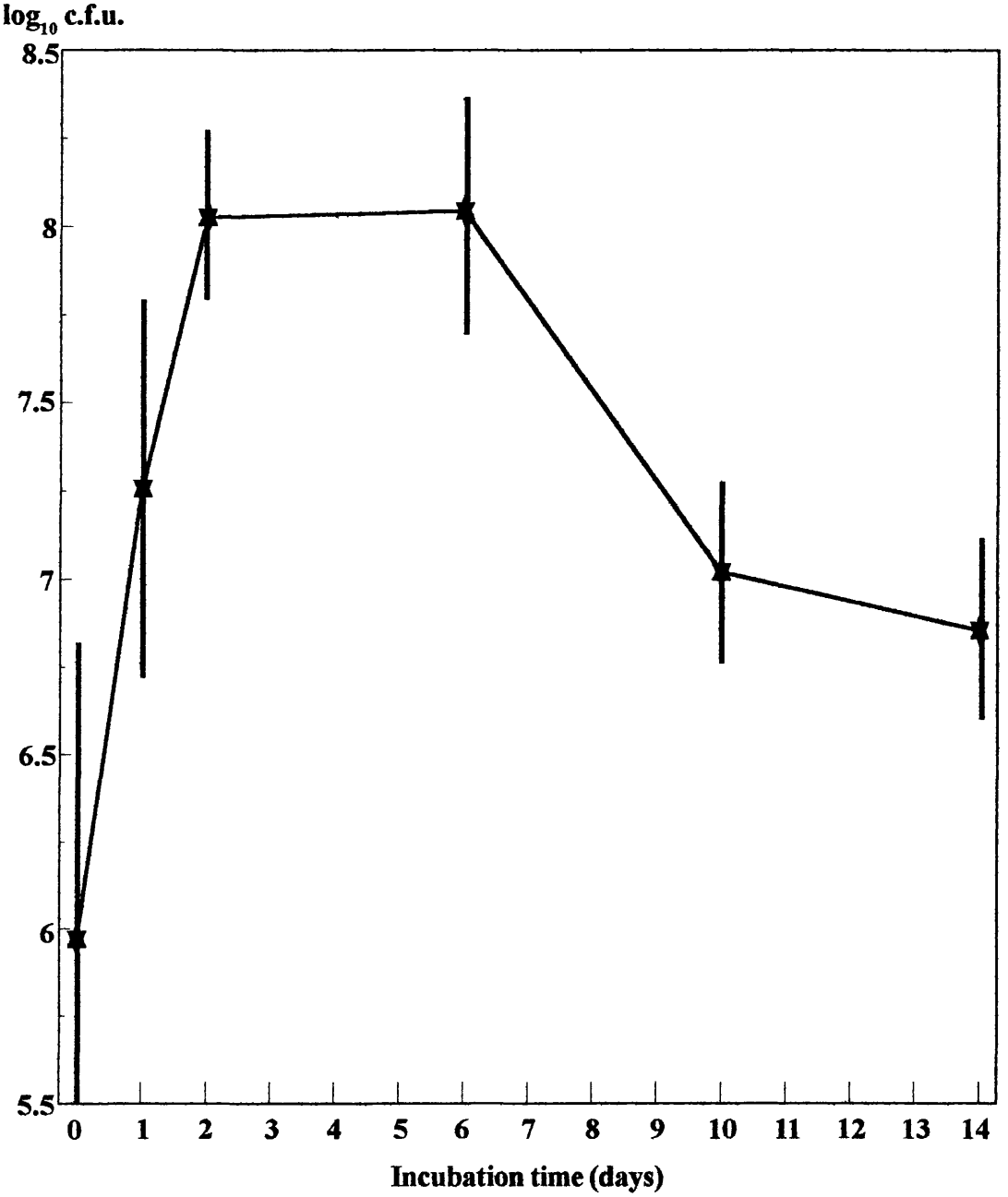


Figure 3.21 The number of bacteria retrieved at different time points from Millicell-HAs inoculated with *L. casei* NCTC 6375 and cultured for a period of 14 days



Error bars represent mean ± 1 S.D. n = 3

Figure 3.22 **The number of bacteria retrieved at different time points from Millicell-HAs inoculated with *A. viscosus* NCTC 10951 and cultured for a period of 14 days**



Error bars represent mean ± 1 S.D. **n = 3**

Figure 3.21 shows the mean number of *L. casei* viable counts inoculated to Millicell-HA units and then retrieved after 1, 2, 6, 10 and 14 days of growth. The viable counts increased by 5 fold to a maximum of approximately 2 x 10^8 per filter membrane (3.16 x 10^8 c.f.u./cm^2) but then declined between 2d and 14d to a density that equalled the initial inoculum. There were no significant differences between the viable counts retrieved on each day of sampling ($p = 0.280$, one-way analysis of variance).

Figure 3.22 shows the mean number of *A. viscosus* viable counts inoculated to Millicell-HA units and then retrieved after 1, 2, 6, 10 and 14 days of growth. The viable counts increased substantially between day 0 and day 2, by 100-fold, to a plateau of approximately 1 x 10^8 per filter membrane (1.66 x 10^8 c.f.u./mm^2) which remained constant between days 2 and 6. However after day 6 (up to day 14) there was a decline in the number of c.f.u. retrieved. Overall there were significant differences in viable counts between days 0 and 14 ($p = 0.001$, one-way analysis of variance), although there was a period of stability between days 2 and 6 when there was no significant difference between the number of c.f.u. ($p = 0.5$, Student's T-test).

3.3 Discussion and conclusions

3.3.1 Preliminary experiments to determine the viability of bacterial cultures in Millicell-HA tissue culture inserts and determination of the optimal conditions for counting the number of bacteria present on the Millicell-HA membranes

In the experiments described in section 3.2.1 it was demonstrated that bacteria could be cultured in Millicell-HA tissue culture inserts without apparent adverse effects. The bacterial viable count at the end of the incubation period was determined by first

separating the membrane filter from the supporting collar and then washing each with diluent. It was felt that washing the filter and its collar separately would give the optimal retrieval of bacteria since there was a possibility that the diluent might not be able to generate sufficient force to shear the bacteria from the filter surface due to disruption of the fluid flow by the collar. Millward & Wilson (1989) employed vortex mixing to retrieve *Streptococcus sanguis* cells from the surface of cellulose nitrate membrane filters after culturing for up to 3d. Therefore it was felt that the vortex mixer would be suitable to retrieve the majority of cells from the surface of the Millicell-HA membrane filters.

In addition to increasing the potential efficiency with which bacteria can be removed from the Millicell-HA inserts, separating the membrane from the collar allowed a comparison of the relative number of bacteria attached to the membrane compared to the collar. In this experiment 71% of *S. mutans* were retrieved from the membrane compared with 29% from the collar. The relative data for *L. casei* were 52% and 48%, whilst those for *A. viscosus* were 98% and 2%. A possible reason for the high number of lactobacilli associated with the collar is that *L. casei* grew as a relatively non-adherent layer on the filter surface and separation of the filter and collar disturbed the cells; this is further supported by the relatively low numbers of cells seen in the scanning electron micrographs. Furthermore *L. casei* has been reported as poorly adherent to enamel, root and mucous membrane surfaces in the mouth (Marsh & Martin, 1992).

One aim of the current experiments, apart from determining whether bacteria could be grown in the Millicell-HA inserts, was to ascertain the optimum length of time that each filter should be vortexed to achieve the maximum possible retrieval of bacteria. When the data were examined it was found that although the number of retrieved bacteria

increased up to 180s of vortexing, over 90% of the viable counts were retrieved within the first 120s, with less than 10% being retrieved in the final 60s. In light of the results of section 3.2.1 it was decided to vortex both filter membrane and collar for 120s to ensure efficient retrieval of bacteria and to estimate viable counts in all future experiments using this model system.

In vitro model systems have been described for the development of bacterial plaques upon various surfaces, some of which expressed their data as viable counts per unit area. For instance Li & Bowden (1994) recorded 5.12 x 10^6 *S. mutans* BM71 and 2.77 x 10^6 *L. casei* BM225 c.f.u./cm^2 on mucin-conditioned glass surfaces exposed to bacterial monocultures in a chemostat for 20hr. Furthermore Millward & Wilson (1989) recorded viable counts of 2 x 10^7 *S. sanguis* c.f.u./cm^2 on cellulose nitrate filters which had been incubated on the surface of Wilkins Chalgren agar plates. The viable counts retrieved from Millicell-HA membrane filters during the current experiments were 2.0 x 10^8, 1.0 x 10^8 and 2.5 x 10^8 c.f.u./cm^2 for *S. mutans*, *L. casei* and *A. viscosus* respectively. The viable counts retrieved from the current experiments were less than 10-fold greater than those recorded by Millward & Wilson, suggesting that there were no major differences between the two. The substantial differences in experimental design between the current study and that of Li and Bowden (e.g. growth surface, culture vessel and growth medium) may well explain the near 100-fold difference in viable counts recorded. However Macpherson, MacFarlane & Stephen (1991) recorded total viable counts of 2.5 x 10^8 c.f.u./cm^2 on enamel surfaces exposed *in situ* in intra-oral appliances for 48hr which was comparable with the current data and indicates that Millicell-HA inserts may be able to sustain films of similar density to those found *in situ*.

3.3.2 Determination of the optimum density of the initial bacterial inoculum for biofilm formation

The second set of experiments (section 3.2.2) was designed to examine the effect of inoculating different densities of bacterial suspension into the model system. This was done to discover if an increase in the density of the initial inoculum could increase the final bacterial density achieved or whether there was a maximum value, as suggested by the data of Donoghue & Perrons (1988). In addition it was hoped to determine the best inoculum to employ in future experiments. When an initial inoculum of either 0.1 or 1 mg/ml (wet w/v) was employed for all three bacterial species the viable counts at the end of the incubation period were significantly higher. There was, however, no observable increase between initial and final viable counts when an inoculum of 100mg/ml was employed. This tends to suggest that there is a limit of some kind upon the maximum bacterial population density which the model can sustain. In addition when the data for viable counts retrieved from membranes in subsequent experiments were converted to c.f.u./cm^2 it was found that, in general organisms reached a maximum density of between 1.0×10^8 and 7.9×10^8 c.f.u./cm^2 for each species which was comparable with the previous experiments in this chapter and therefore with those of Millward & Wilson (1989) and Macpherson, MacFarlane & Stephen (1991), although higher than that of Li & Bowden (1994).

There are a variety of possible reasons for why Millicell-HA inserts can only support a certain density of bacterial mass, none of which is exclusive of the others. The most obvious reason is limitation of an essential trace nutrient; either due to its depletion or due to it being unable to diffuse through the plaque to reach the most distal cells. However, it was not clear whether this occurred or not and would require further study.

In addition there is possibility that the organisms may secrete a pheromone as they grow which is used to indicate population density and could eventually trigger metabolic changes such as stationary phase when a threshold is passed. This phenomenon of 'Quorum sensing' has been reviewed recently by Swift *et al.* (1996). Whatever the underlying reasons, the current study indicates that a bacterial population inoculated to Millicell-HA units will multiply until it achieves the maximum possible density, unless it was already at that maximum density when inoculated.

3.3.3 The ability of mixtures of bacteria to grow on the Millicell-HA membrane surface

As expected from the experiments described in sections 3.2.1 and 3.2.2, axenic cultures of *S. mutans*, *L. casei* and *A. viscosus* showed a significant increase in viable counts from baseline, but there were no differences between the final viable counts of the three species. When each of the three species was grown in binary culture with either of the other two species as described in section 3.2.3, the viable counts once again increased significantly from baseline. There were no significant differences between the final c.f.u. of each species either when grown as pure or mixed cultures; i.e. not only was the total biomass equivalent in units containing pure cultures compared with those containing mixed cultures, but the biomass of the individual species did not differ significantly either. Beckers & van der Hoeven (1984) found that when *S. mutans* and *A. viscosus* were co-infected into gnotobiotic rats, they had the same doubling times as when mono-infected. However, although the growth rate in rats was unaffected when the bacteria were in mixed populations, the final viable count of *A. viscosus* was a little lower than in pure cultures, a reduction which was not noted with *S. mutans*. The authors attributed this difference in final counts of *A. viscosus* to possible competition for nutrients.

The data determined in section 3.2.3 are in broad agreement with those of Beckers & van der Hoeven (1984); i.e. there was no significant interaction between the species although there was a minor reduction in final counts compared with the pure cultures. Glenister *et al.* (1988) employed a chemostat rather than laboratory animals to study the growth of complex mixtures of bacteria and found that addition of mucin led to increased *A. viscosus* counts, whilst those of *S. mutans* and *L. casei* were unchanged (only showing an increase compared with other organisms when the growth pH was low). This gives a somewhat different picture to the data of Beckers & van der Hoeven (1984) or this thesis since mucin was not employed by either. In addition Herles *et al.* (1994), using a chemostat flow-cell, found that *S. mutans* and *S. sanguis* predominated whilst *A. viscosus* viable counts were reduced to very low levels. These studies suggest that the three bacterial types can be co-cultured *in vitro*, although modification of environmental factors such as flow rate, nutrient content or pH could alter the balance of different species (e.g. Donoghue & Perrons 1991). However, since *S. mutans*, *L. casei* and *A. viscosus* could be co-cultured in the Millicell-HA model system apparently as effectively as any of the other model systems it appeared that it had potential for investigating the growth and interactions of oral bacterial species.

3.3.4 Examination of pure and mixed bacterial films in Millicell-HA tissue culture inserts by scanning electron microscopy

In addition to information from viable counts it was considered useful to determine visually how the species interacted. Scanning electron micrographs of the surfaces of two-day mixed cultures indicated that, under the conditions employed, the bacterial

species grew together as a relatively homogeneous mixture with no evidence of discrete micro-colonies. In addition the cell morphology of the bacteria in mixed culture was comparable with the respective morphologies in pure culture. Both of the above points suggest that there is little or no antagonistic interaction between the species. In addition the findings described in section 3.2.3 and also those of Beckers & van der Hoeven (1984) tend to confirm this conclusion.

Simion *et al.* (1994 and in 1995) found that membrane filters, used for guided tissue regeneration, when exposed to the oral environment were colonised by a bacterial plaque; the earlier study revealed plaques composed mainly of rod-shaped and filamentous organisms, whereas the latter showed a preponderance of cocci and short rods. Visually the plaques recorded by Simion *et al.* (1994 and 1995) were similar to each other and also to the mixed plaques produced during the current experiments. However pure culture biofilms formed by *S. mutans* in the current study appeared different to those developed by Zampatti, Roques & Michel (1994) on bovine enamel since there was no evidence of cells coalescing to form clusters possibly because in Zampatti, Roques & Michel's study the cells were exposed to a stream of medium supplemented with sucrose at intervals whereas during the current experiment they were incubated statically with T.H.B.

3.3.5 Determination of bacterial viability in Millicell-HA tissue culture inserts with time

Since the three species were capable of growing in Millicell-HAs and giving similar plaques to those produced by other workers on various surfaces, it was decided to extend the period of incubation (section 3.2.5) as caries induction *in vitro* would

probably require an extended period of incubation. However it was at this stage that a flaw in the model system became apparent. *Streptococcus mutans* was found to be capable of somehow escaping the Millicell-HA tissue culture inserts and contaminating its own broth supply. Since a great deal of care was taken not to generate aerosols when inoculating the inserts and none of the broth supplies for *L. casei* or *A. viscosus* became contaminated, the obvious conclusion was that *S. mutans* grew through either the pores of the membrane filter or penetrated manufacturing flaws (e.g. at the junction between the filter and its supporting collar). Manufacturing flaws can probably be discounted for the same reason as aerosol generation during inoculation; contamination did not occur with either *L. casei* or *A. viscosus*.

An attempt was made to examine transverse sections of Millicell-HA filter membranes for the presence of *S. mutans* cells within the filter structure. Unfortunately, the material from which the filters were composed - cellulose esters - did not lend itself to the sectioning procedure for light microscopy since it tended to crack and disintegrate. Nor did it take up stain adequately to make it discernible for transmission electron microscopy purposes. Bacterial invasion of human tooth root tissue (e.g. Schüpbach, Lutz & Guggenheim, 1992 and Nagaoka *et al.*, 1995) and of guided tissue regeneration filters (Simion *et al.*, 1995) has been described and it seems the most likely explanation for the *S. mutans* results. However, both dentinal tubules in human tooth roots and the pores in guided tissue regeneration are of larger diameter than the pores in Millicell-HA filter membranes (> 1μm and 5 - 20μm (Schüpbach, Guggenheim & Lutz, 1990 and Simion *et al.*, 1994 respectively) compared with 0.45μm) which made the apparent ability of *S. mutans* to penetrate the membrane structure something of a surprise. Nevertheless *S. mutans* has been demonstrated to alter its shape from coccus to rod

when the ratio of bicarbonate or borate to potassium in the environment is changed (Tao, MacAlister & Tanzer, 1993) and it is possible that environmental changes might have led to a sufficient change in shape to allow its passage through the membrane filter.

A one-off experiment was performed to determine whether only *S. mutans* could contaminate its broth supply or whether this was a feature of other streptococci too, since this would have implications for the long-term use of the Millicell-HA model system. It was found the *Enterococcus faecalis* penetrated the filter membrane in 2d, whilst the other strains employed (*S. sobrinus* NCTC 10922, *S. sanguis* NCTC 7863 and a wild-type isolate of *S. mutans*) did so in 4d.

Thus it seemed that since streptococci could penetrate the membrane filters within a few days the Millicell-HA inserts were unsuitable to model root surface caries development. However the Millicell-HA model had many positive features; reproducible films of the test species could be cultured, the units were relatively simple to handle and the fluid phase could be sampled or replaced at will. Therefore it was decided to search for an alternative model which would retain the positive features but overcome the problem of bacterial penetration of the filter membrane.

In conclusion, the experiments described in chapter 3 have shown that a model system for developing bacterial plaques *in vitro* with a membrane filter as the growth surface is practical. Three oral organisms which are believed to play an important role in root surface caries development, *Streptococcus mutans*, *Lactobacillus casei* and *Actinomyces viscosus*, were successfully grown in pure and mixed cultures in this model system. As an *in vitro* model system, Millicell-HA tissue culture inserts were relatively easy to handle

and did not require elaborate preparation or equipment. However the inserts were not able to prevent *S. mutans* from spreading into the broth supply and so it was decided to continue development of the system with a growth vessel that could prevent the spread of this organism.

Chapter 4 The Ultrafree-CL *in vitro* model growth system

4.1 Introduction

In spite of the problems which occurred with Millicell-HA tissue culture inserts in chapter 3 it was felt that growth of bacterial films on filter membranes could still be a useful alternative to the model systems currently available. Inoculation was simple and weakly adherent organisms could be cultured without being washed away by a stream of fluid nutrient medium. Separation of bacteria from the nutrient source means that it should be simple to supply various different types of nutrients. Furthermore, it should be possible to analyse the waste medium for dissolution products from tooth mineral without disrupting the bacterial film or contaminating the medium with bacterial cells. After some investigation it was decided to test the Ultrafree-CL ultrafiltration unit, supplied by Millipore U.K. Ltd., which incorporated a membrane filter with a pore size of 0.22μm.

4.1.1 Description of the Ultrafree-CL ultrafiltration unit

The culture vessels chosen for the experiments which follow were Ultrafree-CL ultrafiltration units supplied by Millipore Ltd., U.K. These units consist of two polypropylene cylinders; an upper filter component which is seated partially within a centrifuge tube component (figure 4.1). One end of the upper filter component is sealed with a Durapore membrane filter (Millipore Ltd., U.K.), constructed of polyvinylidene difluoride (P.V.D.F.) with pores of 0.22μm diameter, whilst the other end is sealed with a press-fit cap. The working area of the membrane filter is 80mm^2. The filter component is partially inserted into the centrifuge tube component and a rim of greater diameter at the open end of the filter component prevents it sliding further than a predetermined

distance into the centrifuge tube. These units are not supplied sterile by the manufacturer but can be sterilised with ethylene oxide.

In order to culture bacteria in the Ultrafree-CL unit, a bacterial suspension was pipetted into the filter component to form a layer on the upper surface of the membrane filter. Meanwhile a volume of broth was pipetted into the centrifuge tube such that when the filter component was inserted, the broth just contacted the underside of the membrane filter without rising above its level; this was determined by trial-and-error to be 5.05ml. An air (and dust) tight seal between the filter and centrifuge tube components was formed by a smear of sterile Vaseline. Figure 4.2 is a diagram and figure 4.3 a photograph of the components of an Ultrafree-CL unit assembled and in use.

4.2 Exploration and evaluation of the potential of Ultrafree-CL ultra-filtration units as an *in vitro* model system for growing bacterial films

4.2.1 A preliminary experiment to determine whether bacteria can grow in Ultrafree-CL ultrafiltration units and to determine the optimum number of serial washes for retrieval of bacteria

(a) Introduction

An initial experiment was performed to determine the ability of *S. mutans, L. casei* and *A. viscosus* to survive and grow in Ultrafree-CL units. In addition the optimal conditions for the retrieval and quantification of bacteria from the filter surface were defined.

(b) Materials and methods

Preparation of culture vessels Each Ultrafree-CL ultrafiltration unit was separated into its

Figure 4.1 Photograph of the separate components of an Ultrafree-CL assembly

Figure 4.2 Diagram of an Ultrafree-CL assembly

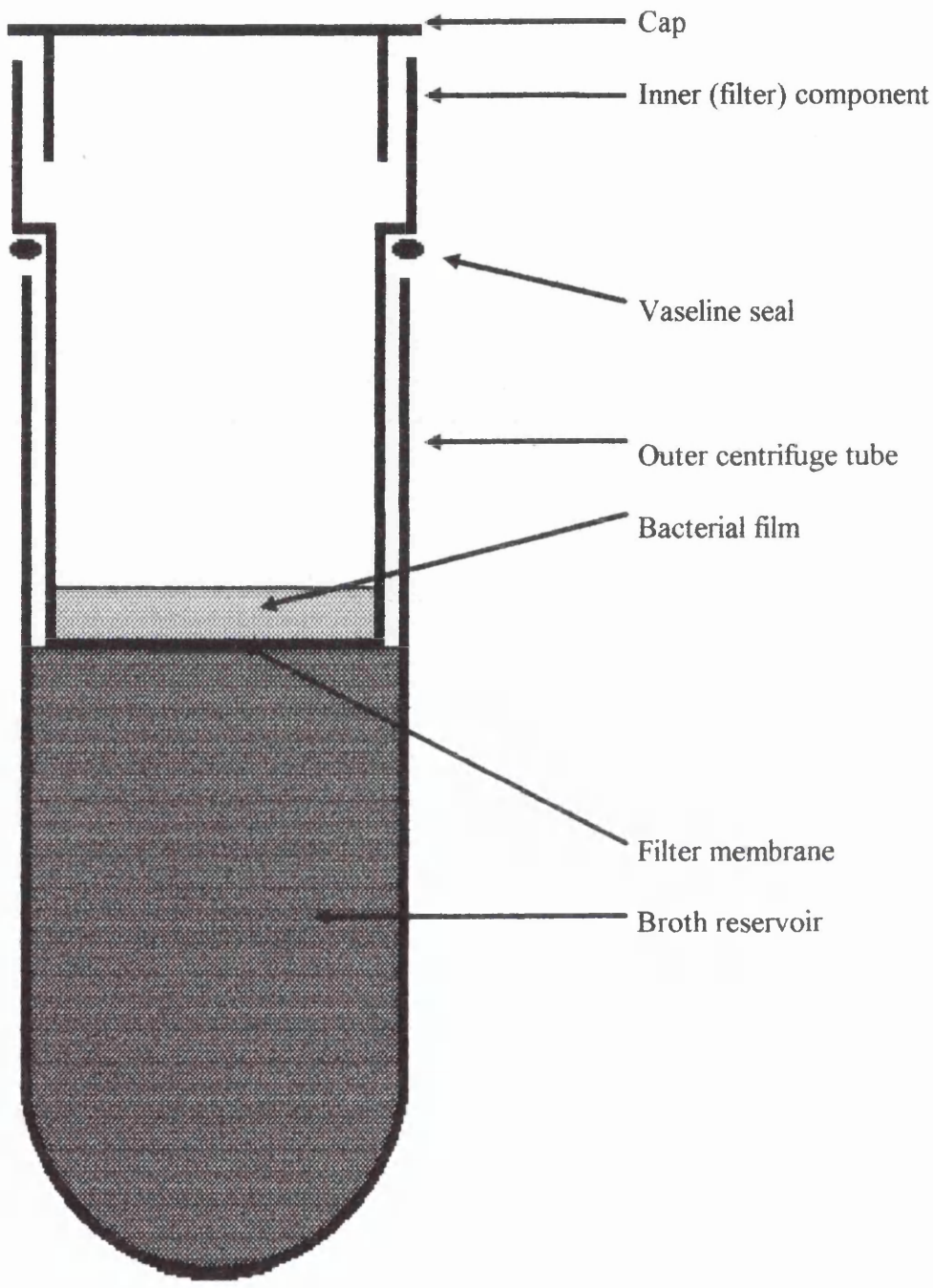


Figure 4.3 Photograph of an Ultrafree-CL unit assembled

two components, sealed into a plastic sterilising packet and commercially sterilised by ethylene oxide. Each unit was then reassembled for use in a sterile-air laminar flow hood which had been running for 5min and the working surface swabbed with an Azowipe (70% isopropanol soaked wipe, Vernaid, Vernon-Carus Ltd., U.K.) prior to use. In addition all manipulations in the sterile-air hood were performed whilst wearing latex gloves (Hand Safe disposable gloves, U.K.) which had been cleaned with an azowipe. Firstly the centrifuge tube was removed with pre-flamed forceps from the sterile pack, placed in a test-tube rack and 5.05ml of sterile T.H.B. added aseptically. The filter component was then removed from the packaging and a smear of autoclave-sterilised Vaseline (Cheseborough-Ponds, U.S.A.) added to the shoulder of the filter component (figure 4.2) with a sterile 'Pastette' (Western Laboratory Service Ltd., U.K.) to ensure a 'bacteria-tight' seal. Finally the filter component was inserted into the centrifuge tube and its top sealed with the plastic cap.

To verify that the air-stream in the sterile air laminar-flow hood remained sterile during assembly of the units, two blood agar plates were sited randomly in the hood and exposed to the air stream. One blood agar plate was incubated anaerobically for 48hr at 37°C, whilst the other was incubated aerobically for the same period. This procedure for preparation of Ultrafree-CL ultrafiltration units was followed in all experiments described in the following chapters. No contamination of 'air-stream' control plates ever occurred.

Organisms and growth conditions employed The bacterial species employed were type culture strains of *Streptococcus mutans* (NCTC 10449), *Lactobacillus casei* (NCTC 6375) and *Actinomyces viscosus* (NCTC 10951). Standardised suspensions of these species were prepared to a cell density of 1mg/ml (wet w/v) as described in section 2.4.

A 100µl volume of each species was inoculated into the filter component of each individual Ultrafree-CL unit. The addition of the bacterial suspensions to the units was randomised, to facilitate statistical analyses of the results, by arbitrarily giving each unit/ bacterium combination a code number and then using a random number table to assign the order of inoculation (Wardlaw, 1987). The inoculated Ultrafree-CL units were incubated anaerobically for 4d at 37°C and the T.H.B. was replaced aseptically with fresh medium in the sterile air laminar-flow hood after 2d incubation as described earlier.

Determination of bacterial viable counts The Ultrafree-CL ultrafiltration unit is constructed from rigid polypropylene and the membrane filter has a supporting structure of the same material beneath it. This made it difficult and time consuming to cut the membrane filter free of its supporting structure, as was performed with the Millicell-HA tissue culture inserts (chapter 3). Therefore it was decided to pipette diluent into the filter component, employ a vortex mixer to detach the bacterial cells from the membrane surface and then to aspirate the resulting cell suspension for estimation of viable counts. Also, since it was uncertain if a single wash could separate most of the bacterial cells it was decided to repeat the washing process a number of times as described below.

At the end of the incubation period 1ml of 135mM KCl was pipetted into each filter component, which was then vortexed for 2min at full power. Afterwards the resulting bacterial suspension was aspirated and transferred to a sterile bijoux. A fresh 1ml volume of sterile 135mM KCl was pipetted into each filter and these were again vortexed for 2min at full power after which the bacterial suspension was transferred to a second sterile bijoux. This process was repeated until 10 consecutive washes had been performed. The order in which the units were washed was randomised. One hundred fold serial dilutions

of the filter washings were prepared and 50µl aliquots of the dilutions used to determine the c.f.u. present in each wash as described before (section 2.5). In order to verify the cultural purity and identity of the bacteria, a loopful of each suspension washed from the filter units was inoculated to blood agar and to the relevant semi-selective medium. The experiment was repeated on three separate occasions.

Statistical tests employed The data generated by this experiment were entered into the Minitab release 9.2 statistical package for personal computer (Minitab Inc., U.S.A.) and then analysed by means of an appropriate statistical test. For comparison between the means of 2 groups of data, Student's T-test was employed. For more complex comparisons between the means of more than two groups of data, one-way analysis of variance was employed to avoid the possibility of spurious significance values arising.

(c) Results

Table 4.1 shows the mean viable counts of *S. mutans*, *L. casei* and *A. viscosus* inoculated into Ultrafree-CL units and the mean number of c.f.u. retrieved by each 2 minute wash. The number of streptococci and lactobacilli retrieved by the first wash was about ten-fold greater than was in the initial inoculum but the number of c.f.u. retrieved by each successive wash decreased. However, although the *A. viscosus* viable counts showed a similar trend, the initial increase in c.f.u. was almost 100-fold. The number of *L. casei* and *A. viscosus* viable counts retrieved by the second wash were significantly lower than those retrieved by the first wash ($p < 0.01$ in both cases, Student's T-test), however it was not until the third wash that the number of *S. mutans* retrieved was significantly less than wash number one ($p < 0.01$, Student's T-test).

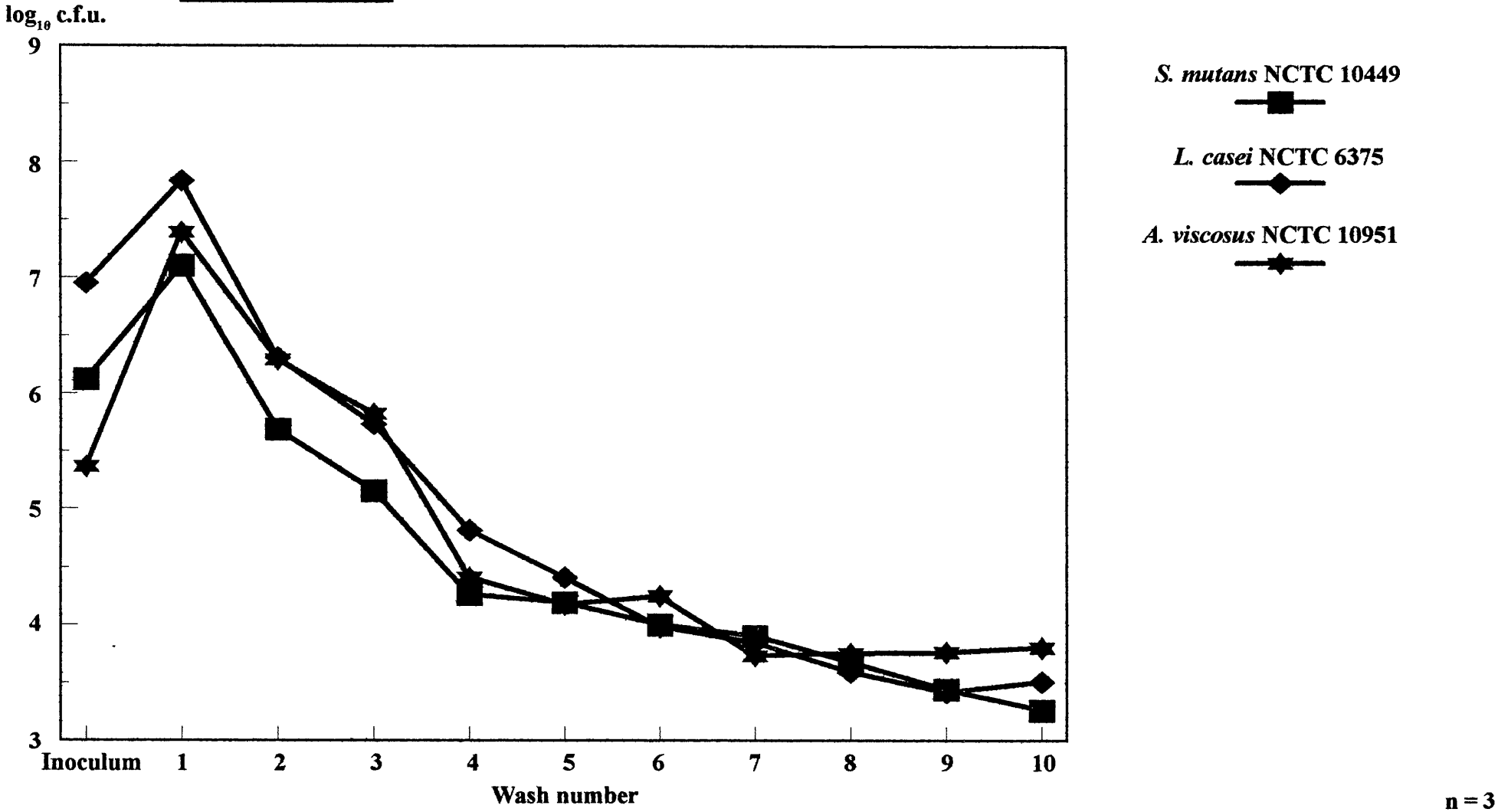
Table 4.1 **The number of colony forming units inoculated to Ultrafree-CL units, incubated for 4 days and retrieved after 10 consecutive washes of 2 minutes length**

Organism	$\log_{10}$ c.f.u. (± 1 S.D.) present in; Wash number;					
	Initial inoculum	**1**	**2**	**3**	**4**	**5**
S. mutans NCTC 10449	6.12 (0.04)	7.10 (0.82)	5.68 (0.51)	5.15 (0.22)	4.26 (0.81)	4.19 (0.68)
L. casei NCTC 6375	6.95 (0.60)	7.83 (0.03)	6.31 (0.35)	5.73 (0.80)	4.82 (0.70)	4.41 (0.47)
A. viscosus NCTC 10951	5.36 (0.21)	7.39 (0.22)	6.29 (0.26)	5.82 (0.68)	4.41 (0.09)	4.18 (0.15)

Organism	$\log_{10}$ c.f.u. (± 1 S.D.) present in; Wash number;				
	6	**7**	**8**	**9**	**10**
S. mutans NCTC 10449	4.00 (0.71)	3.90 (0.72)	3.68 (0.80)	3.44 (0.63)	3.26 (0.95)
L. casei NCTC 6375	3.98 (0.26)	3.84 (0.25)	3.60 (0.14)	3.42 (0.02)	3.51 (0.26)
A. viscosus NCTC 10951	4.25 (0.15)	3.73 (0.07)	3.76 (0.50)	3.77 (0.09)	3.81 (0.57)

n = 3

Figure 4.4 **Comparison of colony forming units inoculated to Ultrafree-CL units, incubated for 4 days and retrieved after 10 consecutive washes for 2 minutes**



n = 3

Figure 4.4 shows a comparison of the results for the three species tested. The number of c.f.u. retrieved after both the first and last washes was approximately equal for all species and the decrease in c.f.u. retrieved after each wash was also approximately equal. There was no significant difference between the final counts of the three test species overall ($p = 0.595$, one-way analysis of variance). In addition more than 99% of the viable bacteria were retrieved by the first four washes and therefore this number was chosen for future experiments.

4.2.2 The long-term population dynamics of the test bacterial species grown in Ultrafree-CL units

(a) Introduction

Caries is generally a chronic disease and the development of root surface caries is usually a slow process, with the plaque requiring to be present on the root surface over a long period. Therefore it was decided to study the viable counts of the test species present in the test system at selected time-points during an extended incubation period.

(b) Materials and methods

Organisms used, growth conditions and determination of viable counts Suspensions of the three test species were prepared to a cell density of 1mg/ml (wet w/v) and a 100µl volume of each was inoculated into the filter component of four individual Ultrafree-CL units standing in T.H.B. The order of addition of bacterial suspensions to the units was randomised as previously described. The inoculated Ultrafree-CL units were incubated anaerobically at 37°C and the T.H.B. was replaced every second day over a period of 21 days. The number of c.f.u. for each species was determined after 1, 6, 14 and 21d of growth. Selection of Ultrafree-CL units for counting at each time point was randomised.

Whilst replacing the broth supply it was examined carefully for evidence of turbidity in case *S. mutans* or one of the other species had managed to penetrate the membrane. The cultural purity and identity of the organisms washed from the Ultrafree-CL units was also determined. This experiment was repeated on three separate occasions and the results analysed statistically as described previously.

(c) Results

Table 4.2 shows the mean numbers of *S. mutans*, *L. casei* and *A. viscosus* inoculated into Ultrafree-CL units and the number retrieved after 1, 6, 10, 14 and 21 days of incubation. The viable counts of each species retrieved after 1d of incubation were significantly greater than the initial inocula ($p < 0.001$, one-way analysis of variance). However, there then followed a slow decline in the number of *S. mutans* c.f.u. retrieved during the subsequent incubation period from the first to the twenty-first day, with the viable counts retrieved during the last week of incubation being significantly lower than the 1d counts ($p < 0.001$, one-way analysis of variance) and very similar to the initial inoculum. In contrast no significant differences were found in the *L. casei* or *A. viscosus* viable counts during the following three weeks of the experiment ($p = 0.283$ and $p = 0.068$ respectively, one-way analysis of variance).

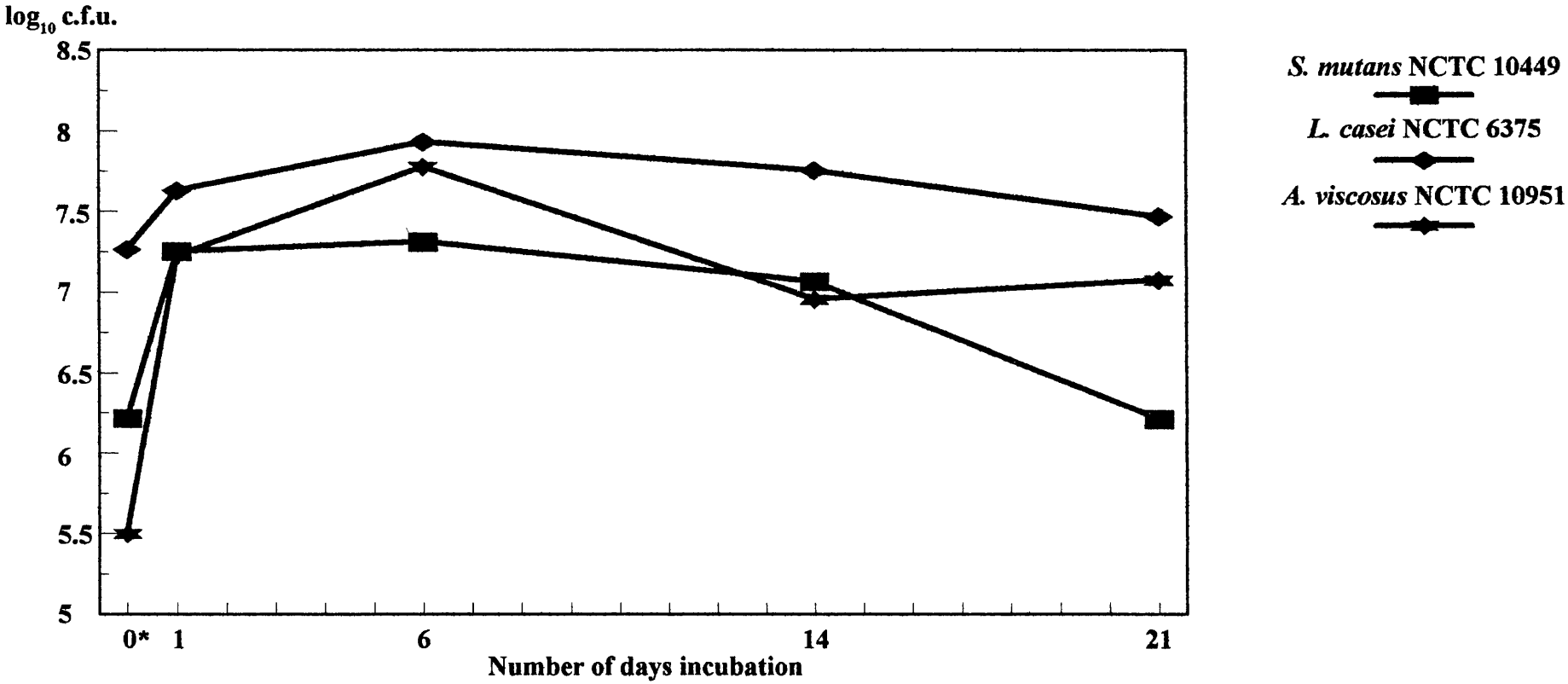
Figure 4.5 shows a comparison of the mean viable counts of each species inoculated into Ultrafree-CL units and also the number of c.f.u. retrieved at each time point during the incubation period. There were no significant differences between the viable counts of the three species on days 1 and 6 ($p > 0.05$ in both cases, one-way analysis of variance), however there were significant differences between the counts of the three species on days 14 and 21 ($p = 0.002$ and $p = 0.001$ respectively, one-way analysis of variance).

Table 4.2 **The change in viable counts of *S. mutans*, *L. casei* and *A. viscosus* during 21 days of incubation**

Organism	$\log_{10}$ c.f.u. (± 1 S.D.) present on day;				
	0 (Initial inoculum)	**1**	**6**	**14**	**21**
S. mutans NCTC 10449	6.21 (0.73)	7.25 (0.21)	7.31 (0.18)	7.06 (0.10)	6.21 (0.26)
L. casei NCTC 6375	7.26 (0.11)	7.62 (0.31)	7.93 (0.36)	7.75 (0.12)	7.47 (0.25)
A. viscosus NCTC 10951	5.50 (0.21)	7.22 (0.52)	7.78 (0.32)	6.95 (0.23)	7.07 (0.16)

n = 3

Figure 4.5 The change in the viable counts of *S. mutans* NCTC 10449, *L. casei* NCTC 6375 and *A. viscosus* NCTC 10951 when cultured separately for 21 days



* = Density of initial inoculum

n = 3

It was noted during routine replacement of the broth supply for *S. mutans* that two thirds of them became turbid, indicating that contamination had occurred. However contamination did not occur on the same day in each case, but haphazardly from day 8 up to day 21. Subsequent analysis revealed the contaminant to be *S. mutans*, whereas no contamination of the broth was noted in units inoculated with either *L. casei* or *A. viscosus*.

4.2.3 Growth of *Streptococcus mutans* in Ultrafree-CL ultrafiltration units with 0.1µm pore size membranes

(a) Introduction

In view of the ability of *S. mutans* to occasionally penetrate membrane filters with 0.22µm diameter pores it was therefore decided to test the ability of filters with 0.1µm pores to retain the species.

(b) Materials and methods

Organisms used, growth conditions and determination of viable counts

Streptococcus mutans NCTC 10449 was prepared to a density of 100mg/ml and 100µl volumes of this were inoculated into the filter component of three Ultrafree-CL units with 0.1µm diameter pores in the filter membrane and which were standing in 5.05ml each of T.H.B. The units were incubated for 21d at 37°C in an anaerobic environment and the broth supply was replenished every second day. The waste broth was examined carefully for turbidity each time it was aspirated. At the end of the incubation period the number of c.f.u. present in the unit was determined, as was the purity and identity of the bacterial culture washed from the filter. This experiment was performed once in triplicate.

(c) Results

The density of the initial inoculum was 6.98 c.f.u. per 100µl, which was effectively unchanged at 7.22 $\pm$ 0.39 $\log_{10}$ c.f.u. per unit on day 21. In addition no turbidity was noted in any of waste broths.

4.2.4 Examination by light microscopy of transverse sections through a film of *S. mutans* NCTC 10449 grown in an Ultrafree-CL unit

(a) Introduction

The ability of *S. mutans* to penetrate the Ultrafree-CL unit filter membrane with a pore size of just 0.22µm was surprising. However it was not clear how this happened and so it was decided to examine transverse sections through a filter membrane harbouring bacteria to determine any similarities with previous observations in dentine (Schüpbach, Guggenheim & Lutz, 1990 and Schüpbach, Lutz & Guggenheim, 1992) and in guided tissue regeneration filters (Simion *et al.*, 1994 and 1995).

(b) Materials and methods

Organisms and growth conditions employed The organism employed was *S. mutans* NCTC 10449 prepared to 1mg/ml (wet w/v), which was inoculated to and incubated in Ultrafree-CL units as before and at the end of the 6d incubation period prepared for microscopy as described below.

Preparation of transverse sections for light microscopy After incubation, the film was fixed to the filter membrane by soaking for 1hr in 2% glutaraldehyde (Agar Aids, U.K.) in 0.1M sodium cacodylate buffer at pH 7.2 (Merck, U.K.). The fixed film was then washed for 3 x 20min using 0.1M sodium cacodylate buffer (pH 7.2). Once the sample

Figure 4.6 Transverse section of an Ultrafree-CL ultrafiltration unit filter membrane (Magnification = 197 x)

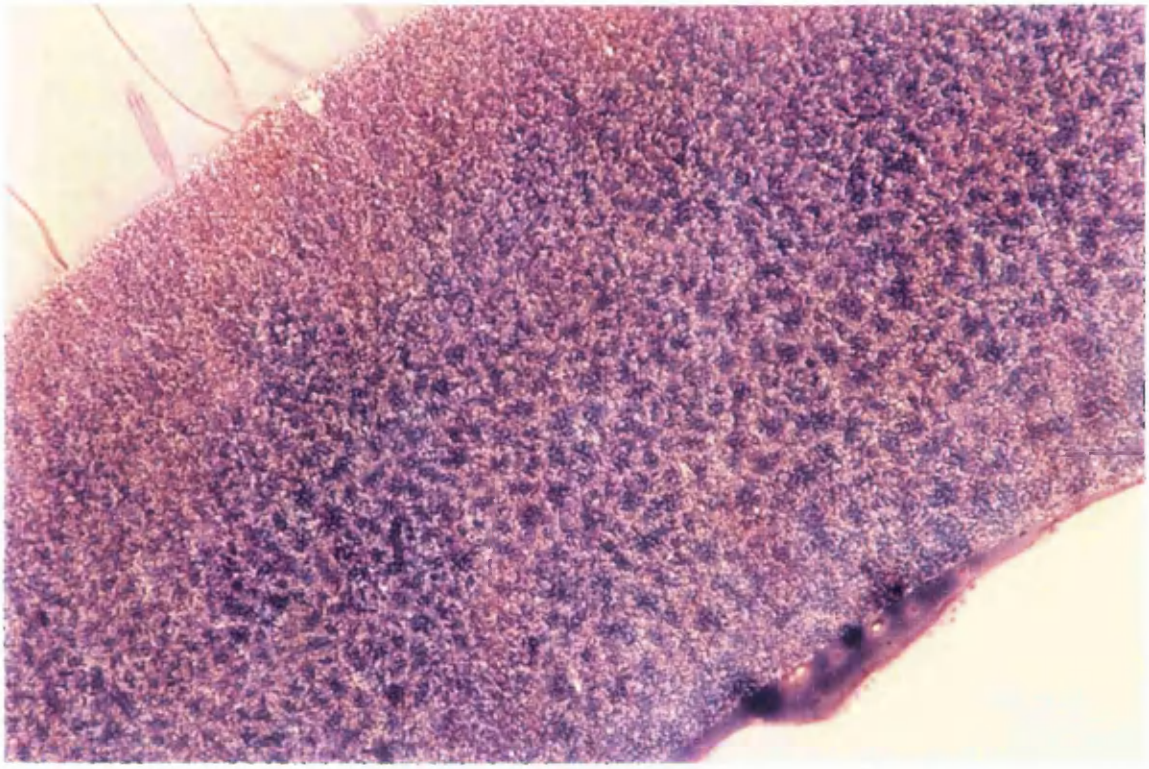
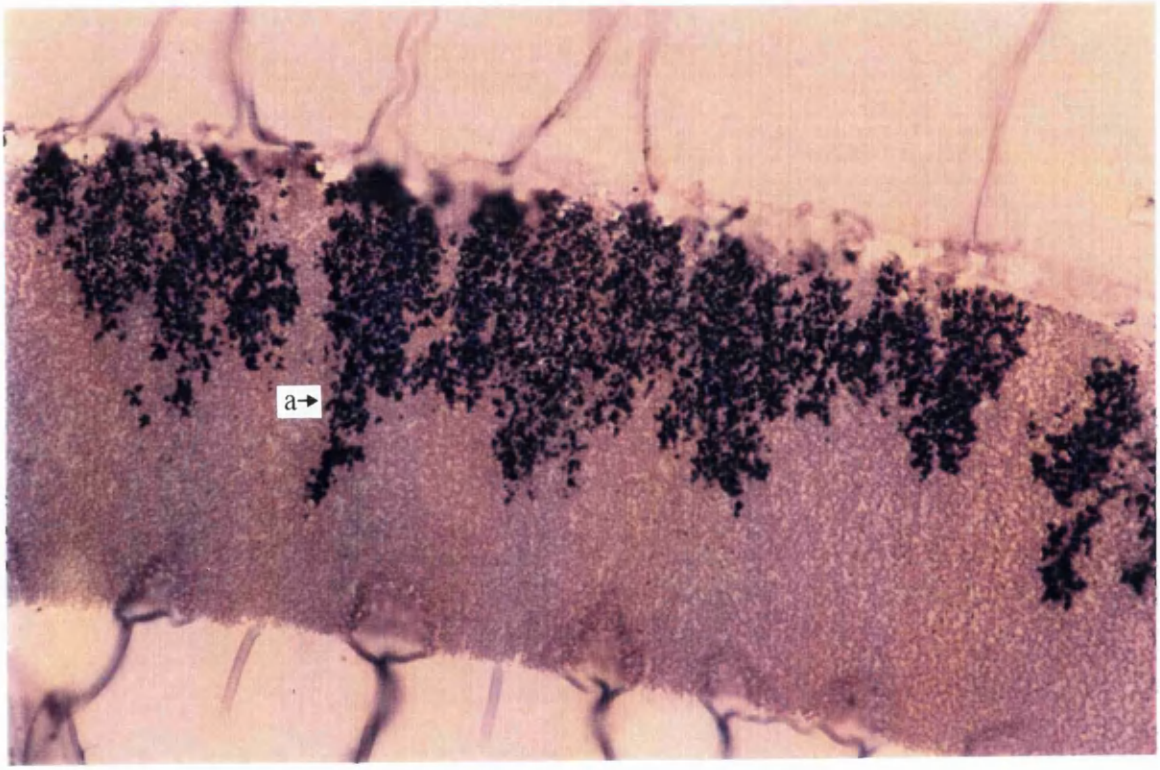


Figure 4.7 Transverse section of an Ultrafree-CL ultrafiltration unit filter membrane with *S. mutans* (Magnification = 125 x)



had been fixed it was dried using a graded series of ethanol concentrations (50, 70, 90, 95 and 100%) for half an hour per step. After drying, the filter was embedded in T.A.A.B. resin (T.A.A.B. Laboratories, U.K.) and then 1μm thick sections were cut using an L.K.B. 3 Ultrotome (L.K.B., U.K.). The cut sections were mounted on poly-L-lysine (Sigma, U.K.) coated glass microscope slides (Chance-Proper, U.K.) and stained with Toluidene blue (Merck, U.K.) prior to examination using a Zeiss Axiophot light microscope (Zeiss, Germany).

(c) Results

Figure 4.6 shows a light micrograph of a transverse section through an uninoculated Ultrafree-CL ultrafiltration filter membrane.

Figure 4.7 shows a light micrograph of a transverse section through a culture of *S. mutans* grown upon an Ultrafree-CL filter membrane for 6 days. The micrograph shows the *S. mutans* cells invading the structure of the membrane (arrowed 'a').

4.2.5 Determination of the effect of initial inoculum density upon final viable counts in Ultrafree-CL ultrafiltration units

(a) Introduction

It was clear from chapter 3 that a difference of up to 1,000-fold in the initial inoculum added to the Millicell-HA unit did not lead to significant differences between the final number of viable bacteria yielded (section 3.2.2). The c.f.u. present in a low density initial inoculum increased significantly during the course of the experiment, whilst those present in a high density inoculum did not. Therefore it was decided to investigate this effect with the Ultrafree-CL unit using a similar experimental protocol.

(b) Materials and methods

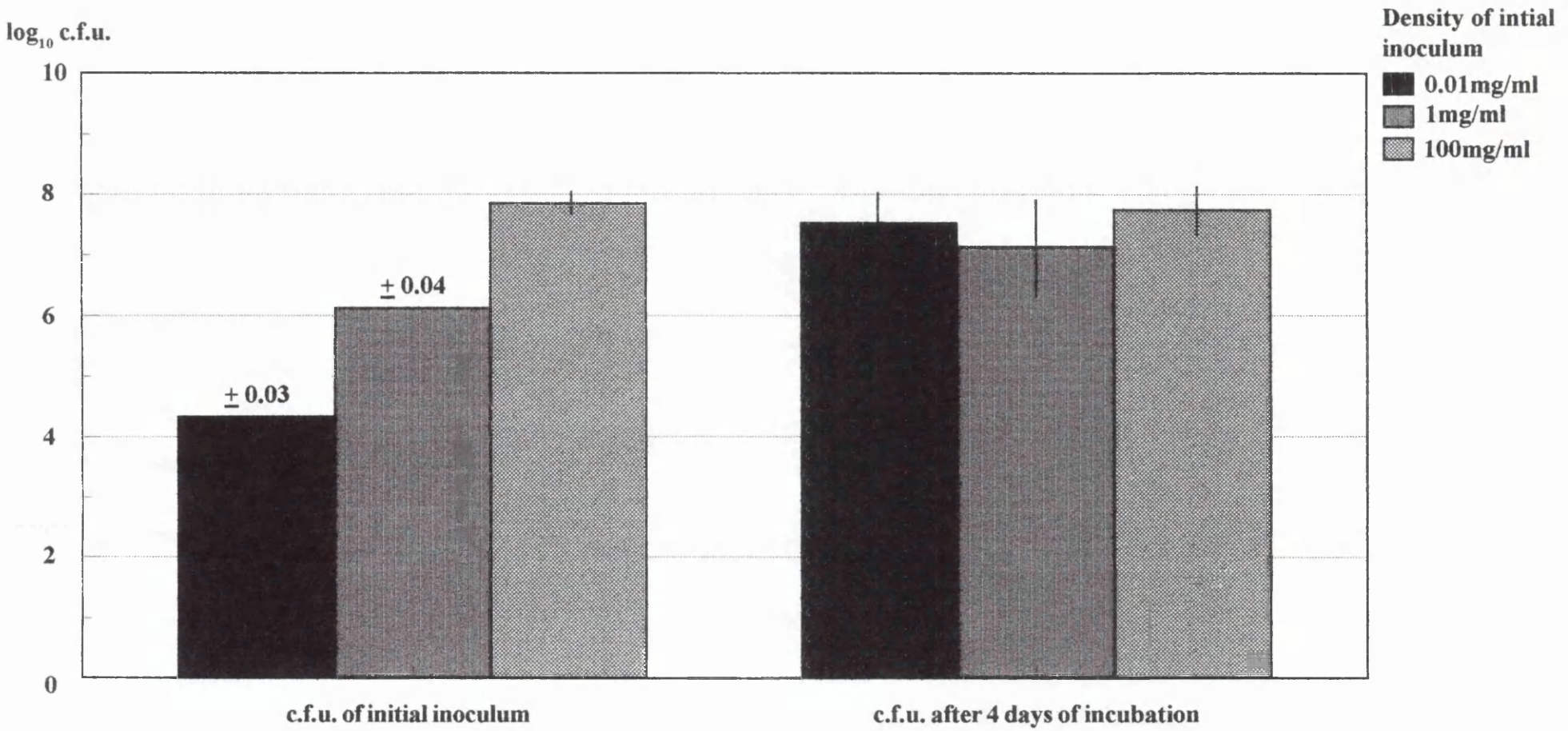
Organisms and growth conditions employed and determination of bacterial viable counts

The bacterial species employed were *S. mutans* NCTC 10449, *L. casei* NCTC 6375 and *A. viscosus* NCTC 10951, suspensions of each were prepared to densities of 0.01, 1 and 100mg/ml (wet w/v)(section 2.4) and 100µl volumes inoculated in a random order (section 4.2.1). Each assembly was incubated anaerobically for 4d at 37°C, with the broth being replaced with a fresh volume (5.05ml) after 2d of incubation. At the end of the incubation period the bacteria were retrieved from each filter unit in a random order with 4 consecutive washes of sterile 135mM KCl (section 4.2.1) and the sum total viable count calculated. The cultural purity and identity of the organisms washed from each Ultrafree-CL unit was also determined. This experiment was repeated on three separate occasions and the data analysed statistically using the Minitab statistical package as before.

(c) Results

Figure 4.8 shows the mean number of *S. mutans* c.f.u. inoculated to Ultrafree-CL units at 0.01, 1 and 100mg/ml (wet w/v) and the mean number of c.f.u. retrieved after 4 days' incubation. The increase in the numbers of *S. mutans* over 4 days from initial inocula of both 0.01 and 1mg/ml was statistically significant ($p < 0.001$, one-way analysis of variance). However, there was no significant difference between the initial and final numbers from an inoculum of 100mg/ml ($p > 0.1$, Student's T-test). Moreover there were no significant differences between the final viable counts which developed from the three initial inocula ($p = 0.471$, one-way analysis of variance). Figure 4.9 shows the results for *L. casei* and Figure 4.10 for *A. viscosus*. A similar trend to the data described for *S. mutans* was found with regard to bacteria cultured at each of the three different

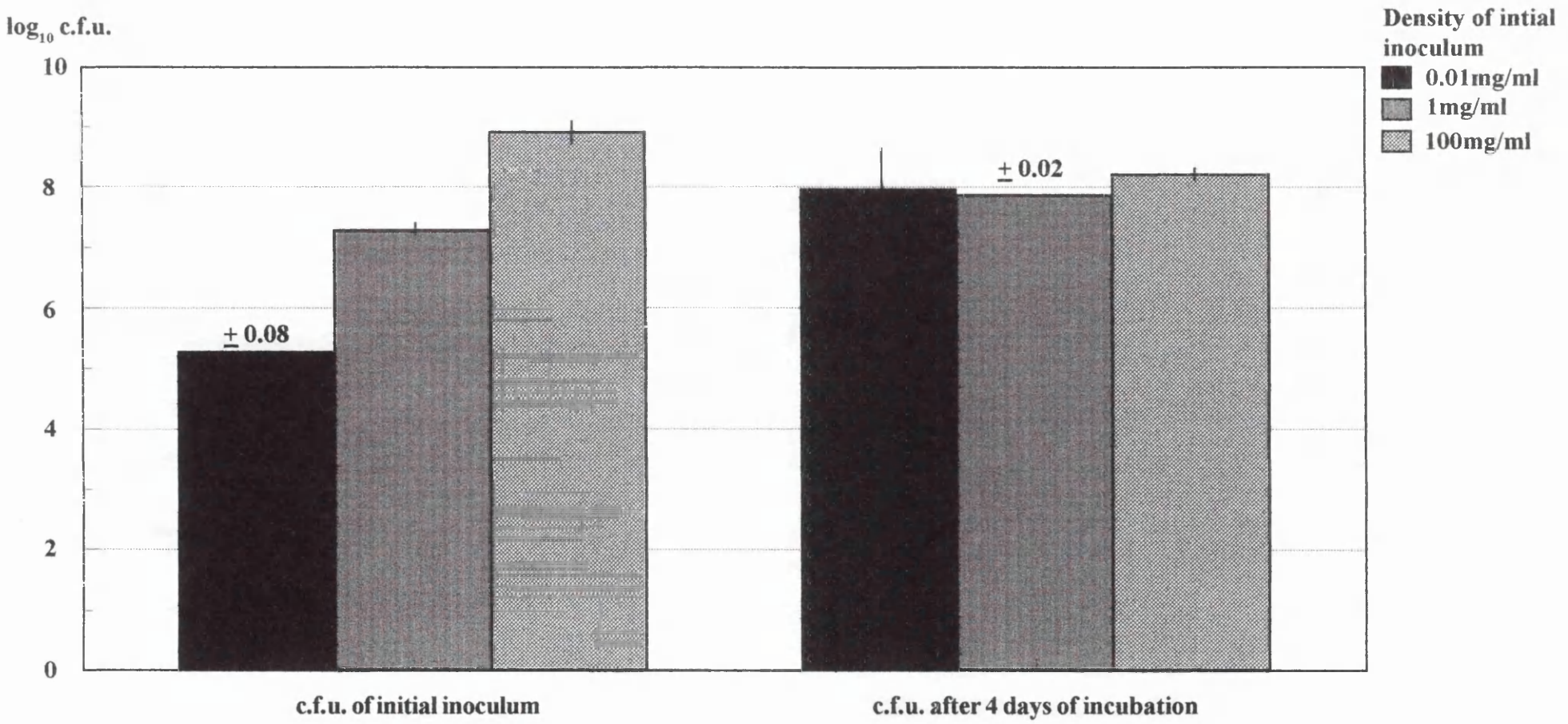
Figure 4.8 **The effect of different densities of initial inoculum on the final numbers of viable *S. mutans* NCTC 10449 after anaerobic incubation for 4 days**



Error bars represent mean ± 1 S.D.

n = 3

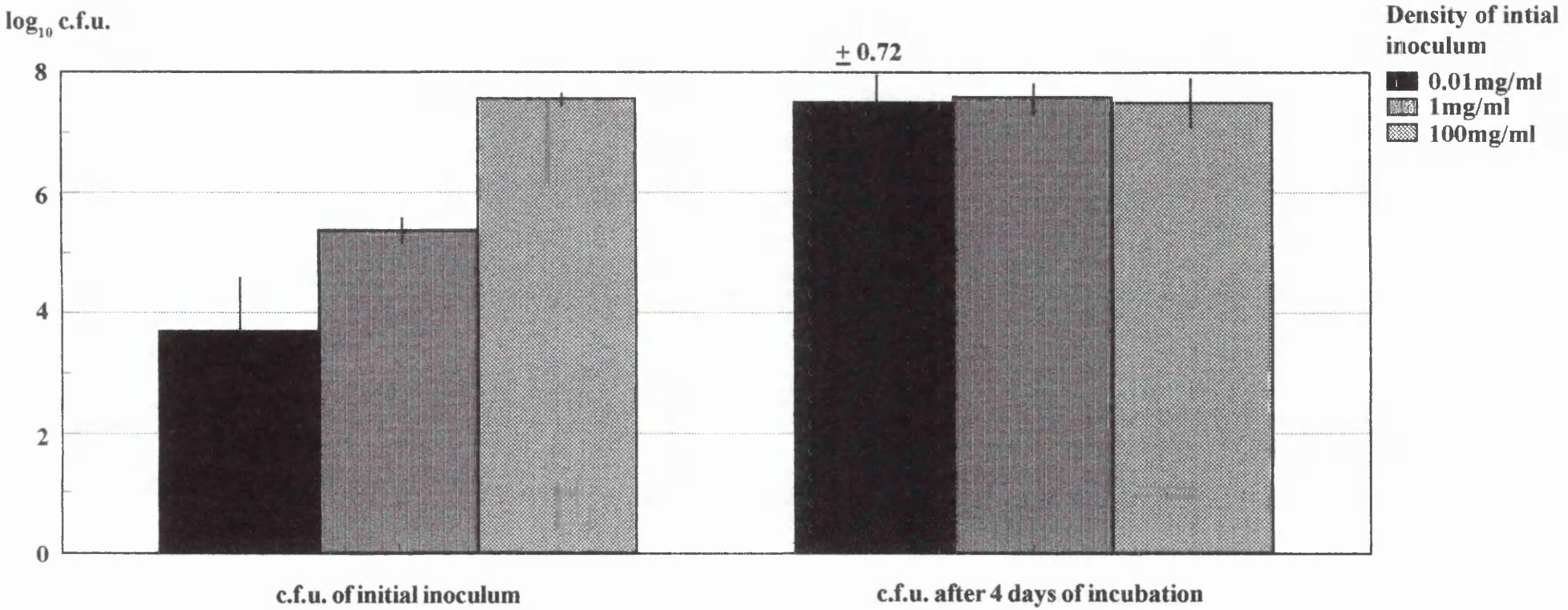
Figure 4.9 **The effect of different densities of initial inoculum on the final numbers of viable *L. casei* NCTC 6375 after anaerobic incubation for 4 days**



Error bars represent mean ± 1 S.D.

n = 3

Figure 4.10 **The effect of different densities of initial inoculum on the final numbers of viable *A. viscosus* NCTC 10951 after anaerobic incubation for 4 days**



Error bars represent mean ± 1 S.D.

n = 3

densities of inoculum. There were no statistically significant differences between the number of c.f.u. of the 3 species retrieved at the end of the experiment ($p = 0.069$, one-way analysis of variance).

4.2.6 Determination of the effect of interacting bacterial species in Ultrafree-CL units on viable counts

(a) Introduction

The bacterial population of the mouth consists of a complex mixture of organisms (Bowden, 1990) and some may have a beneficial or harmful effect on others; *Veillonella alcalescens* which metabolises lactate is an example of the former (Donoghue & Perrons, 1988), whilst *S. mutans* T2 which produces a bacteriocin against *A. viscosus* (Beckers & van der Hoeven, 1984) is an example of the latter. It was therefore decided to examine the overall effect of growing *S. mutans*, *L. casei* and *A. viscosus* together in the units with a view to developing a method to explore the demineralisation of human tooth roots using mixed plaque communities.

(b) Materials and methods

Organisms and growth conditions employed Suspensions of 0.01mg/ml (wet w/v) density were prepared with the three test species as described before and then equal volumes (500µl) were mixed to give the following two- and three-way mixtures; *S. mutans* + *L. casei*, *S. mutans* + *A. viscosus*, *L. casei* + *A. viscosus* and *S. mutans* + *L. casei* + *A. viscosus*. A 100µl volume of each pure and mixed bacterial suspension was inoculated to the filter component of a separate Ultrafree-CL unit standing in 5.05ml of T.H.B. and incubated anaerobically for 4d at 37°C, with the broth being replaced by a fresh volume

(5.05ml) after 2 days. The order of addition of bacterial suspensions to the Ultrafree-CL units was randomised as described in section 4.2.1.

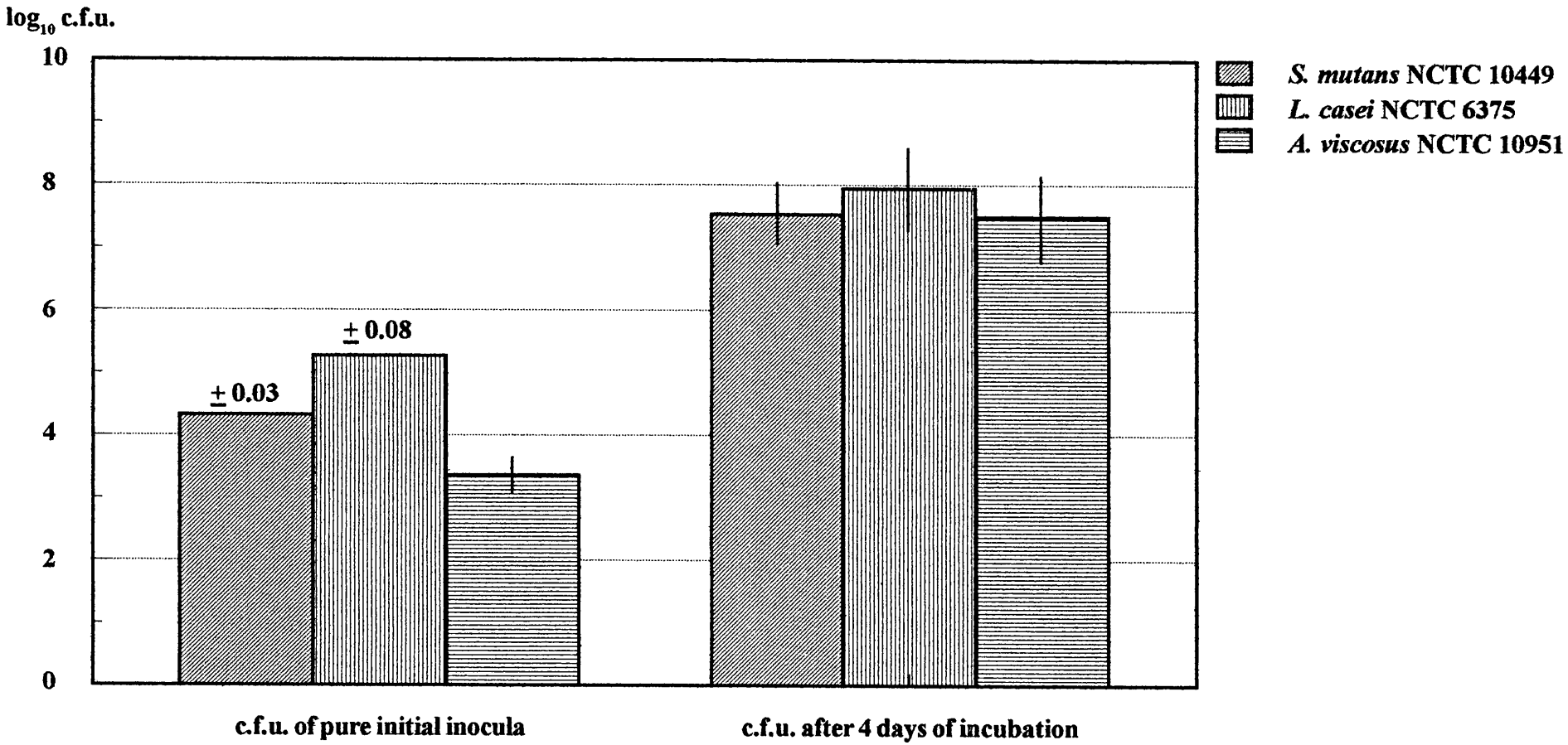
Determination of viable counts At the end of the incubation period the number of c.f.u. in each Ultrafree-CL unit was determined in a random order as described previously. The colonial morphology of each bacterial species from the two- and three-way mixtures was sufficiently distinctive when grown on C.B.A. to allow the viable counts to be determined without resorting to selective media (section 3.2.3). The cultural purity and identity of the organisms washed from the units were also determined. This experiment was repeated on three occasions and the results analysed statistically as before.

(c) Results

Figure 4.11 shows the mean viable counts of the three bacterial species when inoculated in pure culture and retrieved from the Ultrafree-CL units after 4 days' incubation. The number of c.f.u. retrieved from the units after 4 days was significantly greater than in the initial inoculum ($p < 0.001$, one-way analysis of variance), whilst the final c.f.u. of the 3 species was very similar.

Figure 4.12 shows the mean number of c.f.u. in the initial inocula and final populations of the mixture of *S. mutans* + *L. casei*. The viable counts of both species in the initial inocula of the mixtures were approximately equal to those in the corresponding pure cultures. However, the final viable counts of *S. mutans* were approximately 10-fold less in the mixture than in the corresponding pure culture, although this difference was not significant ($p = 0.053$, one-way analysis of variance). In contrast the final number of *L. casei* c.f.u. in the mixture were very similar to those in pure culture. The viable counts

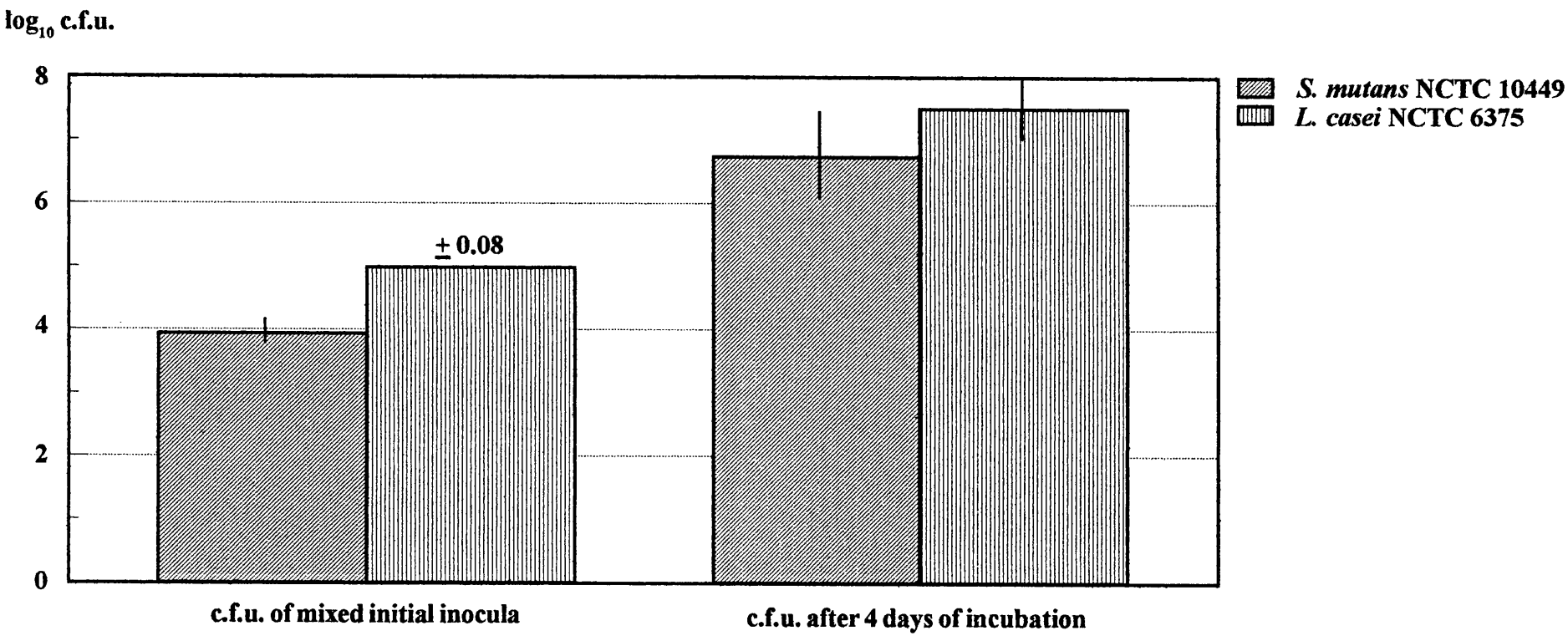
Figure 4.11 **The change in the numbers of *S. mutans* NCTC 10449, *L. casei* NCTC 6375 and *A. viscosus* NCTC 10951 over 4 days when growing in pure culture**



Error bars represent mean ± 1 S.D.

n = 3

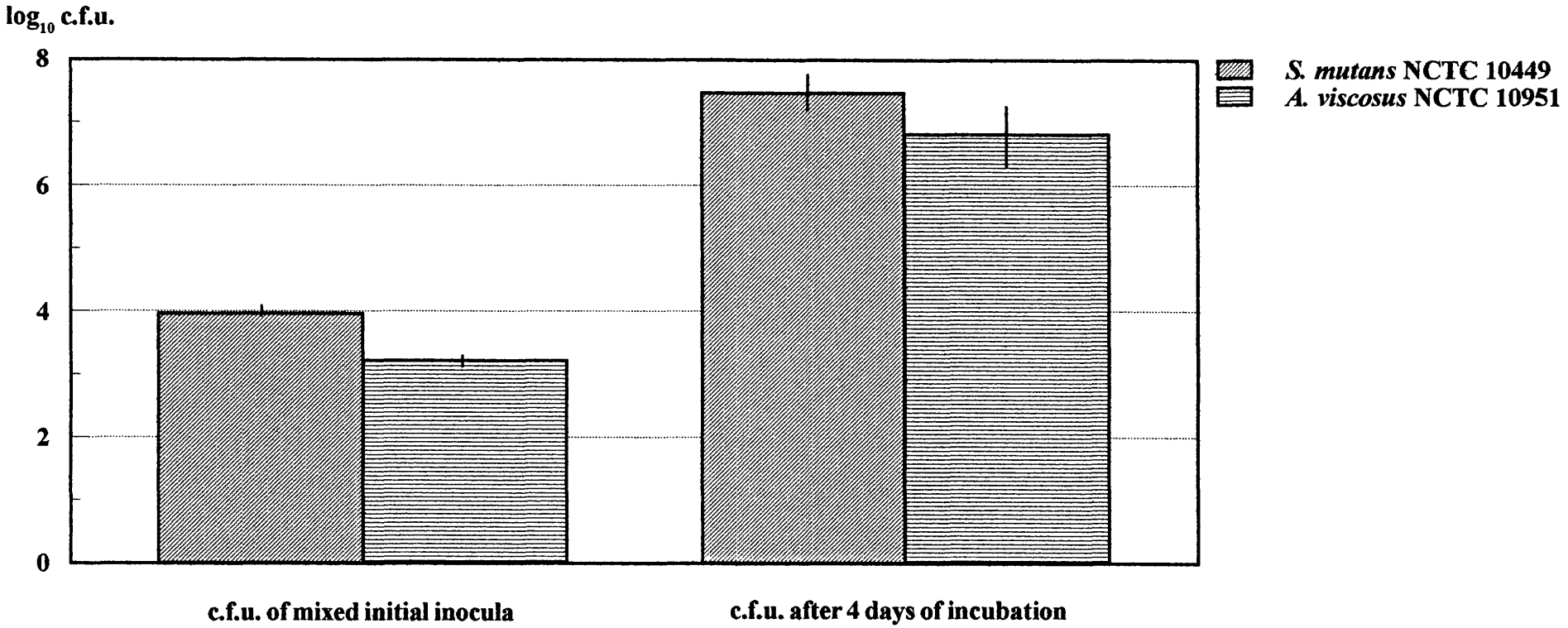
Figure 4.12 **The change in the numbers of *S. mutans* NCTC 10449 and *L. casei* NCTC 6375 over 4 days when cultured together**



Error bars represent mean ± 1 S.D.

n = 3

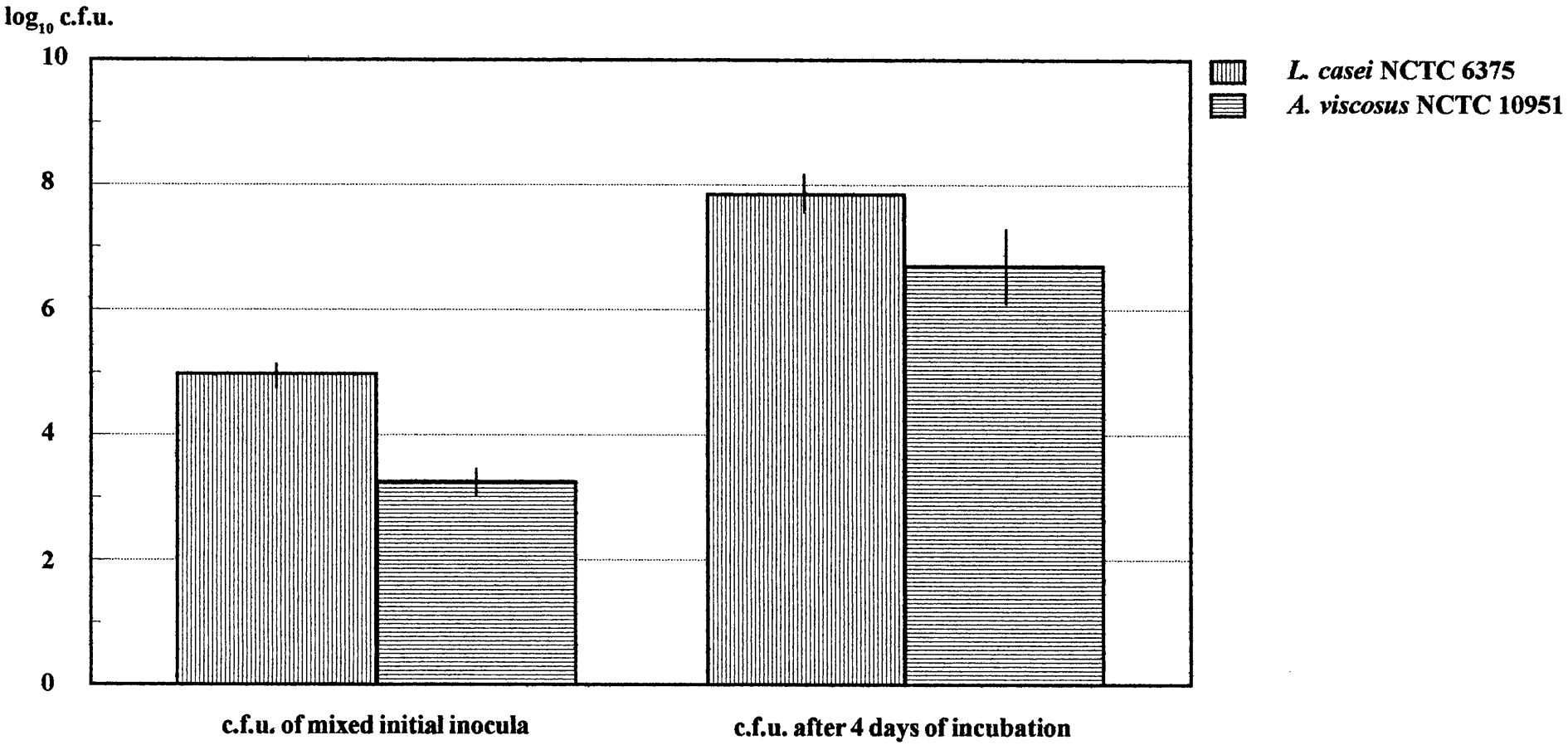
Figure 4.13 **The change in the numbers of *S. mutans* NCTC 10449 and *A. viscosus* NCTC 10951 over 4 days when cultured together**



Error bars represent mean ± 1 S.D.

n = 3

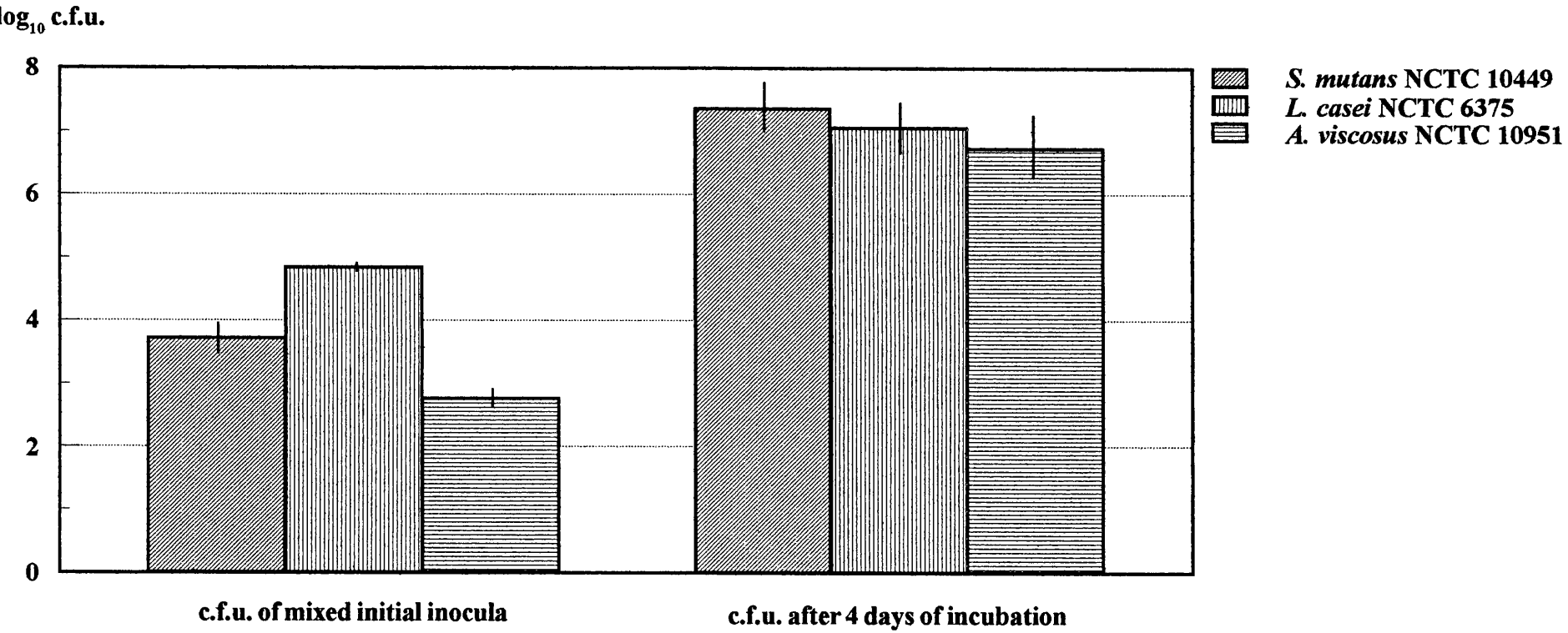
Figure 4.14 **The change in the numbers of *L. casei* NCTC 6375 and *A. viscosus* NCTC 10951 over 4 days when cultured together**



Error bars represent mean ± 1 S.D.

n = 3

Figure 4.15 **The change in the numbers of *S. mutans* NCTC 10449, *L. casei* NCTC 6375 and *A. viscosus* NCTC 10951 over 4 days when cultured together**



Error bars represent mean ± 1 S.D.

n = 3

of both *S. mutans* and *L. casei* retrieved after four days of incubation were significantly greater than in the initial inoculum ($p < 0.001$, one-way analysis of variance).

Figures 4.13 and 4.14 show the mean number of c.f.u. in the initial inocula and final populations of the two-way mixtures of *S. mutans* + *A. viscosus* and *L. casei* + *A. viscosus* respectively. The viable counts of both species in the mixed initial inocula were approximately equal to those in the corresponding pure cultures. In addition the final number of c.f.u. of each species in these mixtures, which were significantly greater ($p < 0.001$, one-way analysis of variance) than in the initial inocula, were approximately equal to the corresponding pure cultures.

Figure 4.15 shows the mean number of c.f.u. in the initial inoculum and final population of a three-way mixture of the organisms (*S. mutans* + *L. casei* + *A. viscosus*). The number of c.f.u. of each species present in the mixed initial inoculum was not significantly lower than the corresponding pure culture ($p = 0.053$, one-way analysis of variance). In addition the final viable counts of each bacterial species were not significantly lower than the corresponding pure cultures ($p = 0.184$, one-way analysis of variance). However the number of c.f.u. retrieved after 4 days of incubation was significantly greater than in the initial inocula ($p < 0.001$, one-way analysis of variance).

4.2.7 Examination of pure and mixed bacterial films in Ultrafree-CL ultrafiltration units by scanning electron microscopy

(a) Introduction

All of the experiments described in the preceding sections of this chapter have been purely interested in what happens to the viable counts of bacteria inoculated into the

Ultrafree-CL units; they provide no information on the appearance of the films that the bacteria form. Therefore it was decided to grow pure and mixed cultures of the chosen bacterial species in Ultrafree-CL units for 6 and 21d and then to examine the surface of these films by scanning electron microscopy to gather some information upon cell and plaque morphology.

(b) Materials and methods

Organisms and growth conditions employed The organisms employed were *S. mutans* NCTC 10449, *L. casei* NCTC 6375 and *A. viscosus* NCTC 10951, which had been prepared to a density of 1mg/ml (wet w/v). In addition a three-way mixture of these organisms was prepared by mixing equal volumes (500µl) of the suspensions. A 100µl volume of each suspension was inoculated into two separate Ultrafree-CL units, standing in 5.05ml of T.H.B. which were incubated anaerobically at 37°C. The T.H.B. supply was replaced with fresh broth every second day.

Preparation of films for scanning electron microscopy After 6 days of incubation one of the two Ultrafree-CL units inoculated with each suspension were taken and prepared for microscopy as follows; the unit was rinsed by removing the broth from the centrifuge tube, replacing it with 5.05ml of sterile phosphate buffered saline (P.B.S.) and incubating for 1hr at 37°C in an orbital incubator, after which the P.B.S. was replaced with fresh buffer and the rinsing process repeated on two further occasions. After the third rinse, 1ml of filter sterilised 2.5% glutaraldehyde (in P.B.S.) was gently layered onto the surface of the bacterial film and incubated statically for 1hr at 37°C to fix the bacterial film. The glutaraldehyde was then carefully aspirated from the Ultrafree-CL unit, which was next soaked overnight in three 5.05ml volumes of fresh P.B.S. at 4°C to remove the

glutaraldehyde residue. The membrane filter was removed from each filter component by cutting the plastic cylinder transversely with a heated scalpel blade (# 11, Swann-Morton, U.K.) and then dissecting the filter from its supporting mount. The membrane filter was manipulated with forceps at its edges and transferred to 1ml of P.B.S. for storage at 4°C prior to preparation for microscopy as described in section 3.2.4. The prepared filters were examined by scanning electron microscopy as in section 3.2.4.

After a total incubation period of 21d the second Ultrafree-CL unit containing each species was prepared for microscopy as above. Furthermore, an un-inoculated filter membrane was also prepared for microscopy as in section 3.2.4.

(c) Results

Figure 4.16 shows an un-inoculated Ultrafree-CL membrane filter. The filter appears to be formed of irregular shaped nodes connected haphazardly by filamentous 'bridges' of uneven length and breadth, whilst the pores have variable shape and diameter.

Figures 4.17, 4.19 and 4.21 show the typical surface morphology of *S. mutans*, *L. casei* and *A. viscosus* plaques (respectively) grown as pure cultures upon Ultrafree-CL membrane filters for 6d. *Streptococcus mutans* plaques appeared to be composed of a mat of interwoven chains of cocci, although there were some areas of low cell density which looked like deep 'chasms' extending into the film. In addition there was some evidence of extracellular matrix (arrowed 'a') in the *S. mutans* cultures. In contrast, very few cells were visible on the surface of filters inoculated with *L. casei*, although those which were present had a typical bacilliary shape. The *A. viscosus* film comprised branching chains of cells with irregular length and shape. Although the *A. viscosus* cells

generally formed a thick dense film, they also displayed zones of low density which looked like 'chasms' into the depth of the film. However it is not clear whether the 'chasms' were naturally occurring features or artefacts that arose during sample preparation.

Figures 4.18, 4.20 and 4.22 show the typical surface morphology of *S. mutans*, *L. casei* and *A. viscosus* plaques (respectively) grown as pure cultures upon Ultrafree-CL membrane filters for 21d. The *S. mutans* film was composed of long interwoven chains of cells which generally formed a dense mat although it was interspersed with some less densely populated areas ('chasms'). There was some evidence of intracellular matrix (arrowed 'a') and a small number of cells appeared to have lysed, leaving just the cell wall (arrowed 'b'). As before very few *L. casei* cells could be found attached to the filter membrane and those which were present tended to be elongated compared with those at 6d. *Actinomyces viscosus* formed a thick interwoven mat on the filter surface with fewer of the less dense regions ('chasms') than at 6d. The cells formed long branching chains of irregular shape and length which appeared similar to those observed in the 6d culture.

Figures 4.23 and 4.24 show representative micrographs of the surface of a mixed culture of *S. mutans*, *L. casei* and *A. viscosus* grown in an Ultrafree-CL ultrafiltration unit for 6d and 21d respectively. After 6d the bacteria formed an evenly mixed plaque showing cells typical of each species ('a' = coccoid, 'b' = bacilliary, 'c' = pleomorphic). It was interesting to note that all of the *S. mutans* cells had a ridge which appeared to extend right around the circumference of the cell at its midriff (arrowed 'd'). At 6d the film of cells appeared to be a thick interwoven layer of cell chains which had a number of less dense 'chasms'. At 21d (Figure 4.24) the film of cells was still comprised of a random

Figure 4.16 An Ultrafree-CL ultrafiltration membrane (Magnification = 5,000x) ——— 5µm

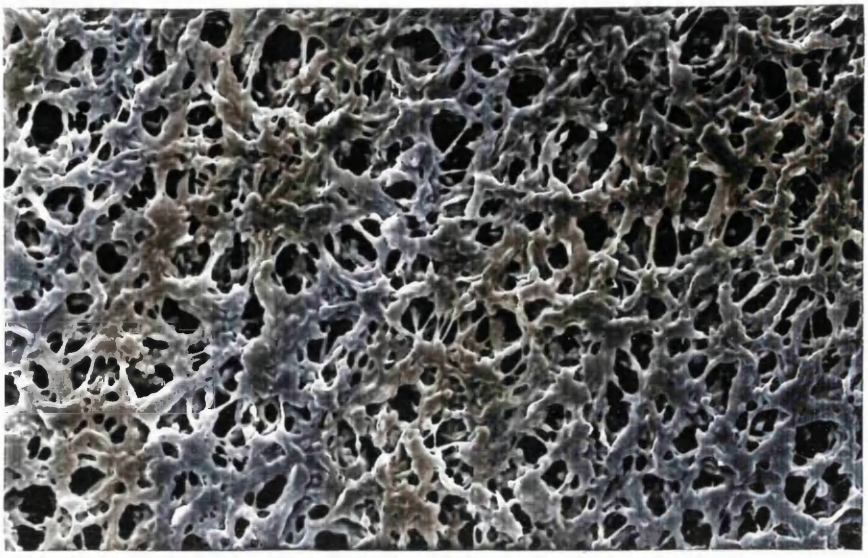


Figure 4.17 A plaque of *S. mutans* grown upon an Ultrafree-CL ultrafiltration membrane for 6d (Magnification = 5,000x)

——— 5μm

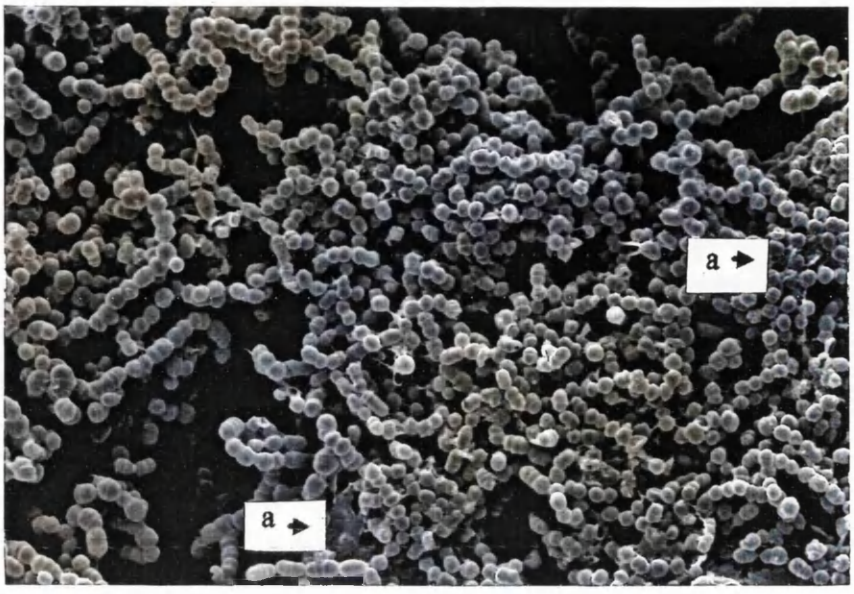


Figure 4.18 A plaque of *S. mutans* grown upon an Ultrafree-CL ultrafiltration membrane for 21d (Magnification = 5,000x)

——— 5μm

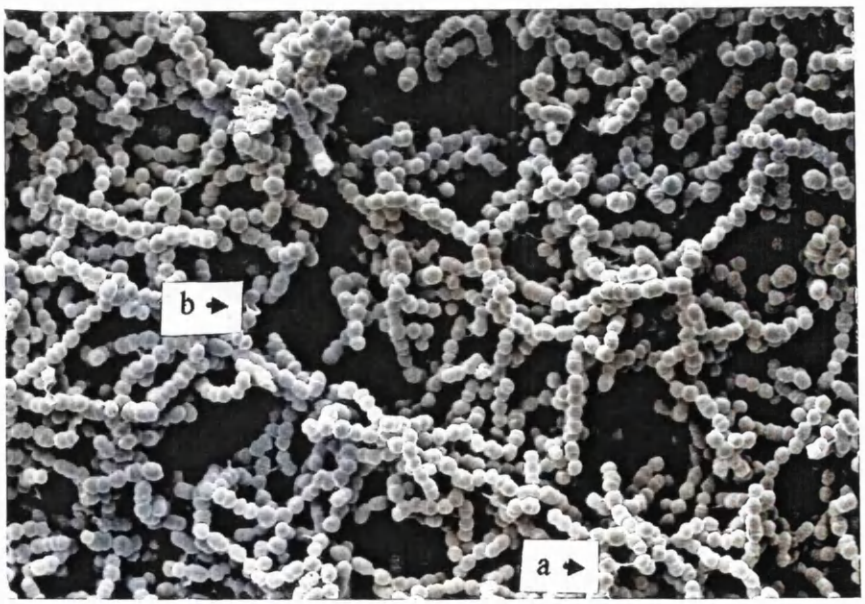


Figure 4.19 *L. casei* grown upon an Ultrafree-CL ultrafiltration membrane for 6d (Magnification = 5,000x) ——— 5µm

Figure 4.20 *L. casei* grown upon an Ultrafree-CL ultrafiltration membrane for 21d (Magnification = 5,000x) ——— 5µm

Figure 4.21 A plaque of *A. viscosus* grown upon an Ultrafree-CL ultrafiltration membrane for 6d (Magnification = 5,000x)

5µm

Figure 4.22 A plaque of *A. viscosus* grown upon an Ultrafree-CL ultrafiltration membrane for 21d (Magnification = 5,000x)

5µm

Figure 4.23 A mixed plaque of *S. mutans*, *L. casei* and *A. viscosus* grown upon an Ultrafree-CL ultrafiltration membrane for 6d

(Magnification = 10,000x) ——— 2μm

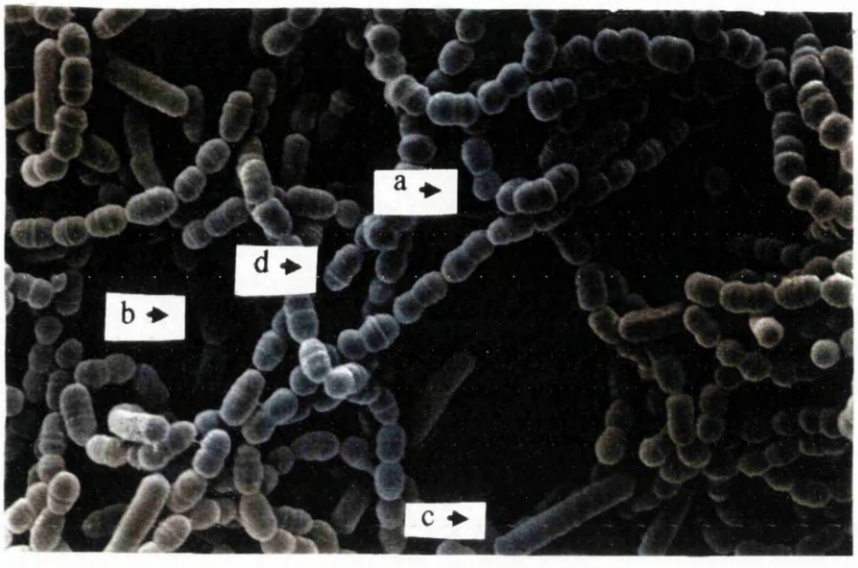
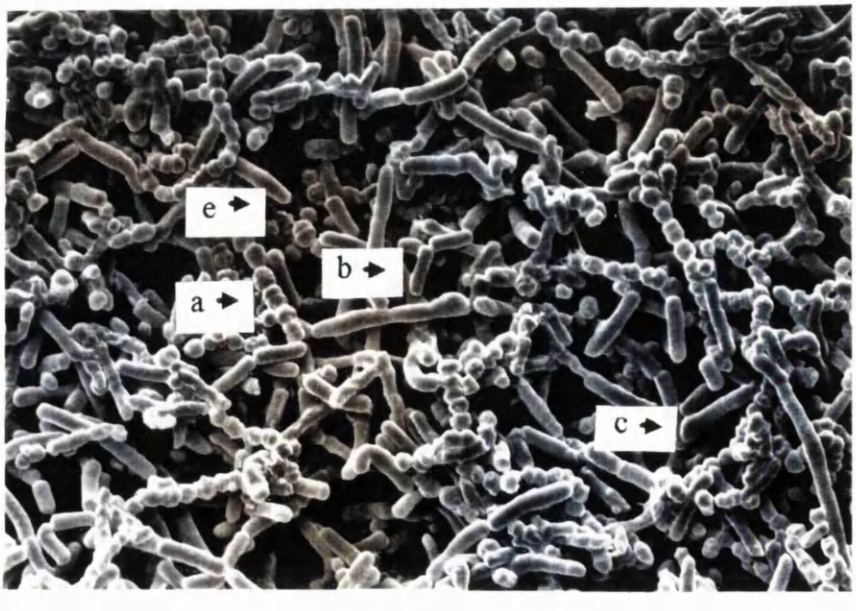


Figure 4.24 A mixed plaque of *S. mutans*, *L. casei* and *A. viscosus* grown upon an Ultrafree-CL ultrafiltration membrane for 21d

(Magnification = 5,000x) ——— 5μm



mixture of the different cell types ('a' = coccoid, 'b' = bacilliary and 'c' = pleomorphic), although it did not display so many low cell density areas ('chasms'). In general the bacteria did not appear different to the ones observed after 6d of culture, however a small number of cells did appear to be damaged (arrowed 'e').

4.2.8 Determination of the effect of various nutrient sources upon bacterial viable counts in Ultrafree-CL ultrafiltration units

(a) Introduction

Growth of the three experimental species of bacteria with Todd Hewitt broth as the nutrient source gave relatively reproducible populations in Ultrafree-CL units. However, Todd Hewitt broth contains a variety of compounds which are likely to complicate and compromise the detection and quantification of minerals and organic acids that will be produced during demineralisation experiments with root surface dentine. In addition, T.H.B. is buffered, which will reduce the magnitude of any pH reduction induced by bacterial fermentation products, with a reduced likelihood of demineralisation occurring. Furthermore the major buffering component of T.H.B. is phosphate, which could overwhelm the detection of any phosphate released from root sections during demineralisation. This medium also contains meat extracts which could make it more difficult to detect any proteins that might be released from the root structure.

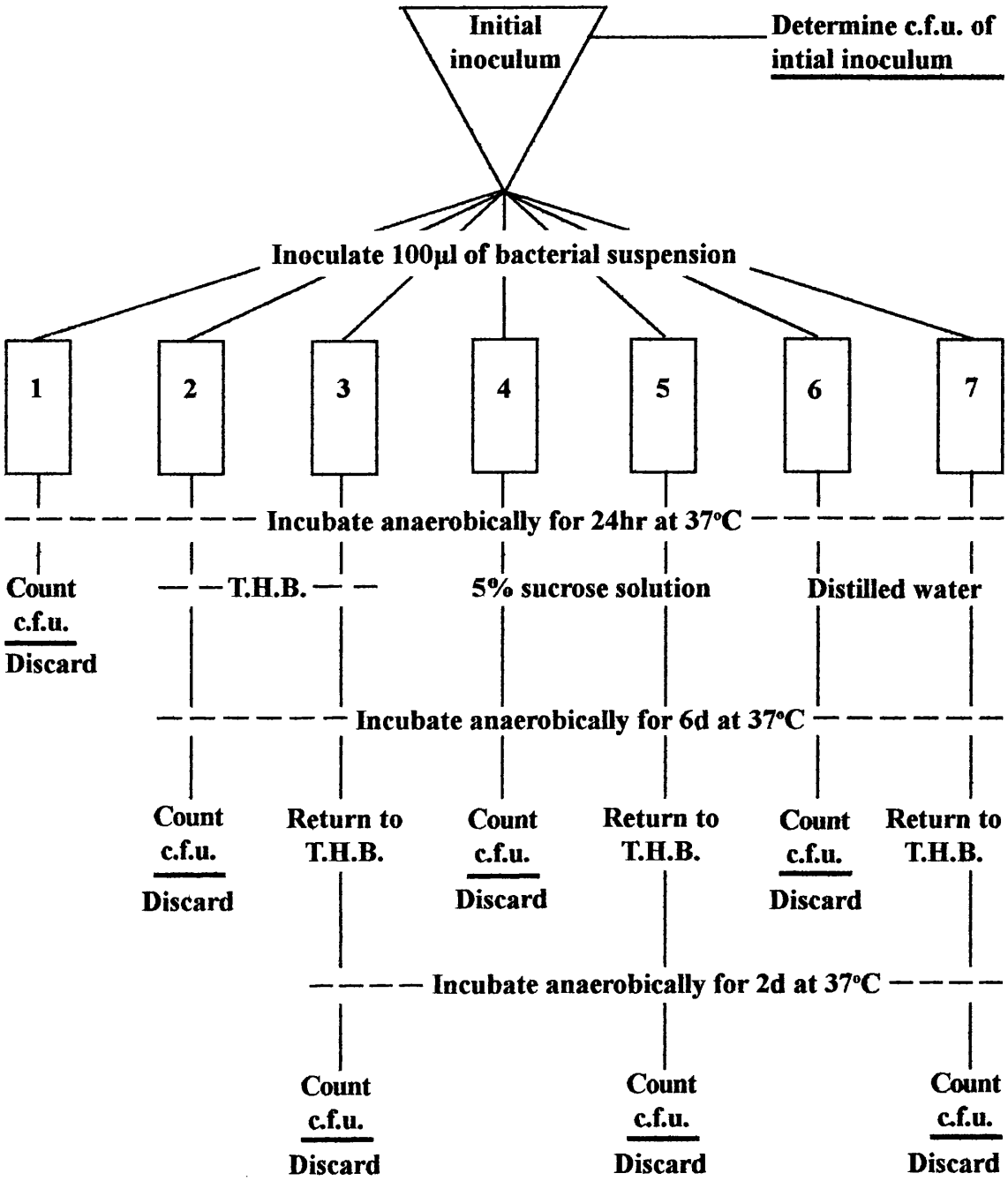
When they grow in the oral environment bacteria are exposed to fluctuating levels of nutrients from salivary flow and their host's diet (Donoghue & Perrons, 1988). In the time between their host's meals the major external nutrient source is likely to be saliva (and perhaps gingival crevicular fluid if near the gingival margin) although some are known to ferment stored carbohydrate (Wijeyweera & Kleinberg, 1989). Since

cariogenic oral bacteria are exposed to a changing environment it would be reasonable to model the caries process by supplying bacterial films with periods of high carbohydrate concentration and then to return them to nutrient medium. Therefore, it would be useful to determine the effect of a variety of nutrient sources upon the viable counts of the test species. The chosen nutrients were; T.H.B. since this has been employed in previous experiments and was capable of sustaining growth of all three organisms in the units, 5% sucrose solution since this will be used later in demineralisation experiments to mimic a high carbohydrate meal and finally distilled water as a negative control.

(b) Materials and methods

Organisms used, growth conditions and determination of bacterial viable counts The bacterial species used were *S. mutans* NCTC 10449, *L. casei* NCTC 6375 and *A. viscosus* NCTC 10951 prepared to a density of 1mg/ml (wet w/v). A 100µl volume of each bacterial suspension was inoculated into the filter component of 7 Ultrafree-CL units standing in T.H.B. Table 4.3 shows an example of the experimental protocol. The inoculated Ultrafree-CL units were incubated anaerobically for 1d at 37°C and one was used to determine the viable count, whilst the broth was aspirated from the other 6 centrifuge tubes in a sterile air laminar flow hood and discarded. The units were then rinsed by pipetting 5.05ml of sterile 135mM KCl into each centrifuge tube and incubating aerobically for 20min at 37°C in an orbital incubator (Gallenkamp, U.K.), after which the KCl was discarded and the process repeated a further two times. After the third incubation the KCl was discarded and 5.05ml volumes of T.H.B., sterile 5% sucrose solution (in distilled water) and sterile distilled water itself were each added to the centrifuge tube components of two separate Ultrafree-CL units (table 4.3). The selection of Ultrafree-CL units for addition of nutrients was randomised. The Ultrafree-CL units

Table 4.3 Protocol for experiment to determine the effect of various nutrient sources upon bacterial viable counts



were then incubated anaerobically for 6d at 37°C, with the medium (T.H.B., 5% sucrose or water) being replaced with a fresh volume of the same solution every second day.

After 6d of incubation the number of c.f.u. in the filter component of one of each pair of Ultrafree-CL units incubated in T.H.B., 5% sucrose and distilled water was determined (table 4.3). The cultural purity and identity of the organisms washed from each Ultrafree-CL was also determined. The second filter of each pair of Ultrafree-CL units was placed in T.H.B. and incubated for a further 2d at 37°C in an anaerobic environment. At the end of this incubation period the number of c.f.u. in the remaining filter unit was determined, as was the cultural purity and identity of the organisms. As in previous experiments the addition of bacteria to the units was randomised and so was the selection of units for sampling at each time point.

This experiment was performed on three separate occasions and the results analysed statistically as described in section 4.2.1. To permit statistical analyses, the absence of retrievable viable counts was arbitrarily recorded as 1×10^1 c.f.u. per unit since this was 10% of the minimum detection limit.

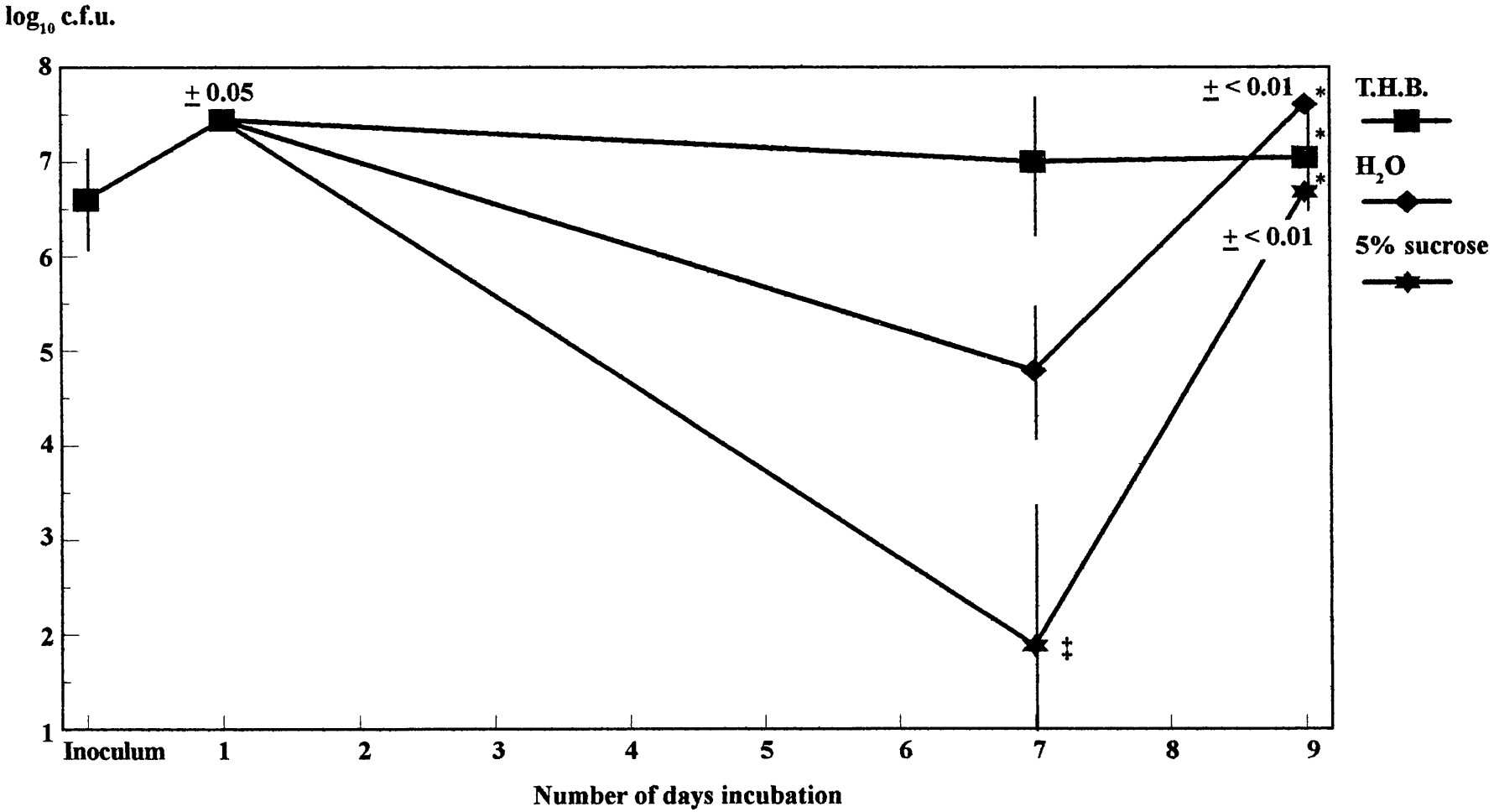
(c) Results

Figure 4.25 shows the mean viable counts of *S. mutans* inoculated into each Ultrafree-CL and also the mean number of c.f.u. retrieved after 1d (T.H.B.), 7d (1d T.H.B. followed by 6d T.H.B., water or 5% sucrose) and 9d (1d T.H.B. followed by 6d T.H.B., water or 5% sucrose followed by a further 2d T.H.B.). The number of c.f.u. of this organism were at least 10 fold higher on the first day of incubation compared with the initial inoculum. There was little difference in the viable counts obtained on day 1

compared with day 7 when the bacteria were cultured with T.H.B. However, when the organisms were exposed to distilled water for 6 days the numbers retrieved were 100-fold less than when exposed to T.H.B. In the case of sucrose solution the numbers were more than 100,000-fold lower (there were no retrievable counts in 2 out of 3 repeats). There were statistically significant differences in the viable counts of films on day 7 between T.H.B., sucrose and water ($p = 0.003$, one-way analysis of variance). However, when *S. mutans* cultures were returned to T.H.B. for 2d the number of viable bacteria returned to a level similar to the cultures exposed solely to T.H.B. (no statistical analyses could be performed since there were insufficient replicate data for this time point).

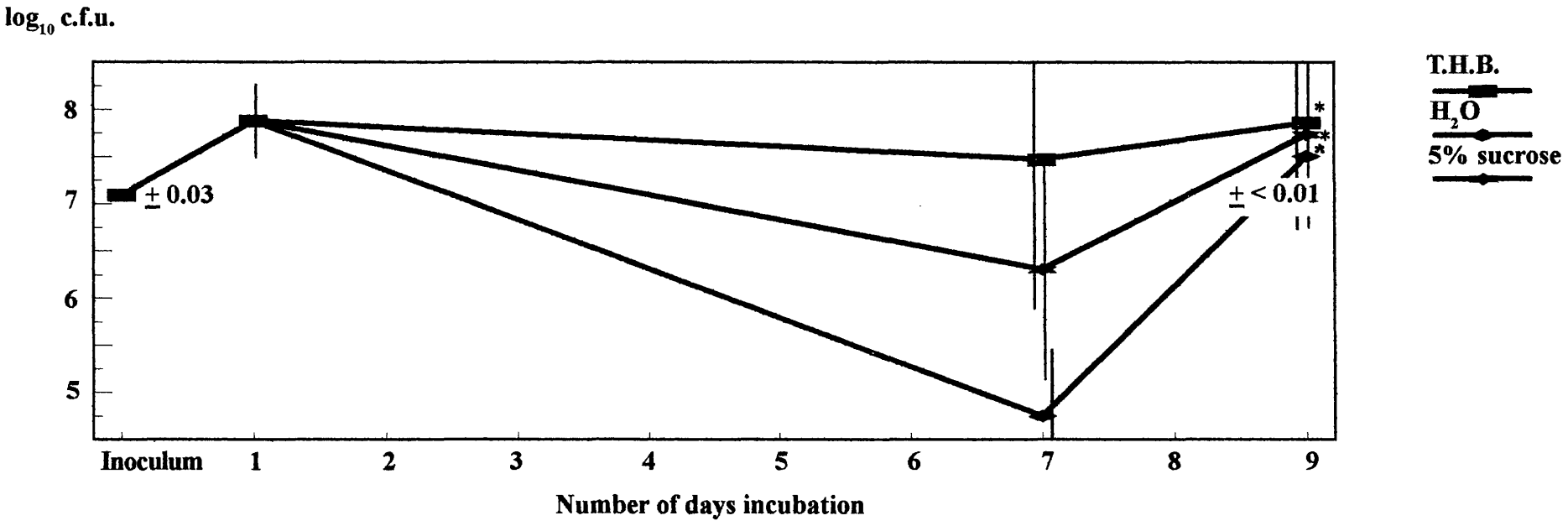
Figure 4.26 shows the mean viable counts of *L. casei* inoculated into each Ultrafree-CL and also the mean number of c.f.u. retrieved after 1d (T.H.B.), 7d (1d T.H.B. followed by 6d T.H.B., water or 5% sucrose) and 9d (1d T.H.B. followed by 6d T.H.B., water or 5% sucrose followed by a further 2d T.H.B.). The number of c.f.u. increased by at least 10-fold during the first day of incubation. Under the influence of T.H.B, the viable counts remained relatively stable during the complete incubation period. When the *L. casei* cultures were exposed to distilled water for 6 days, the number of c.f.u. declined by 1,000-fold compared to cultures supplied with T.H.B. A 10-fold reduction occurred when sucrose was the nutrient source for 6d. There were no significant differences in the viable counts retrieved on day 7 between films supplied with T.H.B., sucrose or water ($p = 0.075$, one-way analysis of variance). When the *L. casei* culture was returned to T.H.B. for 2d the number of viable bacteria increased to a level similar to that which developed when exposed solely to T.H.B. (the difference could not be analysed statistically since there were insufficient replicates at this time point).

Figure 4.25 **The change in numbers of *S. mutans* NCTC 10449 over a total of 9 days incubation when supplied with different nutrients**



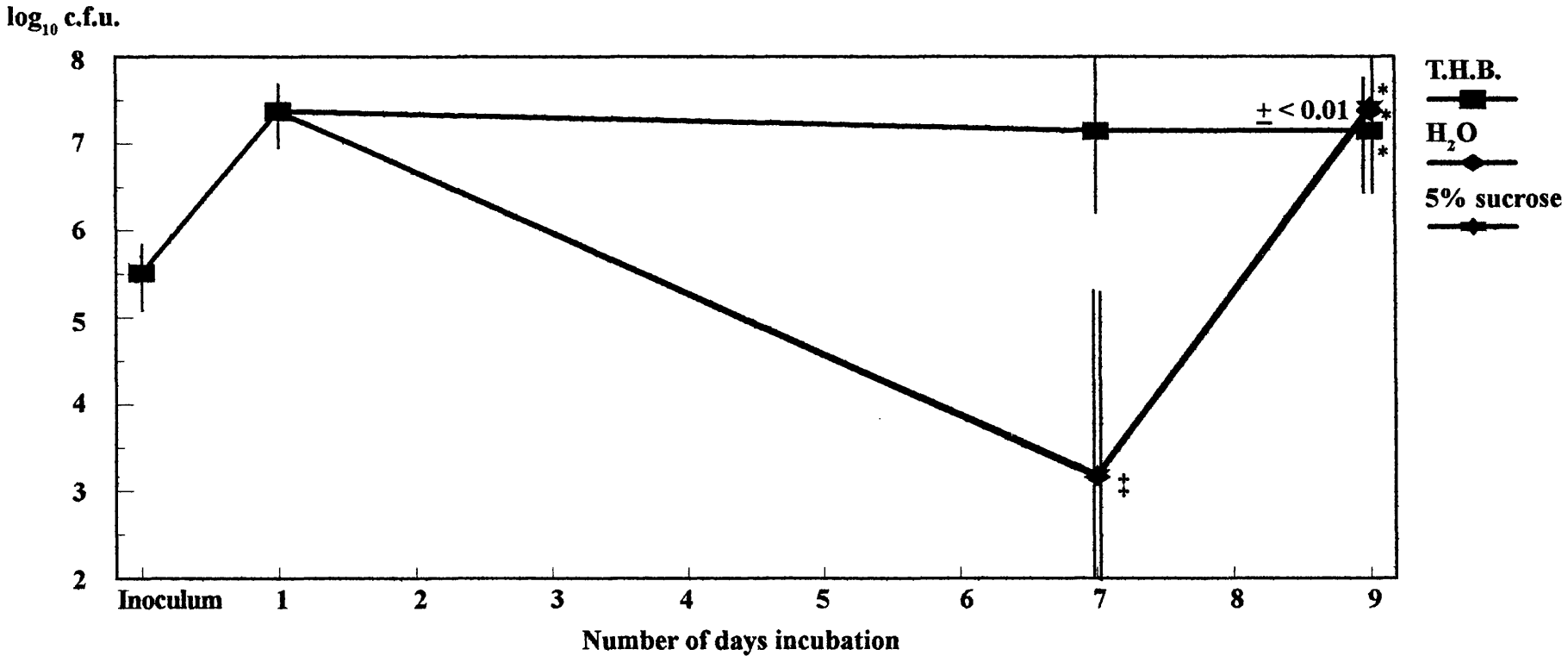
Error bars represent mean ± 1 S.D. n = 3, except for * where n = 2 ‡ = no counts retrieved on two occasions

Figure 4.26 **The change in numbers of *L. casei* NCTC 6375 over a total of 9 days incubation when supplied with different nutrients**



Error bars represent mean ± 1 S.D. n = 3, except for * where n = 2

Figure 4.27 **The change in numbers of *A. viscosus* NCTC 10951 over a total of 9 days incubation when supplied with different nutrients**



Error bars represent mean ± 1 S.D. **n = 3, except for * where n = 2** **‡ = no counts recovered on one occasion**

Figure 4.27 shows the mean viable counts of *A. viscosus* inoculated into each Ultrafree-CL and also the mean number of c.f.u. retrieved after 1d (T.H.B.), 7d (1d T.H.B. followed by 6d T.H.B., water or 5% sucrose) and 9d (1d T.H.B. followed by 6d T.H.B., water or 5% sucrose followed by a further 2d T.H.B.). The number of *A. viscosus* increased by at least 10 fold during the first day of incubation and little change took place over the next 8 days' incubation in T.H.B. When supplied with either distilled water or sucrose for 6d, the numbers of c.f.u. declined by 10,000-fold compared to cultures supplied with T.H.B. (there were no detectable c.f.u. in 1 of 2 repeats using either sucrose or water). Moreover, there were significant differences in the viable counts retrieved between films supplied with T.H.B., sucrose or water ($p = 0.039$, one-way analysis of variance). When the *A. viscosus* cultures were returned to T.H.B. the number of viable bacteria recovered to a level similar to cultures supplied with T.H.B. for all 9d, although statistical significance could not be determined since there were insufficient replicate data.

There were no significant differences between the viable counts of each species retrieved after 1d of incubation with T.H.B. (i.e. day 1)($p > 0.1$, one-way analysis of variance), 1d with T.H.B. followed by a further 6d in T.H.B. (i.e. day 7)($p > 0.1$, one-way analysis of variance) or 1d in T.H.B. followed by 6d of incubation with water (i.e. day 7)($p > 0.1$, one-way analysis of variance). In contrast there were significant differences between the three species in viable counts retrieved after incubation for 1d in T.H.B. followed by 6d with sucrose (i.e. day 7)($p = 0.033$, one-way analysis of variance).

4.3 Discussion and conclusions

4.3.1 Introduction

Chapter 3 described a series of experiments that aimed to elucidate some experimental parameters of a novel *in vitro* model system for the growth of bacterial films. However, it was found that one of the selected bacterial species, *Streptococcus mutans*, could penetrate the supporting membrane after several days' growth. Since an experimental system would be required to retain bacteria for considerably longer than 4 days if root surface caries is to be modelled, it was decided to employ a growth vessel with smaller pores in the filter membrane. Ultrafree-CL ultrafiltration units were selected as an alternative to the Millicell-HA tissue culture inserts employed in chapter 3 as they are supplied with pore sizes of 0.22μm or less. The aims of the initial experiments described in this chapter were similar to those of chapter 3; namely to determine the feasibility of producing bacterial films in Ultrafree-CL ultrafiltration units and to clarify some of the associated experimental parameters.

4.3.2 Preliminary experiments to determine whether bacteria can grow in Ultrafree-CL ultrafiltration units and to determine the optimum number of serial washes for retrieval of bacteria

The three test species were successfully cultured for four days without any evidence that they had penetrated the membrane to contaminate their broth supplies (section 4.2.1). In addition each species yielded at least a 10-fold increase in viable numbers compared with the original inoculum and since more than 99% of the viable counts were retrieved in the first four washes it was decided that four consecutive washes should be employed in future experiments. Furthermore, for the purpose of comparing the data from this experiment with the results of other workers and with the Millicell-HA inserts (results

described in chapter 3), the data were also converted to viable counts per cm^2 of filter membrane.

The three test bacterial species were retrieved from the Ultrafree-CL units at a mean density of 1 x 10^7 (*S. mutans*), 6 x 10^7 (*L. casei*) and 4 x 10^7 c.f.u. per unit (*A. viscosus*). When the sum total viable counts retrieved from each Ultrafree-CL ultrafiltration unit were converted to c.f.u. per cm^2 it was then possible to compare the population density on the filters with the data of others. The values were as follows; *S. mutans* 1.66 x 10^7 c.f.u./cm^2, *L. casei* 1.00 x 10^8 c.f.u./cm^2 and *A. viscosus* 5.01 x 10^7 c.f.u./cm^2. Experiments performed *in vitro* by Li & Bowden (1994) to investigate the accumulation of *S. mutans*, *L. casei* and *A. naeslundii* on mucin conditioned glass surfaces indicated counts of 5.01 x 10^6, 2.75 x 10^6 and 3.39 x 10^7 c.f.u./cm^2 respectively in stable biofilms. Considering the differences in experimental design between the experiments of Li & Bowden and the current ones, such as growth vessel (chemostat compared with Ultrafree-CL units respectively), growth surface (glass compared with P.V.D.F. respectively) and bacterial strains employed (*S. mutans* BM71 compared with NCTC 10449, *L. casei* BM225 compared with NCTC 6375 and *A. naeslundii* compared with *A. viscosus* respectively) the data are remarkably similar. Millward & Wilson (1989) reported approximately 1 x 10^7 c.f.u./cm^2 *Streptococcus sanguis* cells adherent to nitro-cellulose membrane filters which were incubated on the surface of an agar-based medium for 24, 44 and 72hr. The design of Millward & Wilson's study was similar to the current one in that the bacteria were cultured as a film upon a membrane filter. However it differed in that the membrane was composed of cellulose nitrate compared with P.V.D.F. in the current experiments and the nutrients were supplied in an agar base rather than

broth since their aim was to study antibacterial activity of chlorhexidine as opposed to mineral dissolution.

The current data are also compatible with those of van Strijp *et al.* (1994) who recorded 2.82 x 10^6 to 2.88 x 10^8 total c.f.u./cm^2 colonising demineralised dentine *in situ* after 49d. Likewise Leonhardt, Olsson & Dahlén (1995) recorded 7.41 x 10^7 total c.f.u./cm^2 on hydroxyapatite blocks exposed *in situ* in intra-oral appliances for 24hr. Furthermore Macpherson, MacFarlane & Stephen (1991) reported 3.31 x 10^6 and 2.75 x 10^8 total c.f.u./cm^2 adherent to enamel exposed *in situ* in intra-oral appliances after 24 and 48hr respectively. Moreover a recent report by Bradshaw, Marsh, Schilling and Cummins (1996) indicated 3.09 x 10^6 total c.f.u./cm^2 adherent to hydroxyapatite disks exposed to 10 mixed oral bacterial species in a chemostat for 1d, whilst Bradshaw, Marsh, Allison & Schilling (1996) in a similar study (differing only in that the reaction chamber was aerated) recorded 5.93 x 10^6 total c.f.u./cm^2 after 1d. It is noteworthy that, in spite of the very different design of the current model system and that of Millward & Wilson (1989) on the one hand and those of van Strijp *et al.* (1994), Leonhardt, Olsson & Dahlén (1995), Macpherson, MacFarlane & Stephen (1991), Bradshaw, Marsh, Allison & Schilling (1996), Bradshaw, Marsh, Schilling & Cummins (1996) or Li & Bowden (1994) on the other, there was such similarity in the total cell density adherent per cm^2. The similarity between the viable counts per cm^2 of the current model system and other ones of different design indicates that the Ultrafree-CL ultrafiltration unit could prove a useful growth vessel for future experiments.

4.3.3 The long-term population dynamics of the test bacterial species grown in Ultrafree-CL units

Since the test species (*S. mutans*, *L. casei* and *A. viscosus*) could be grown in Ultrafree-CL units for 4 days it was decided to extend the length of the incubation period (section 4.2.2). This was intended both to demonstrate the stability of the films over an extended period of time and also to indicate whether any of the species could penetrate the filter with a pore size that is often used to sterilise heat labile media. However *S. mutans* did penetrate the filter membrane haphazardly in 60% of Ultrafree-CL units incubated for more than 8 days, and so it is likely that filters with pores smaller than 0.22μm diameter will be required for long-term demineralisation experiments. As anticipated, the viable counts of each test species increased significantly during the first 24hr and then remained stable for up to 14d. There are no directly comparable data, although Bradshaw, Marsh, Allison & Schilling (1996) and Bradshaw, Marsh, Schilling & Cummins (1996) also determined the changes in viable counts of a biofilm (albeit containing a mix of 10 species unlike the current experiment which contained individual single species) over a total of 21d of incubation. The ability of Ultrafree-CL units to maintain relatively stable viable biofilms for at least 14d was encouraging, since it was comparable with both of the above reports and indicated that the biofilm viable counts could remain stable for up to 21d.

In contrast to the relative stability of viable counts up to 14d of incubation, there was a tendency in some cases for the number of c.f.u. retrieved to be lower at 21d. Whilst this tendency towards reduced counts was not statistically significant with either *L. casei* or *A. viscosus*, it did become significant between 14 and 21d in the case of *S. mutans*. It is not entirely clear why the number of c.f.u. of *S. mutans* should be significantly lower

after 21d than after 14d. One possibility is that *S. mutans* is simply dying, although this is most unlikely since it was demonstrated in section 4.2.8 that any reduction in viable counts was soon reversed when the cultures were returned to T.H.B. This suggests that even if *S. mutans* died in the 48hr between 'feeds' its numbers ought to increase again when supplied with fresh nutrients. On the other hand it is possible that the *S. mutans* cells were either growing into the Ultrafree-CL filter membrane surface such that although the same overall cell density was present it became less easy to retrieve them all by the standard protocol (a possibility lent some credence by the observation in transverse section light micrographs that *S. mutans* invaded the membrane structure; section 4.2.4). Another possibility, although still controversial, is that some of the cells entered a viable-but non-culturable state as has been reported for *Micrococcus luteus* during long-term starvation by Kaprelyants & Kell (1993). However, there is as yet no direct evidence that *S. mutans* enters a viable-but-non-culturable state nor was starvation prolonged during the current experiments. Nevertheless it would be of interest to determine the underlying reasons for this tendency towards decreased *S. mutans* viable counts between days 14 and 21, since this could have a bearing upon the design of any future long-term demineralisation studies.

4.3.4 Growth of *Streptococcus mutans* in Ultrafree-CL ultrafiltration units with 0.1μm pore size membranes

Penetration of the 0.22μm membrane by *Streptococcus mutans* was prevented by employing membranes with pores of 0.1μm diameter and there were no significant differences in the number of c.f.u. retrieved after 21d when the two pore sizes were compared ($p = 0.034$, Student's T-test), suggesting that the smaller pores do not have a major impact upon the attainable cell density at this time point. Furthermore equivalence

of viable counts with previous experiments using this model system indicates equivalence with the data in the available literature. However to make best use of the stocks of units with a pore size of 0.22μm and also the available time (since another series of exploratory experiments would be required) it was decided to continue to employ Ultrafree-CL units with 0.22μm pores in the membrane filters, since it was felt that preliminary experiments designed to determine the ability of the test species to generate demineralisation in Ultrafree-CL units would not need to continue for more than 8d if biochemical analyses were performed. Moreover it was decided that if the preliminary experiment proved successful and there was time remaining then the 0.1μm membranes could be characterised more fully and then employed for extended demineralisation experiments if all proved well.

4.3.5 Examination by light microscopy of transverse sections through a film of *S. mutans* NCTC 10449 grown in an Ultrafree-CL unit

Transverse sections of Ultrafree-CL ultrafilter membranes (0.22μm diameter pores) which had been inoculated with *S. mutans* were observed by transmission light microscopy (section 4.2.4) to determine whether this organism was escaping from the units by penetrating the membrane filter. It was noted that the *S. mutans* cells had indeed invaded the structure of the membrane filter across much of its surface.

Bacterial penetration of polytetrafluoroethylene (P.T.F.E.) guided tissue regeneration membranes after periodontal surgery has been recognised as a problem and a number of studies have explored this phenomenon. For instance Simion *et al.* (1990 and 1992) exposed P.T.F.E. membranes *in situ* in intra-oral appliances and then examined them by both light microscopy and scanning electron microscopy at weekly intervals. Both

studies indicated that bacterial contamination of the inner (unexposed) surfaces of the membranes commonly occurred between the first and fourth weeks of incubation. However the quoted pore size of 'less than 8μm' suggests that the diameter of the pores is probably greater than the 0.22μm of the Ultrafree-CL membrane filters and therefore bacterial penetration could have been predicted. The ability of some but not all species of oral bacteria to penetrate the filter membranes is interesting and Ultrafree-CL units may represent an experimental model that could be developed further to study bacterial invasion of dentine since a number of studies have demonstrated bacterial spread through dentine both *in situ* (e.g. Schüpbach, Guggenheim & Lutz, 1990 and Schüpbach, Lutz & Guggenheim, 1992) and *in vitro* (e.g. Nagaoka *et al.*, 1995 and van Strijp *et al.*, 1994).

4.3.6 Determination of the effect of initial inoculum density upon final viable counts in Ultrafree-CL ultrafiltration units

Once satisfied that the test strains could be cultured in Ultrafree-CL units for a period of several weeks, experiments were performed to determine whether different densities of initial inocula would result in different final cell densities (section 4.2.5). It was also decided to decrease the most dilute initial inoculum from 0.1mg/ml (wet w/v)(as used in Millicell-HA units) to 0.01mg/ml to determine whether further dilution of the inoculum affected the final density of cells which could be achieved. In spite of a range of 10,000-fold between the most dilute and the most concentrated initial inoculum there were no significant differences between the viable counts retrieved after 4 days of incubation.

The mean viable counts retrieved from units at the end of the experiment ranged from 1.26×10^7 to 1.00×10^8 per unit and they did not appear to be correlated with the density of the initial inoculum. When the viable counts per unit were expressed as a

function of filter membrane surface area a density of 1.59×10^7 to 1.26×10^8 c.f.u./cm^2 was found that equalled the values reported in previous experiments in this chapter and, by analogy, the viable cell densities observed by other workers *in vitro* and *in situ* (van Strijp *et al.*, 1994, Leonhardt, Olsson & Dahlén, 1995 and Millward & Wilson, 1989) whose findings have been described previously.

It is not clear why cell density should be restricted in this model system since the mechanical limitations of the mouth do not apply and the nutrient supply was replaced every second day meaning that any chemical changes (e.g. altered pH, redox potential or loss of essential nutrients) would be reversed for at least some of the time. Furthermore there was little obvious indication of cell death or lysis in scanning electron micrographs (section 4.2.7). However, a body of data is amassing which indicates that some Gram-negative bacterial species liberate a small molecule, or pheromone, which acts as a signal to indicate cell density (see review by Swift *et al.*, 1996). This ability to 'communicate' between the members of a bacterial community, which has been dubbed 'quorum sensing', is involved with the regulation of cellular functions which are dependent upon cell population density (and environmental factors). Quorum sensing appears to operate by the expression of a small, easily diffusible molecule which acts as an autoregulator of its own expression. Once a critical concentration of this molecule is achieved (indicative of a critical cell density) expression of dependent functions occurs. This phenomenon has been described by Winson *et al.* (1995) in relation to the expression of a battery of 'pathogenic' proteases by the human opportunist pathogen *Pseudomonas aeruginosa.* Although there is currently no evidence that this occurs with the (Gram-positive) test species, it is possible that they may also be able to communicate by a similar means and thus regulate their activity in response to cell density. It will be interesting to see whether

improvements in detection techniques and in understanding of the molecular biology of *S. mutans*, *L. casei* and *A. viscosus* reveals that they too have 'quorum sensing' abilities.

4.3.7 Determination of the effect of interacting bacterial species in Ultrafree-CL units on viable counts

In vivo, many different bacterial species are present in dental plaques (see review in section 1.3.3). Furthermore it seems likely that different consortia of species, although having different cariogenic potentials, might all be capable of producing human tooth root surface caries (Nyvad & Kilian, 1990). For this reason the ability of *S. mutans*, *L. casei* and *A. viscosus* to grow together in the Ultrafree-CL model system (section 4.2.6) was investigated since it will be necessary to perform a demineralisation experiment employing all three species.

There were no statistically significant differences between the viable counts of each of the three species grown as either pure or mixed cultures, which suggests that they were able to co-exist under the current experimental conditions. There does not appear to be any directly comparable data in the literature concerning growth of cultures containing mixtures of only the three test species currently employed. Furthermore the novel nature of this model growth system means that it differed from any previous *in vitro* studies of bacterial interaction in the type of growth surface employed and in the way that the nutrients were supplied which makes valid comparisons difficult. Donoghue & Perrons (1988) reported that the numbers of *A. viscosus* and *S. mutans* c.f.u. retrieved from plaques grown on enamel in a model mouth were significantly less in mixed cultures compared with pure. Nevertheless there was a significant increase in *A. viscosus* viable counts in mixed cultures as the incubation progressed from 45 to 90hr (no data was

given for any change in *S. mutans* viable counts between 45 and 90hr). However Donoghue & Perrons employed *Streptococcus mitior*, *Veillonella alcalescens* and *S. mutans* as opposed to *S. mutans* and *L. casei* in the current experiments. Furthermore a complex medium was supplied in a static fashion to the bacteria in the current experiments whereas synthetic saliva was dripped onto the bacterial film by Donoghue & Perrons (1988) which may have tended to dislodge any loosely adherent cells and so affect the population dynamics of the plaque.

In contrast Bradshaw, Marsh, Schilling & Cummins (1996) found that the proportions of *S. mutans* and *L. casei* both increased in 10 species mixed plaques allowed to adhere to hydroxyapatite disks in a chemostat. However the current model system differed from that of Bradshaw, Marsh, Schilling & Cummins in numerous ways; for instance the number of species employed (3 compared with 10 respectively), bacterial strains employed (*S. mutans* NCTC 10449 compared with R9, *L. casei* NCTC 6375 compared with AC413 respectively), growth vessel (Ultrafree-CL unit as opposed to a chemostat respectively) and growth surface (P.V.D.F. membrane filter and hydroxyapatite disk respectively). Thus a comparison of changes in the proportions of bacteria in the two model systems is of limited value. Nevertheless it was encouraging that the three test species could co-exist in the Ultrafree-CL units as this suggests that it should be possible to develop mixed plaques of at least these species for demineralisation of root sections.

4.3.8 Examination of pure and mixed bacterial films in Ultrafree-CL ultrafiltration units by scanning electron microscopy

The surface upon which the bacterial films were grown in this model system differed from human tooth root surfaces (i.e. in its chemistry) and the environment in Ultrafree-

CL units was different to that encountered *in situ* (i.e. in nutrient supply and shear forces acting upon the film). Therefore it was possible that the bacterial films which developed were physically different to plaques grown upon cementum or dentine surfaces *in situ*. The current experiments (section 4.2.7) were thus designed to give an indication of the appearance of bacterial films grown in Ultrafree-CL units which could be compared with films grown *in situ* since this would be the natural environment.

Streptococcus mutans cells grown in the current model system for both 6 and 21d appeared similar to those grown on the surface of mucin-conditioned glass in a chemostat by Li & Bowden (1994); i.e. interwoven chains of cocci with no obvious sign of intercellular matrix. In addition there were no lysed *S. mutans* cells (i.e. cell walls) after 6d of incubation and only very limited numbers after 21d which tends to indicate that the cells retained vitality throughout the incubation period. Moreover the 6 and 21 day-old cultures of *A. viscosus* had a similar cell morphology to those of the very closely related organism *A. naeslundii* noted by Li & Bowden; i.e. branching, pleomorphic filaments. Furthermore there was no sign of *A. viscosus* cell lysis during the current experiments which would have indicated a loss of vitality. However, during the present experiments only a few *L. casei* cells could be observed scattered across the surface of the membrane filters after either 6 or 21d; this is most likely a result of cells becoming dislodged during preparation for electron microscopy since *L. casei* does not have a reputation for strong adherence to surfaces such as enamel (Marsh & Martin, 1992).

A number of studies have explored bacterial colonisation of P.T.F.E. guided tissue regeneration membranes exposed *in situ*; although they are not directly comparable with the current data (since it was generated *in vitro*) there remain some interesting

comparisons. For instance the current scanning electron micrographs of a mixture of *S. mutans*, *L. casei* and *A. viscosus* grown for 6d showed a relatively homogeneous distribution of cell types (i.e. cocci, rods and filaments) rather than segregation into microcolonies, which corresponds with the observations of Simion *et al.* (1994) after 1 week of growth *in situ*. However after longer incubation periods Simion *et al.* noted a shift towards a greater proportion of rod and filamentous-shaped bacteria; a trend which was not observed in the current experiments. Most likely the absence of any succession of bacterial populations during the current experiments results from the absence of any selective pressures such as shear forces (from salivary flow and mastication), changes in nutrients (from meals) and colonisation by extraneous bacteria (from saliva) which would be encountered *in situ*.

It was encouraging to note that some of the bacterial surface features observed during the current experiments have also been recorded *in situ*. For instance Nyvad & Fejerskov (1987a) found that root surfaces exposed *in situ* were colonised with a heterogeneous plaque which showed little evidence of extracellular matrix; it was suggested that this may have been due to removal of extracellular polysaccharides by the preparative processes employed. There was little sign of matrix during the current experiments and this may have been for the same reason. Moreover the cocci observed in the current study had a granular layer on their cell surface in some cases (at high magnification) and also a circumferential constriction which were both noted by Nyvad & Fejerskov (1987a). Therefore scanning electron micrographs taken during the current experiments indicate that the bacteria have similar morphologies to those observed previously *in vitro* and *in situ*.

4.3.9 Determination of the effect of various nutrient sources upon bacterial viable counts in Ultrafree-CL ultrafiltration units

Up to this point the experiments were designed to explore whether stable biofilms of pure or mixed cultures of *S. mutans, L. casei* and *A. viscosus* could be produced in Ultrafree-CL units with the potential to demineralise tooth root surfaces. However, it was not clear what would happen to the numbers of viable bacteria when the films were supplied with carbohydrates and so an experiment was designed to explore this area (section 4.2.8). In order to make any potential changes in viable counts obvious it was felt that the bacterial films should be exposed to each of the test solutions (T.H.B., 5% sucrose or distilled water) for a substantial period of time rather than try to mimic the *in vivo* situation. Since previous experiments had determined viable counts after 6d of incubation this period of exposure to the test solution was selected to allow comparisons with the past experiments at this time point.

The first experiment indicated that the number of bacteria retrieved from units supplied with sucrose solution or distilled water alone was much lower than from those supplied with T.H.B. Since it was not known if any reduction in numbers due to incubation with either sucrose solution or distilled water would be reversed by the addition of medium containing fresh nutrients and buffering components the experiment was made more sophisticated by returning units which had been exposed to sucrose or water for 6d to T.H.B. for a further 2d. Unfortunately, a lack of time meant that it was not possible to perform a third repeat of the extended experiment which is why the results for that sampling point are in duplicate whilst the others are in triplicate.

After the initial 24hr incubation period the viable counts retrieved (at 3.16×10^7 to 6.31×10^7 c.f.u./cm^2) were comparable with previous experiments in this chapter and therefore with other workers which suggests that the bacterial films were behaving as expected during this time. However subsequent exposure to sucrose solution or distilled water led to significant reductions in viable counts of all three species during the following 6d. It is likely that reduced viable counts in the presence of sucrose was due to the build up of acids in the environment by bacterial fermentation of sucrose. In contrast prolonged incubation with distilled water (6d in the current experiment) could have resulted in either cell death or possibly development of a viable-but-non-culturable state as mentioned before. Since no information is currently available concerning viable-but-non-culturable states in *S. mutans*, *L. casei* or *A. viscosus* this is only speculative and requires further study.

The three test species reacted in different ways to incubation with sucrose or distilled water; whilst *S. mutans* viable counts were lower with sucrose than water, those of *L. casei* were lower with water than sucrose and those of *A. viscosus* were equally low with either. However there appears to be little mention in the literature of the effect of sucrose or water upon viable counts of oral bacterial films cultured *in vitro*. Furthermore cycling of nutrient media and its impact upon subsequent viable counts does not seem to have been examined to any great extent in its own right, rather it tends to have been used to more accurately simulate the situation within the mouth (e.g. Zampatti, Roques & Michel, 1994).

Whatever the underlying reasons for reduction in viable counts when the bacterial films were supplied with sucrose or water (compared with the T.H.B. control culture) it was

encouraging to find that subsequent incubation with T.H.B. led to a recovery in counts to a density that approximated to the T.H.B. control cultures. This suggests that, for a potential demineralisation experiment employing the Ultrafree-CL model system, the number of viable bacteria would recover after return to T.H.B. even though they had decreased during incubation with sucrose or water. This aspect of the model should be an advantage since the cell density present at the start of sequential incubation periods in sucrose could be standardised to that present at the initiation of the experiment. Thus one potential source of variation (i.e. different counts between the species and between incubation periods) should be controllable. Overall the degree of variation in the data between repeats of the experiment was within acceptable limits. However there was substantial variation in the viable counts between repeats after the films had been incubated with either sucrose or water, indeed the viable counts had been reduced to below the detection limit of the culture method employed in a number of cases.

4.3.10 Comparison of Ultrafree-CL units with Millicell-HA inserts

Millicell-HA inserts were generally easier to use than Ultrafree-CL units since fewer, less intricate, preparatory steps were required. For example Millicell-HA units were supplied pre-sterilised and preparation did not involve the assembly of a series of components that required to be sealed with a smear of Vaseline. Furthermore replacement of the Millicell-HA culture medium was simple and did not require the same degree of manipulation as Ultrafree-CL units (e.g. the need to break and re-establish the seal). In contrast retrieval of bacteria from Millicell-HA units was more complicated than Ultrafree-CL units since the membrane and supporting collar had to be separated prior to retrieval of from Millicell-HAs whereas wash buffer could simply be pipetted into Ultrafree-CL units. However the overall handling advantages of Millicell-HA units over Ultrafree-CL units

would have made them the units of choice if they had been available with 0.22μm diameter pores.

A comparison of both model systems showed similarity in the way that bacterial viable counts developed. For instance there was a substantial increase in the number of c.f.u. retrieved from both types of unit when inoculated with a low density of bacterial cells initially. In contrast there was no increase in bacterial counts from the highest density of initial inoculum in either culture vessel which indicates that both have a maximum population density or 'climax community'. Finally a comparison of the scanning electron micrographs indicated no discernible difference between bacterial films grown upon both type of membrane, although the membranes themselves were different since Millicell-HA membrane filters appeared to be globules or nodes randomly linked by strands of structural material whereas Ultrafree-CL membranes appeared much more fibrous. In addition whilst Millicell-HA membrane filters are composed of cellulose esters, those of Ultrafree-CL membranes are composed of P.V.D.F. Taken together, the above indicates that although Millicell-HA units would have been preferred, Ultrafree-CL units were an acceptable alternative to them since films with similar viable counts and appearance could be cultured.

4.3.11 Conclusions

To conclude, the *in vitro* model system based upon Ultrafree-CL ultrafiltration units was capable of supporting the growth of bacterial films which could remain relatively stable for 2 weeks and more. Furthermore the maximum density of bacteria which could be cultured appeared to be relatively constant irrespective of the initial inoculum or test species employed. The viable counts of films developed in these units were also

comparable with the results recorded with Millicell-HA units and also the results of other workers. In addition, mixtures of the test species could be grown in the model system just as effectively as pure cultures. Moreover, although *Streptococcus mutans* was occasionally able to penetrate the Ultrafree-CL membrane filter with 0.22μm diameter pores in a rather haphazard manner, units with 0.1μm pores prevented such bacterial penetration in long-term experiments. Furthermore it was found that exposure of bacterial films to T.H.B. subsequent to either sucrose solution or distilled water resulted in the recovery of depressed viable counts to a level comparable with that of films exposed only to T.H.B. In contrast, the three species employed (*S. mutans*, *L. casei* and *A. viscosus*) reacted in very different ways to being supplied with either sucrose solution or distilled water. However, the fact that recovery of viable counts was possible and that the retrievable number of c.f.u. tended to equilibrate at between 1.59×10^7 and 7.08×10^7 c.f.u. per cm^2 suggested that the Ultrafree-CL model system could be employed in experiments to study the dissolution of root surfaces.

Chapter 5 Preliminary experiments to explore demineralisation of root sections in the Ultrafree-CL model system

5.1 Introduction

In general terms, human dental caries occurs as a result of dissolution of tooth mineral by the organic acids produced by bacteria in dental plaque as fermentation by-products (Dashper & Reynolds, 1996). Much is known about the ability of different micro-organisms to produce organic acids during metabolism (e.g. Distler *et al.*, 1992 and Hudson, Donoghue & Perrons, 1986) and in addition there is a large body of data concerning the involvement of various bacterial species in coronal caries. However information about the precise role of bacteria in root surface caries is currently relatively poor. There are a number of problems inherent in the study of human root surface caries which have contributed to the relative paucity of data. Firstly, as with all biomaterials, human tooth roots can vary in their chemical composition (Selvig & Selvig, 1962). Secondly, little is known about the importance of an intact cemental layer in the caries process. Thirdly, it is relatively difficult to obtain sufficient human teeth of an acceptable quality and it is even more difficult to obtain all of them from the same individual to reduce inter-sample variations. Finally tooth root surfaces tend to be exposed to the mouth progressively with age due to gingival recession (Fure & Zickert, 1990) and exposed surfaces can develop a hypermineralised surface layer (Nakata, Stepnick & Zipkin, 1972) which can affect the dissolution characteristics of roots from the elderly compared with unexposed (young) roots. However there is little data about the mechanisms involved, or if the processes of exposure and hypermineralisation vary among individuals.

To overcome some of the problems noted above, some investigators have resorted to employing bovine rather than human roots as their experimental material since they are relatively easy to obtain (e.g. Klont & ten Cate, 1991). Moreover, with bovine material it is possible to harvest a relatively large number of samples from one donor and therefore inter-individual variations can be avoided. However, studies which employed bovine dentine have, on the whole, examined the biochemical processes involved in dissolution with organic acids of non-bacterial origin, rather than with bacterial plaques (section 1.4.5). In contrast other workers have chosen to employ hydroxyapatite as their test material (e.g. Chestnutt, MacFarlane & Stephen, 1994 modelled enamel caries with hydroxyapatite powder) since it should be relatively homogeneous in chemical composition and easy to obtain. However hydroxyapatite is chemically quite different to tooth root tissue, for instance it does not contain a protein structural matrix. Human tooth root tissue has been employed for demineralisation studies, but most studies have involved the use of non-bacterial acids, whilst those studies which did employ bacteria to induce carious lesions in human material tended to examine the physical rather than biochemical changes that occurred (e.g. microradiographic analyses were performed by Clarkson, Wefel & Miller, 1984 and Clarkson *et al.*, 1987).

Since data concerning the biochemical events that occur in human tooth root surface caries are limited, experiments were devised to measure demineralisation of human tooth root blocks and also of hydroxyapatite powder (as a more consistent positive control). Bacterial films were grown on membrane filter surfaces using the model system developed in chapter 4 as the culture vessel and then root slices or hydroxyapatite powder and sucrose were introduced into the system. At the end of the incubation period the spent culture fluid was analysed for calcium ions and a range of selected acid anions.

5.2 Materials and methods

5.2.1 Preparation of human tooth root slices

Human tooth root annular slices ('root blocks') for use in the demineralisation experiment were prepared as follows. Firstly, human premolar teeth which had been extracted for orthodontic reasons were collected by dental practitioners in the Glasgow area and stored in a saturated solution of thymol (BDH, U.K.) at 4°C. About a month after collection the teeth were washed for 24hr in three changes of sterile distilled water (20ml). The periodontal ligament was then gently scraped from the surface of each tooth root using a clean scalpel blade (#11, Swann-Morton, U.K.) and each tooth was halved longitudinally using a Microslice II precision slicing machine (Malvern Instruments, U.K.) mounted with a diamond coated circular saw (Malvern Instruments, U.K.) at 300r.p.m. with aqueous cooling (Aquagrind, Malvern Instruments, U.K.). Next the central third of each tooth root was excised using the diamond coated saw to give a section of approximately 7 x 7mm.

The cemental surface of each section was masked with a 16mm^2 section of masking tape and then all surfaces of the block varnished twice with nail varnish (Max Factor nail enamel # 29, U.K.) which was allowed to dry overnight at room temperature. Subsequently the masking tape was removed leaving a varnish-free window and each section was disinfected for 1hr at 4°C in a standard solution of Titan Chlor-Tabs disinfectant (2 tablets per litre = 1,000p.p.m. chlorine)(Lever Industrial Ltd., U.K.) followed by washing for 24hr in 3 x 20ml volumes of sterile distilled water at 4°C. Next a 25mm long sterile glass rod was attached to the back of each root section (figure 5.1a) with a smear of nail varnish (this was performed in the sterile air laminar flow hood to maintain sterility). To verify sterility two of the prepared blocks were incubated

Figure 5.1a **Diagram of a root block mounted on a glass rod (Not to scale)**

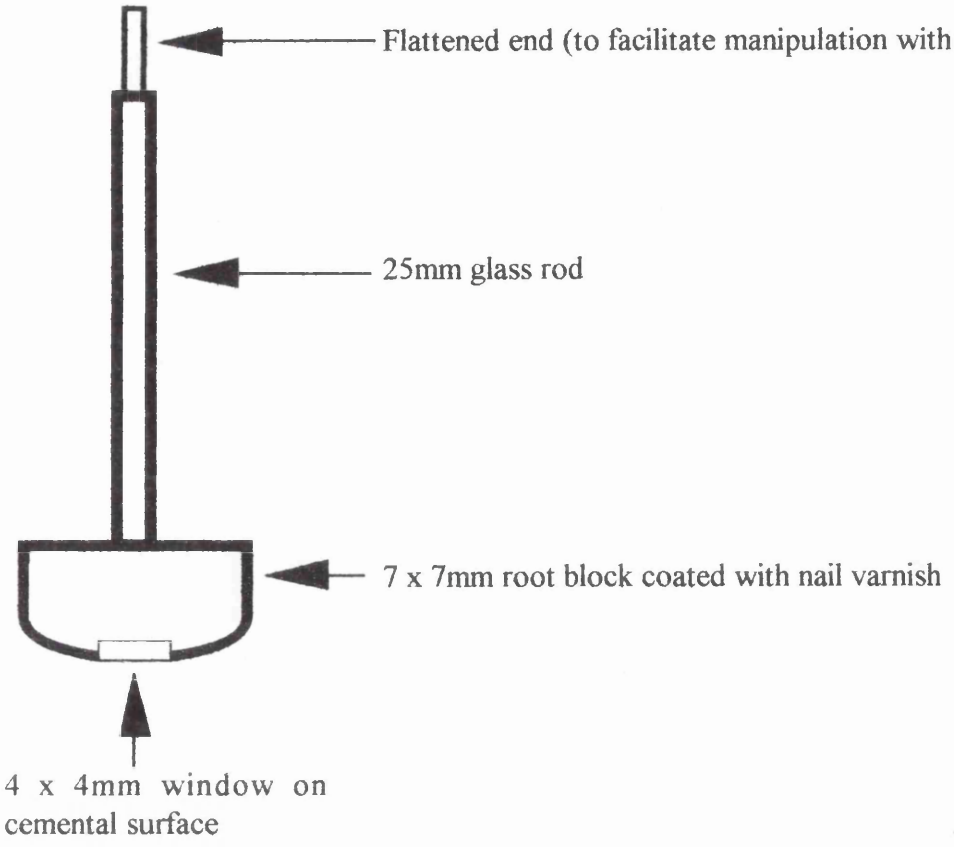
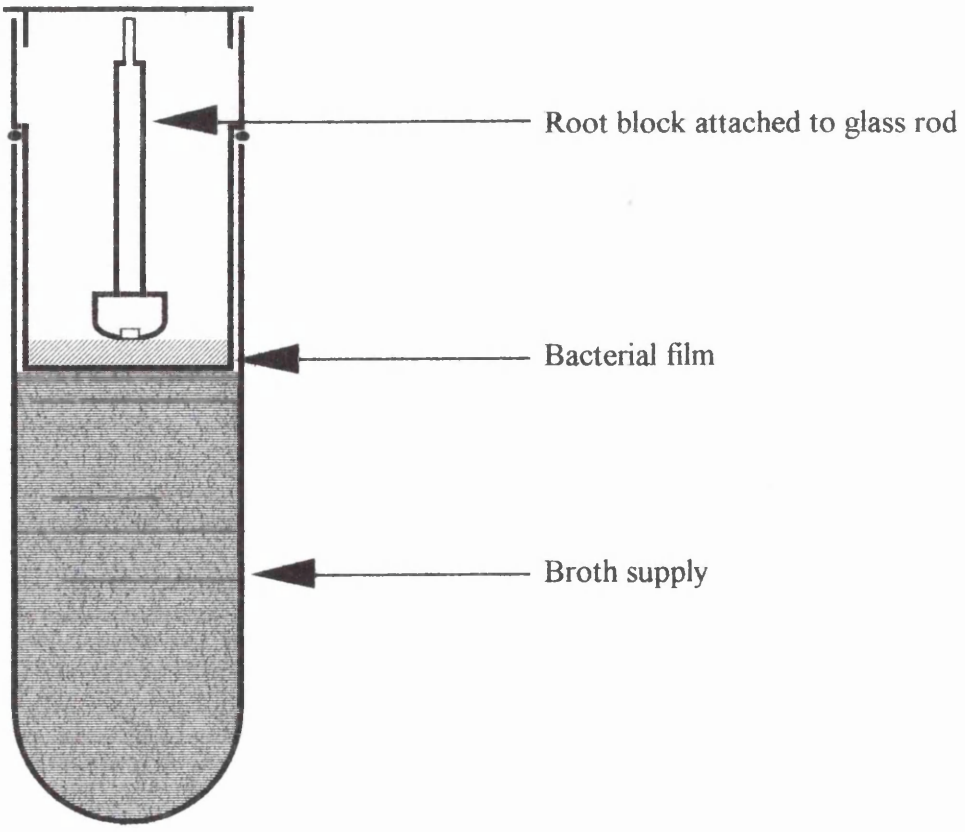


Figure 5.1b **Diagram of a root block *in situ* (Not to scale)**

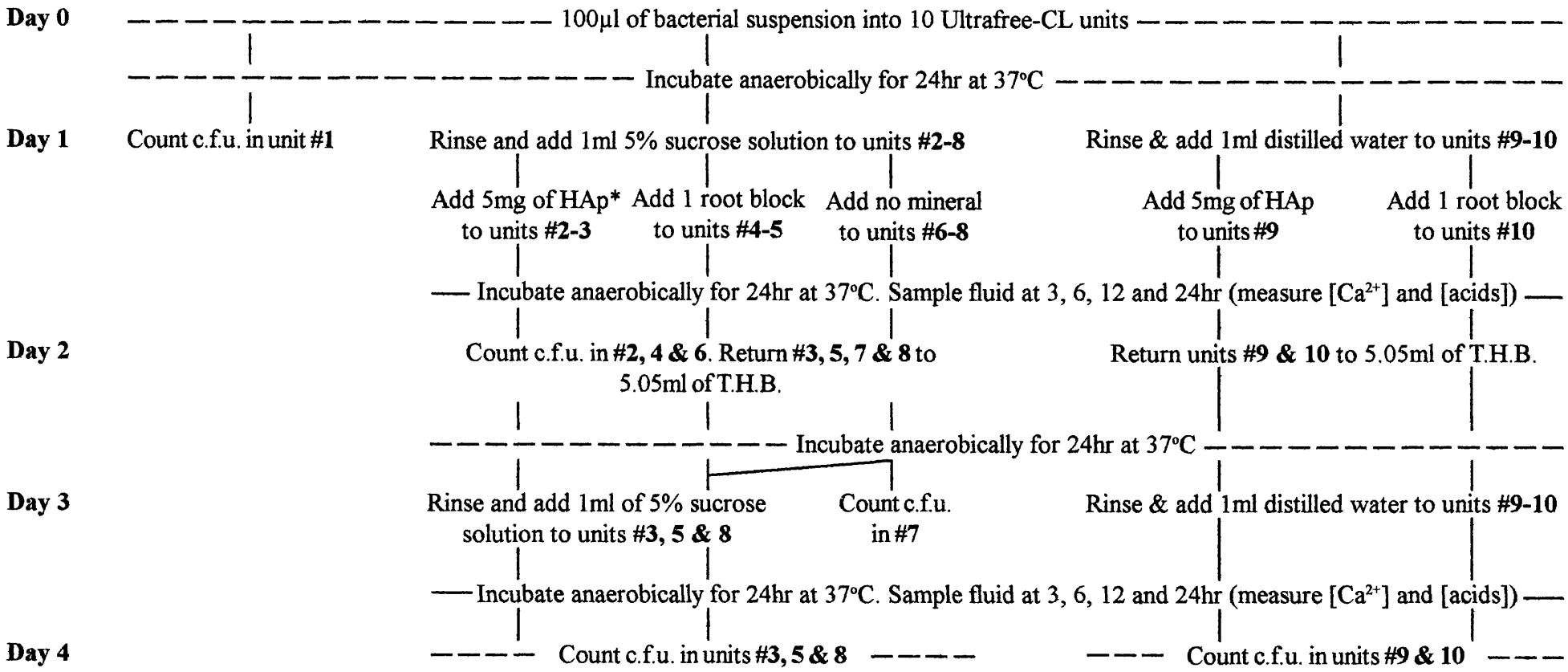


anaerobically for 72hr at 37°C in 5ml of T.H.B., after which a loopful of *S. mutans* NCTC 10449 was inoculated to one to ensure that no disinfectant residues were present which could inhibit bacterial growth.

5.2.2 Organisms used, growth conditions and determination of bacterial viable counts

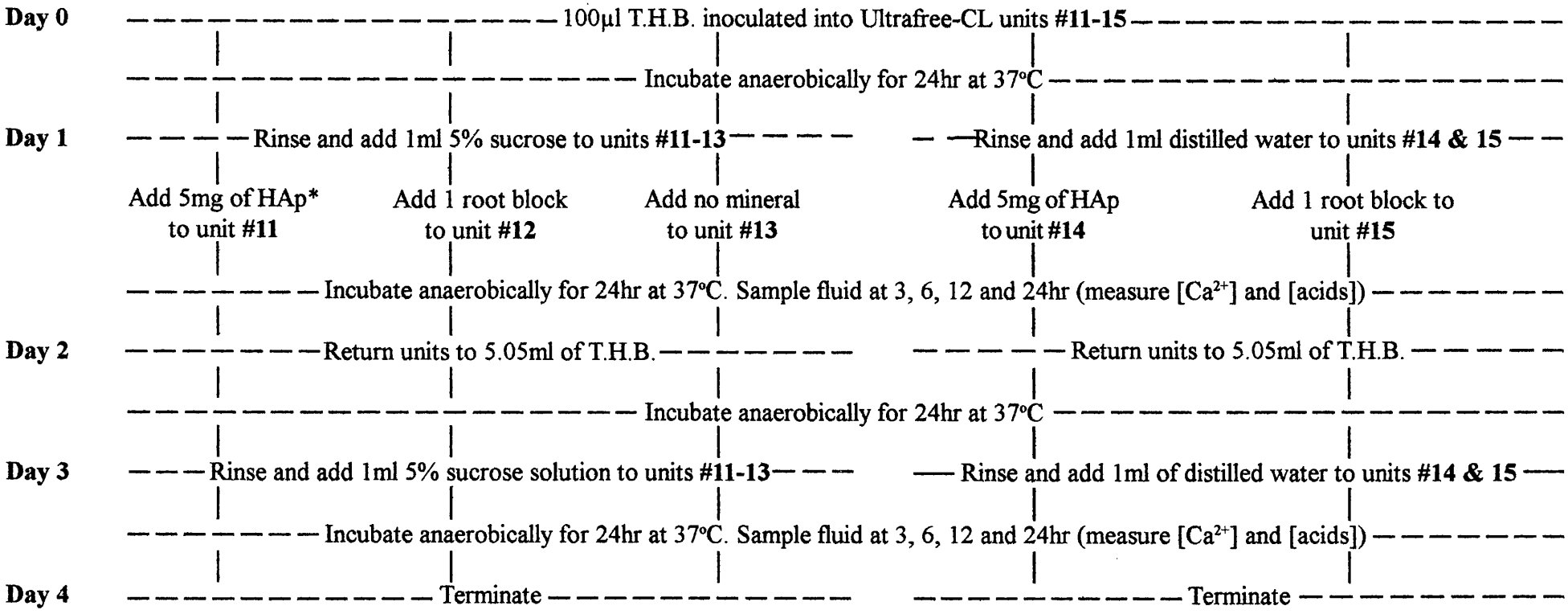
Figures 5.2 and 5.3 show the protocol for the following experiment. The species employed were *S. mutans* NCTC 10449, *L. casei* NCTC 6375 and *A. viscosus* NCTC 10951 which had been prepared to a density of 1mg/ml (wet w/v). A 100µl volume of each bacterial suspension was inoculated into the filter component of 10 Ultrafree-CL units (numbered **#1 - #10**) standing in 5.05ml of T.H.B. (figure 5.2). In addition five bacteria-free negative controls (numbered **#11 - #15**) were each inoculated with a 100µl volume of sterile T.H.B. (figure 5.3). The inoculation of each bacterial suspension to the test filter units was randomised (Wardlaw, 1987) as described in chapter 4. It should be noted that the unit numbering system employed in the text is for illustrative purposes to make description of the experimental protocol simpler; in the actual experiments the mineral/ sucrose combinations were added to randomly selected units. The inoculated Ultrafree-CL units were incubated anaerobically for 1d at 37°C, after which time the number of c.f.u. present in unit **# 1** was determined in turn for each species (figure 5.2). The number of viable counts present in each unit was determined by the method developed in Section 4.2.1. If no c.f.u were obtained then 10% of the minimum detection limit (i.e. 1.00 $\log_{10}$ c.f.u.) was inserted to allow statistical analyses to be performed.

Figure 5.2 **Experimental protocol for demineralisation experiment; repeated for each bacterial species**



* HAp = hydroxyapatite powder

Figure 5.3 **Experimental protocol for demineralisation experiment; bacteria free controls**



* HAp = hydroxyapatite powder

5.2.3 Demineralisation conditions

The following protocol was repeated for each bacterial species. After 1 day of incubation the remaining Ultrafree-CL units (**#2 - #10**) were rinsed with 3 volumes (5.05ml) of sterile 135mM KCl, as described in section 4.2.8. After the units had been rinsed, 5mg of sterile hydroxyapatite was poured carefully onto the bacterial film in 3 filters (**#2, #3**, and **#9**). A human tooth root block, prepared as described, was placed onto the film in a further 3 units (**#4, #5** and **#10**). Figure 5.1b shows a root block *in situ*. No mineral was added to the remaining Ultrafree-CL filter units (**#6** and **#8**), which acted as negative controls (figure 5.2). Addition of mineral to the filter units was randomised.

Following addition of mineral to the Ultrafree-CL units, 1ml of 5% sucrose was gently layered onto the surface of the bacterial film in 3 filters with no mineral (units **#6, #7** and **#8**), 2 filters to which 5mg of hydroxyapatite powder had been added (units **#2** and **#3**) and 2 filters to which root blocks had been added (units **#4** and **#5**). Sucrose free negative controls were also prepared by gently pipetting 1ml of sterile distilled water onto the surface of the bacterial film in 1 filter to which 5mg of hydroxyapatite powder had been added (unit **#9**) and 1 filter to which a root block had been added (unit **#10**)(figure 5.2). Addition of water or sucrose to each filter unit was randomised.

The bacteria-free negative controls (units **#11 - #15**) were treated as outlined in table 5.3. After 1 day of incubation the Ultrafree-CL units were rinsed with 3 x 5.05ml volumes of sterile 135mM KCl. Next 5mg of sterile hydroxyapatite powder was poured carefully onto the surface of 2 filters (**#11** and **#14**). A human tooth root block, prepared as described, was placed onto the surface of a further 2 Ultrafree-CL units (**#12** and **#15**). The remaining Ultrafree-CL filter unit (**#13**) remained mineral free and acted as a

negative control (figure 5.3). Addition of the different mineral sources to filter units was randomised.

Following addition of mineral to the bacteria-free negative controls, 1ml of 5% sucrose was gently layered onto the surface of 1 filter without mineral, 1 filter to which 5mg of hydroxyapatite powder had been added and 1 filter to which a root block had been added (units **#11**, **#12** and **#13** respectively). Sucrose-free negative controls were prepared by gently pipetting 1ml of distilled water onto the surface of 1 filter to which 5mg of hydroxyapatite powder had been added and 1 filter to which a root block had been added (units **#14** and **#15** respectively)(figure 5.3). Addition of water or sucrose to each filter unit was randomised.

Once all the additions had been made, each unit was incubated anaerobically for 24hr at 37°C. During the incubation period, fluid percolated down through the bacterial film and membrane filter and dripped into the empty (lower) centrifuge tube component. Working in the sterile-air laminar flow-hood all the fluid was aspirated from each (lower) centrifuge tube after 3, 6, 12 and 24hr of incubation and stored at -70°C prior to biochemical analyses being performed. After 24hr of incubation any fluid that remained covering the bacterial film in the upper filter component was also aspirated, centrifuged for 5min at 13,500r.p.m. in a microcentrifuge (M.S.E. Microcentaur, M.S.E., U.K.) to remove particulate matter and stored at -70°C prior to analysis.

After incubation for 24hr with the various nutrient and mineral sources, the viable counts present in one unit containing sucrose only (unit **#6**), one containing sucrose plus hydroxyapatite powder (unit **#2**) and one containing sucrose plus root section (unit **#4**)

were determined and all were screened for cultural purity. This was performed for each bacterial species. The remaining Ultrafree-CL units were returned to 5.05ml of T.H.B. (which was added to the (lower) centrifuge tube) and incubated anaerobically at 37°C for a further 24hr. At the end of this incubation period the viable counts in another randomly selected Ultrafree-CL unit (unit #7), using units which had been supplied with sucrose solution but not mineral, were determined along with the cultural purity and identity of the organisms (figure 5.2). Again this was performed for each of the three bacterial species.

The Ultrafree-CL units left (units **#3**, **#5**, **#8**, **#9** and **#10** respectively) were rinsed as above. A 1ml volume of sterile 5% sucrose solution was then gently pipetted onto a previously sucrose fed film (i.e. units **#3**, **#5** and **#8** respectively) whilst sterile distilled water was gently pipetted onto a previously water fed film (i.e. units **#9** and **#10** respectively)(figure 5.2 and 5.3). The units were then incubated anaerobically for 24hr at 37°C and the protocol for collection of spent culture fluid and determination of viable counts was followed as described above.

The bacteria-free negative controls (units **#11 - #15**) were treated in the same fashion as the experimental units (units **#2 - #10**) but since no bacteria had been added, none of the units was required to determine c.f.u. (figure 5.3).

5.2.4 Analyses performed on samples collected

Volume:- The volume of the stored samples was determined as follows. Firstly the volume pre-set control of a micropipette (Volac high precision micropipettor, John Poulten Ltd., U.K.) was adjusted to show a volume greater than the estimated volume of

the sample. The sample was then drawn into the tip (Alpha Labs., U.K.) of the pipette in the normal manner. After the sample fluid had been drawn into the pipette tip the excess air was expelled by adjusting the volume pre-set control until the fluid filled only the most distal portion of the pipette tip. The volume indicated on the volume pre-set scale corresponded to the volume of fluid contained in the pipette tip.

Calcium:- The Calcium ion concentration in each sample was estimated using the Sigma Calcium assay (Sigma Diagnostics, U.K.) as employed by Chestnutt (1992). The working reagent was prepared by mixing equal volumes of 'Calcium binding reagent' (0.024% o-cresolphthalein complexone and 0.25% 8-hydroxyquinoline) and 'Calcium buffer' (0.5M 2-amino-2-methyl-1,3-propanediol). One millilitre volumes of working reagent were pipetted into polystyrene cuvettes with a 1cm light path (Elkay Ultra-Vu microsquare disposable cuvettes, Elkay Laboratory Products Ltd., U.K.) and their absorbance measured at 575nm with a Pye-Unicam SP8-100 Spectrophotometer (Pye-Unicam, U.K.), using distilled water to set zero. Ten microlitre volumes of each test sample and also of a 10mg/dl Calcium standard solution (Sigma Diagnostics, U.K.) were pipetted into duplicate cuvettes of working reagent and thoroughly mixed by repeated aspiration with a micropipette. The absorbance of each mixture was then measured at 575nm and the concentration of calcium calculated as follows:-

$$\frac{Abs_{575}\ T - Abs_{575}\ I}{Abs_{575}\ S - Abs_{575}\ I} \times C = \frac{[Ca^{2+}]\ mg/dl}{k} = [Ca^{2+}]\ mM$$

Where;

Abs_{575} T = the absorbance of the working reagent + the test sample,

Abs_{575} S = the absorbance of the working reagent + the Calcium standard,

Abs_{575} I = the absorbance of the working reagent alone,

C = the concentration of the Calcium standard (10mg/dl) and

k = 4.008; a constant which corrects for the molecular weight of Calcium.

Acid anions:- The concentration of formate, pyruvate, lactate, succinate, acetate and propionate in each sample after 24hr of incubation was determined by isotachophoresis using an L.K.B. Tachophor (L.K.B. Bromma, U.K.)(Geddes & Weetman, 1981) by injecting a 2μl volume of each into the capillary of the Tachophor apparatus at the junction between two buffers; 5mM HCl (Aristar grade, BDH, U.K.) containing 0.1% hydroxypropylmethylcellulose (Sigma, U.K.) adjusted to pH 4.2 using 6-amino-N-hexanoic acid (G.P.R. grade, BDH, U.K.) as the leading buffer and 4mM octanoic acid (G.P.R. grade, BDH, U.K.) adjusted to pH 5.5 using Tris (Analar grade, BDH, U.K.) as the terminating buffer. An initial current of 150μA was applied across the length of the teflon capillary tube (of 30cm length and 0.5mm internal diameter) for 3min, followed by a final current of 50μA. In addition the temperature of the liquid in the capillary was maintained at 12°C. Each acidic anion was detected by the change in resistance that it caused between two electrodes in the detection chamber of the apparatus as it passed the electrodes. Recording of the resistance changes that occurred was initiated 10min into the analysis and continued for at least 5min, with the chart recorder (Bryans 28000 chart recorder, Bryans, U.K.) running at a rate of 0.5mm/s. The length of zone for each acid on the chart was measured using a peak-scale loupe measuring device, with a graticule with 0.1mm divisions and 7x magnification (Aldrich, U.K.), and used to extrapolate the concentration of that acid from a previously prepared standard curve of zone length versus concentration (Appendix 10) using the following formula:-

Figure 5.4 The Tachophor apparatus

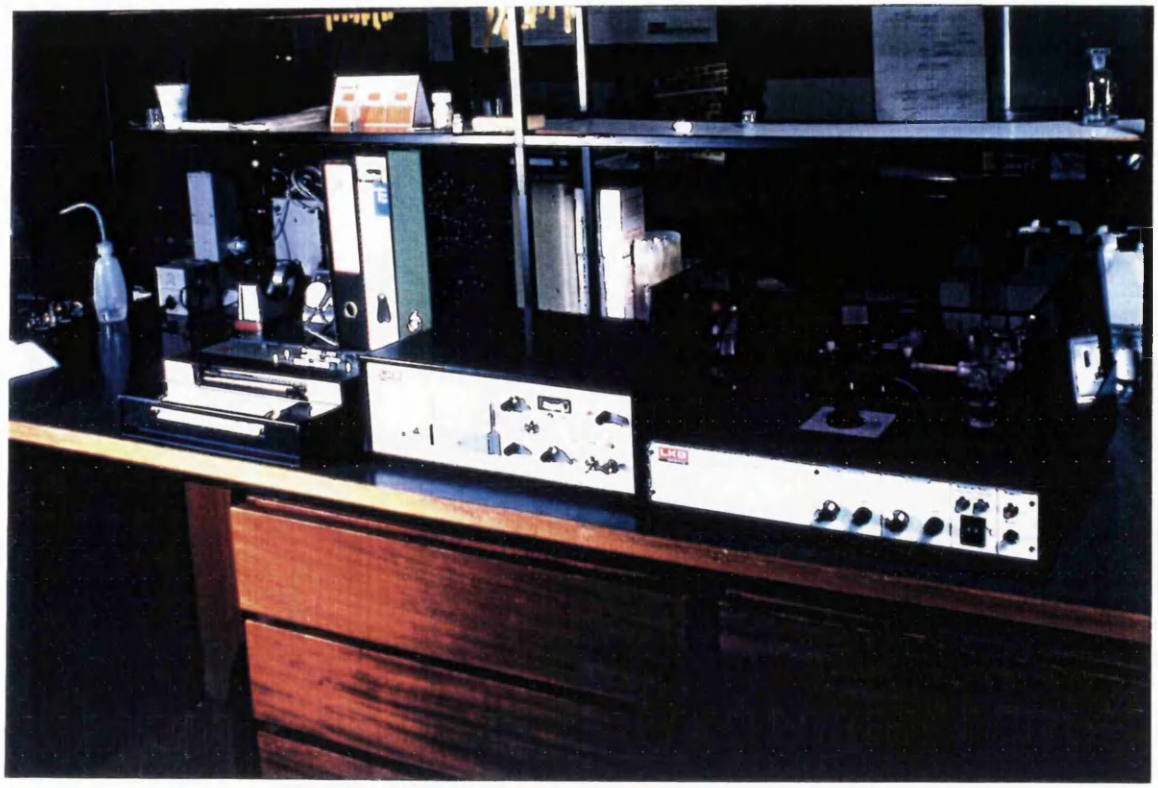
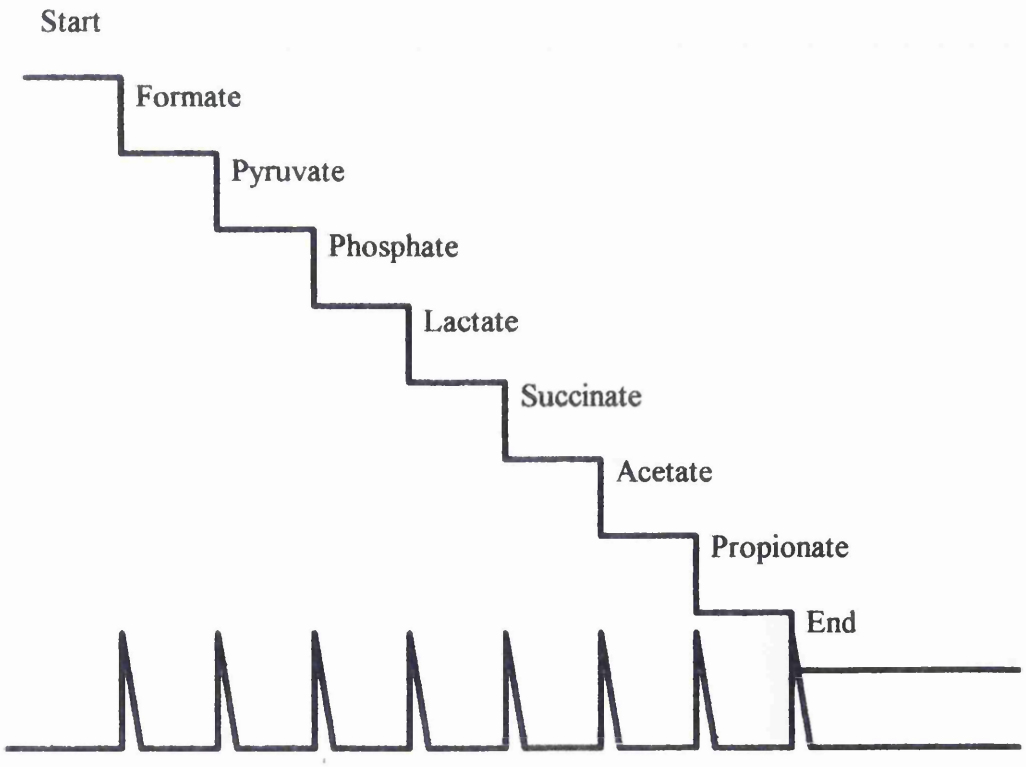


Figure 5.5 Example isotachogram of mixed acid standards



$$\frac{z - i}{s} \text{ / volume of sample loaded onto capillary} = [\text{acid anion}]\ \text{mM}$$

Where;

z = length of acid zone,

i = intercept of standard curve for each acid anion on ordinate and

s = slope of standard curve for each acid anion.

Figure 5.4 shows the Tachophor apparatus and figure 5.5 shows an example of a recording (isotachogram) of 5mM standard mixed acid anions (formate, pyruvate, phosphate, lactate, succinate, acetate and propionate) detected using the Tachophor.

The data generated by the these experiments were analysed statistically using the Minitab statistical analysis package (version 9.2, Minitab Inc., U.S.A.) for Windows (version 3.1, Microsoft Corp., U.S.A.). For simple comparisons between 2 groups of data Student's T-test was employed, whilst one-way analysis of variance was employed to compare more than 2 groups of data.

5.3 Results

At no time was growth of the quality control cultures adversely affected by the disinfectant used during preparation of the root blocks (Section 5.2; Materials and methods; preparation of human tooth root slices).

5.3.1 Viable counts

Streptococcus mutans Table 5.1 shows the mean viable counts of *S. mutans* NCTC 10449 retrieved on each of the four days of incubation. The viable counts of *S. mutans*

Table 5.1 **The change in numbers of *S. mutans* NCTC 10449 over a total of 4 days' incubation with different nutrient and mineral sources**

Ultrafree-CL number	Nutrient/ mineral source supplied on day:- 0	1	2	3	Day of counting	Total log_{10} c.f.u. after repeat 1	2	3	Mean log_{10} c.f.u. (± 1 S.D.)
	Inoculum				0	6.44	5.76	6.52	6.24 (0.42)
1	Inoculum	T.H.B.			1	6.97	6.68	7.25	6.97 (0.29)
6	Inoculum	T.H.B.	Suc‡/0		2	4.38	1.00†	1.00†	2.13 (1.95)
7	Inoculum	T.H.B.	Suc/0	T.H.B.	3	5.27	5.62	5.74	5.54 (0.24)
8	Inoculum	T.H.B.	Suc/0	T.H.B.	Suc/0	4	1.00†	3.90	1.00†	1.97 (1.67)
2	Inoculum	T.H.B.	Suc/HAp*			2	5.46	6.30	6.21	5.99 (0.46)
3	Inoculum	T.H.B.	Suc/HAp	T.H.B.	Suc/HAp	4	7.53	6.27	5.69	6.50 (0.94)
4	Inoculum	T.H.B.	Suc/root			2	5.97	5.06	6.19	5.74 (0.60)
5	Inoculum	T.H.B.	Suc/root	T.H.B.	Suc/root	4	6.06	6.40	6.29	6.25 (0.17)
9	Inoculum	T.H.B.	H_2O/HAp	T.H.B.	H_2O/HAp	4	6.10	5.87	6.68	6.22 (0.42)
10	Inoculum	T.H.B.	H_2O/root	T.H.B.	H_2O/root	4	1.00†	1.00†	6.45	2.82 (3.15)

† no viable cells were detected in these cultures; therefore a value of 10% of the minimum detection limit was chosen for statistical analyses

* HAp = hydroxyapatite powder

‡ suc = sucrose solution

increased 81% during the first day of incubation (unit **#1**) but then decreased markedly by 99.99% when exposed to the sucrose solution alone during the second day of incubation (unit **#6**). Moreover, there were no detectable counts from unit **#6** in two repeats, whilst those from the third were barely 1% of unit **#1**. When returned to T.H.B. during the third day (unit **#7**) the viable counts recovered substantially, however upon re-exposure to sucrose solution during the fourth day (unit **#8**) the c.f.u. had again decreased substantially. There were significant differences between the viable counts retrieved on successive days from the initial inoculum and from units **#1**, **#6**, **#7** and **#8** ($p = 0.001$, one-way analysis of variance).

In contrast, when either hydroxyapatite powder or a root block was added to the culture vessels along with sucrose solution during the second and fourth days (units **#2**, **#3**, **#4** and **#5**) the number of c.f.u. retrieved did not differ significantly from those of the initial inoculum or films exposed to T.H.B. alone on days 1 and 3 (units **#1** and **#7**)($p = 0.06$, one-way analysis of variance). Furthermore the viable counts on day 4 of films supplied with distilled water plus hydroxyapatite powder (unit **#9**) were not significantly different from those on day 1 of films supplied with T.H.B. only (unit **#1**)($p > 0.05$, Student's T-test). However, films supplied with a root block and distilled water (unit **#10**) on day 4 yielded barely 0.01% of the c.f.u. retrieved from films incubated with T.H.B. alone on day 1 (unit **#1**), although no significance could be assigned to this difference due to substantially more bacteria being retrieved during one repeat of unit **#10** than from the other two experimental runs ($p > 0.5$, Student's T-test).

Lactobacillus casei Table 5.2 shows the viable counts of *L. casei* NCTC 6375 retrieved during the experiments. The viable counts from unit **#1** (after 1d of growth in T.H.B.)

Table 5.2 **The change in numbers of *L. casei* NCTC 6375 over a total of 4 days' incubation with different nutrient and mineral sources**

Ultrafree-CL number	Nutrient/ mineral source supplied on day:- 0	1	2	3	Day of counting	Total log_{10} c.f.u. after repeat 1	2	3	Mean log_{10} c.f.u. (± 1 S.D.)
	Inoculum				0	6.97	8.19	7.19	7.45 (0.65)
1	Inoculum T.H.B.				1	6.96	7.67	7.78	7.47 (0.45)
6	Inoculum T.H.B.	Suc‡/0			2	7.11	7.47	6.13	6.90 (0.69)
7	Inoculum T.H.B.	Suc/0	T.H.B.		3	7.66	8.15	7.82	7.88 (0.25)
8	Inoculum T.H.B.	Suc/0	T.H.B.	Suc/0	4	6.82	7.74	7.72	7.43 (0.53)
2	Inoculum T.H.B.	Suc/HAp*			2	6.79	7.61	7.46	7.29 (0.44)
3	Inoculum T.H.B.	Suc/HAp	T.H.B.	Suc/HAp	4	6.86	8.15	8.02	7.68 (0.71)
4	Inoculum T.H.B.	Suc/root			2	5.59	7.50	7.53	6.87 (1.11)
5	Inoculum T.H.B.	Suc/root	T.H.B.	Suc/root	4	6.52	7.75	7.44	7.24 (0.64)
9	Inoculum T.H.B.	H_2O/HAp	T.H.B.	H_2O/HAp	4	4.42	6.51	6.35	5.76 (1.16)
10	Inoculum T.H.B.	H_2O/root	T.H.B.	H_2O/root	4	1.00†	5.21	6.26	4.16 (2.78)

† no viable cells were detected in these cultures; therefore a value of 10% of the minimum detection limit was chosen for statistical analyses

* HAp = hydroxyapatite powder

‡ suc = sucrose solution

were no different to the initial inoculum, nor did they decrease substantially after a subsequent day of incubation with sucrose solution alone (unit **#6**). There was a 10-fold decrease in the counts obtained from unit **#6** (compared with **#1**) during repeat 3 of the experiment but this did not affect the mean viable counts compared with units **#1**, **#7** and **#8** since the other two repeats showed no real changes. When the films were returned to T.H.B. for a further day of incubation (unit **#7**) there was a tendency towards increased viable counts which was minimal and reversed upon return to sucrose solution during the fourth day of the experiment (unit **#8**). The viable counts retrieved from units **#1**, **#6**, **#7** and **#8** (when cycled between T.H.B. and sucrose solution) were not significantly different ($p = 0.334$, one-way analysis of variance).

There were no significant differences between the number of c.f.u. retrieved on days 2 and 4 after exposure to sucrose solution plus either hydroxyapatite powder or a root block (units **#2**, **#3**, **#4** and **#5** respectively) compared with the initial inoculum or units supplied with T.H.B. on days 1 and 3 (units **#1** and **#7**)($p = 0.636$, one-way analysis of variance). In addition there were no significant differences between the viable counts retrieved on day 4 from units supplied with water plus hydroxyapatite powder (unit **#9**) compared with those on day 1 from unit **#1** (T.H.B. alone)($p > 0.05$, Student's T-test). However, the viable counts retrieved on day 4 from units supplied with water plus root (unit **#10**) were less than 0.05% of those retrieved on day 1 from unit **#1** (T.H.B.), although no statistical significance could be attached to this difference due to the variation in the results from unit **#10**.

Actinomyces viscosus Table 5.3 shows the number of viable *A. viscosus* NCTC 10951 which developed over the four days of incubation. After a substantial increase in the

Table 5.3 **The change in numbers of *A. viscosus* NCTC 10951 over a total of 4 days' incubation with different nutrient and mineral sources**

Ultrafree-CL number	Nutrient/ mineral source supplied on day:- 0	1	2	3	Day of counting	Total log_{10} c.f.u. after repeat 1	2	Mean log_{10} c.f.u. (± 1 S.D.)
	Inoculum				0	4.90	5.42	5.16 (0.37)
1	Inoculum	T.H.B.			1	6.18	7.82	7.00 (1.16)
6	Inoculum	T.H.B.	Suc‡/0		2	5.63	5.88	5.76 (0.18)
7	Inoculum	T.H.B.	Suc/0	T.H.B.	3	5.41	4.86	5.14 (0.39)
8	Inoculum	T.H.B.	Suc/0	T.H.B.	Suc/0	4	4.48	5.25	4.87 (0.54)
2	Inoculum	T.H.B.	Suc/HAp*			2	7.53	7.02	7.28 (0.36)
3	Inoculum	T.H.B.	Suc/HAp	T.H.B.	Suc/HAp	4	6.96	5.20	6.08 (1.24)
4	Inoculum	T.H.B.	Suc/root			2	2.02	6.69	4.36 (3.30)
5	Inoculum	T.H.B.	Suc/root	T.H.B.	Suc/root	4	4.68	4.80	4.74 (0.08)
9	Inoculum	T.H.B.	H_2O/HAp	T.H.B.	H_2O/HAp	4	6.15	6.21	6.18 (0.04)
10	Inoculum	T.H.B.	H_2O/root	T.H.B.	H_2O/root	4	1.00†	1.00†	1.00 (0.00)

† no viable cells were detected in these cultures; therefore a value of 10% of the lower detection limit was selected for statistical analyses

* HAp = hydroxyapatite powder

‡ suc = sucrose solution

viable counts during the first day of incubation with T.H.B (unit **#1**) the number of c.f.u. retrieved on subsequent days declined when cycled between sucrose solution, T.H.B. and back again (units **#6**, **#7** and **#8**). However, the data from unit **#8** showed opposite trends between repeats 1 and 2; there was a 10-fold reduction in viable counts in repeat 1 (compared with unit **#7**) whilst there was an almost 2-fold increase in counts in repeat 2.

In contrast to the viable counts that developed in the absence of mineral, those retrieved after incubation with sucrose solution plus hydroxyapatite powder on days 2 and 4 were almost 100-fold greater (units **#2** and **#3**) than on the corresponding days when incubated with sucrose solution alone (units **#6** and **#8**) and were also greater than retrieved on days 1 and 3 after incubation with T.H.B. (units **#1** and **#7**). Nevertheless, the viable counts from units **#2** and **#3** showed the same tendency towards lower viable counts after the second incubation period (unit **#3**) compared with the first (unit **#2**) as was observed with units **#6** and **#8**. However, when the films were incubated with sucrose plus a root block (units **#4** and **#5**), the viable counts retrieved on days 2 and 4 were several log values lower than when sucrose solution alone (units **#6** and **#8**) or sucrose and hydroxyapatite powder (units **#2** and **#3**) were supplied on the corresponding days. The data from repeats 1 and 2 of unit **#4** were contradictory, with repeat 1 being 4-log lower than repeat 2, which substantially affected the mean result and meant that there did not appear to be any differences between days 2 and 4.

Furthermore, the viable counts of films exposed to distilled water along with mineral showed a similar trend to those supplied with sucrose solution and mineral. The number of c.f.u. retrieved on day 4 from films given distilled water and hydroxyapatite powder (unit **#9**) were less than 10-fold lower than those retrieved on day 1 from T.H.B.-fed

films (unit **#1**). In contrast, no viable counts could be detected on day 4 from films incubated with distilled water and a root block (unit **#10**). Since it was only possible to repeat the demineralisation experiment with *A. viscosus* twice in the time available, the data gathered with this organism could not be analysed statistically with confidence.

Comparison of viable count data In comparison *S. mutans*, *L. casei* and *A. viscosus* interacted in very different ways with the various combinations of T.H.B., sucrose solution, distilled water and mineral that were employed in this experiment. The mean viable counts of *S. mutans* and *L. casei* showed no significant differences after 1 day of growth in T.H.B. ($p > 0.1$, Student's T-test) and both appeared similar to *A. viscosus*. However, once the different reaction mixtures were introduced the viable counts of the three organisms diverged. For instance the counts of *S. mutans* decreased significantly in sucrose alone compared with T.H.B., whilst those of *L. casei* did not; the *S. mutans* counts recovered to a large extent when returned to T.H.B. but those of *A. viscosus* continued to decline. When the number of *S. mutans* and *L. casei* c.f.u. from units **#1**, **#6**, **#7** and **#8** (i.e. cycled from T.H.B. to sucrose to T.H.B. and back to sucrose) were compared there were statistically significant differences between them ($p < 0.001$, one-way analysis of variance).

However when either hydroxyapatite powder or a root block was present with sucrose *S. mutans* viable counts were reduced much less than with sucrose alone. The presence of hydroxyapatite powder (but not root blocks) with sucrose was also associated with higher *A. viscosus* counts than sucrose alone, although the counts from unit **#3** tended to be higher than those of *S. mutans*, whilst those from unit **#4** were comparable. In contrast when mineral was added there were no substantial changes in *L. casei* counts

compared with cultures not supplied with mineral and the viable counts tended to be higher than the other two organisms at each time of sampling. Furthermore films of all three organisms yielded greater viable counts with hydroxyapatite powder and distilled water (unit **#9**) than root blocks and distilled water (unit **#10**). However the viable counts of all three organisms in unit **#9** were comparable, whilst in contrast those of *S. mutans* and *L. casei* tended to be greater than *A. viscosus* in unit **#10**.

5.3.2 Calcium release data

Bacteria-free negative controls There was no detectable calcium in any of the samples aspirated from the bacteria free negative controls (units **#11 - #15**).

Streptococcus mutans Table 5.4 shows the concentration of calcium ions (mM) in the spent culture fluid aspirated from the lower (centrifuge tube) and upper (filter) chambers of Ultrafree-CL units inoculated with *S. mutans* NCTC 10449 and supplied with 1ml of 5% sucrose solution and 5mg of hydroxyapatite powder (units **#2** and **#3**) or a root block (units **#4** and **#5**). If there were no detectable calcium ions in a sample then a figure of 0.03mM (10% of the minimum detection limit) was inserted into the table to facilitate statistical analyses. Furthermore there were occasions when insufficient fluid was present in the chamber for a sample to be aspirated and analysed; these are shown as 'n.d.' (not determined).

There were no calcium ions in the majority of negative control samples (units **#6, #8, #9** and **#10**). However, calcium was detected at low levels (0.31 - 0.37mM) in five out of twelve samples from unit **#10** (distilled water and a root block) during the three repeats of the experiment. There was a trend towards increased calcium ion concentration with

Table 5.4 **Calcium concentration (mM) in lower (centrifuge) tube and upper (filter) component during incubation of mineral with *S. mutans* NCTC 10449**

Ultrafree-CL number	Sample source	Time of sampling (hours)	Calcium ion concentration (mM) during repeat 1	2	3	Mean $[Ca^{2+}]$ mM (± 1 S.D.)
First (24hr) incubation period (sucrose solution + hydroxyapatite powder)						
2	Lower	3	0.51	0.03‡	0.31	0.28 (0.24)
		6	1.09	0.30	0.52	0.64 (0.41)
		12	2.44	n.d.†	n.d.	2.44 (/)
		24	4.21	1.39	1.86	2.49 (1.51)
	Upper	24	5.71	1.77	3.00	3.50 (2.02)
3	Lower	3	0.26	0.03‡	0.03‡	0.11 (0.13)
		6	0.58	0.41	n.d.	0.50 (0.12)
		12	n.d.	0.31	n.d.	0.31 (/)
		24	4.98	1.55	0.43	2.32 (2.37)
	Upper	24	6.00	1.96	0.92	2.96 (2.68)
Second (24hr) incubation period						
3	Lower	3	1.16	0.85	1.15	1.05 (0.17)
		6	1.77	n.d.	1.45	1.61 (0.22)
		12	2.49	1.29	1.66	1.82 (0.62)
		24	4.55	n.d.	2.48	3.51 (1.46)
	Upper	24	5.03	1.18	2.43	2.88 (1.97)
First (24hr) incubation period (sucrose solution + a root block)						
4	Lower	3	0.27	0.03‡	0.03‡	0.11 (0.14)
		6	0.63	0.31	0.76	0.57 (0.23)
		12	1.08	0.38	n.d.	0.73 (0.49)
		24	5.28	0.68	2.90	2.95 (2.31)
	Upper	24	4.92	0.71	3.90	3.18 (2.20)
5	Lower	3	0.03‡	0.27	0.03‡	0.11 (0.14)
		6	0.31	0.34	0.41	0.35 (0.05)
		12	0.69	0.03‡	n.d.	0.36 (0.47)
		24	0.70	3.19	2.60	2.17 (1.31)
	Upper	24	0.66	n.d.	2.43	1.55 (1.26)
Second (24hr) incubation period						
5	Lower	3	0.03‡	0.03‡	0.53	0.20 (0.29)
		6	0.03‡	0.49	0.40	0.31 (0.24)
		12	0.28	1.09	0.62	0.67 (0.41)
		24	1.38	4.94	1.53	2.62 (2.01)
	Upper	24	1.18	3.90	1.35	2.14 (1.53)

† n.d. = not determined, insufficient sample volume

‡ Calcium ion concentration below minimum detection limit of assay; therefore a value of 10% of the minimum detection limit was selected for statistical analyses

time both when *S. mutans* was incubated with sucrose solution plus hydroxyapatite powder (units **#2** and **#3**) and when it was incubated with sucrose plus a root block (units **#4** and **#5**) during the first incubation period. For instance there were significant differences in the calcium concentration at 3, 6, 12 and 24hr from units **#2**, **#4** and **#5** ($p = 0.044$, $p = 0.044$ and $p = 0.034$ respectively, one-way analysis of variance). However the differences between 3, 6, 12 and 24hr for unit **#3** were not significant due to the large variation in the concentrations at 24hr.

The calcium ion concentration did not show any significant differences between 3, 6, 12 and 24hr in unit **#3** during the second incubation period due to the high level of calcium already released by 3hr ($p = 0.125$, one-way analysis of variance), although there was a significant increase in calcium concentration with time in unit **#5** ($p = 0.04$, one-way analysis of variance). The calcium concentration did not differ significantly between the upper and lower chambers of any unit at any time during the second incubation period ($p > 0.9$ in all cases, one-way analysis of variance). In addition there were no significant differences between hydroxyapatite powder or root blocks in calcium release (at 24hr) after either incubation period ($p > 0.1$ in both cases, Student's T-test). Moreover there were no significant differences in the calcium ion concentrations (at 24hr) after the first incubation period compared with the second incubation period in units supplied with sucrose solution plus hydroxyapatite powder, nor were there any differences (at 24hr) between the two incubation periods when the films were supplied with sucrose plus root ($p > 0.1$ in both instances, Student's T-test).

Lactobacillus casei Table 5.5 shows the calcium ion concentration (mM) in effluent reaction mixture aspirated from the lower (centrifuge tube) and upper (filter)

components of Ultrafree-CL units inoculated with *L. casei* NCTC 6375 and supplied with 1ml of 5% sucrose solution and 5mg of hydroxyapatite powder (units **#2** and **#3**) or 1ml of 5% sucrose solution and a root block (units **#4** and **#5**).

There were no detectable calcium ions in the majority of negative control samples (sucrose solution alone, distilled water plus hydroxyapatite powder and distilled water plus a root block; units **#6** - **#8**, **#9** and **#10** respectively; data not presented). However, low concentrations of calcium ions (0.29 - 0.39mM) were found in three out of eight samples from unit **#10** (distilled water plus a root block) during repeat 2 (second incubation period) of the experiment. Moreover a higher concentration of calcium ions (0.56mM) was found in one sample, taken at 12hr from unit **#9** (distilled water plus hydroxyapatite powder) during repeat 2 (first incubation period) of the experiment, although the explanation for this is not clear.

The calcium ion concentration in the effluent reaction mixture from *L. casei* films increased with time during the first incubation period and there were significant differences between the concentrations at 3, 6, 12 and 24hr both when supplied with sucrose solution plus hydroxyapatite powder (units **#2** and **#3**) and when supplied with sucrose plus a root block (units **#4** and **#5**)($p = 0.03$ and $p < 0.001$ respectively, one-way analysis of variance). The calcium ion concentration also tended to increase with time during the second incubation period when films were supplied with either sucrose plus hydroxyapatite (unit **#3**) or a root block (unit **#5**) although this increase was only significant with respect to unit **#5** ($p = 0.051$ and $p = 0.012$ respectively, one-way analysis of variance). Moreover the calcium released from hydroxyapatite powder (units **#2** and **#3**) was significantly greater than from root blocks (units **#4** and **#5**) after the first

Table 5.5 **Calcium concentration (mM) in lower (centrifuge) tube and upper (filter) component during incubation of mineral with *L. casei* NCTC 6375**

Ultrafree-CL number	Sample source	Time of sampling (hours)	Calcium ion concentration (mM) during repeat 1	2	3	Mean $[Ca^{2+}]$ mM (± 1 S.D.)
First (24hr) incubation period (sucrose solution + hydroxyapatite powder)						
2	Lower	3	0.03‡	0.03‡	0.03‡	0.03 (0.00)
		6	0.89	0.27	0.25	0.47 (0.36)
		12	6.10	2.03	n.d.†	4.07 (2.88)
		24	17.01	4.63	2.81	8.15 (7.73)
	Upper	24	19.52	6.06	3.36	9.65 (8.67)
3	Lower	3	0.03‡	0.03‡	0.03‡	0.03 (0.00)
		6	0.03‡	0.25	0.03‡	0.10 (0.13)
		12	3.35	1.53	n.d.	2.44 (1.29)
		24	6.04	5.42	1.95	4.47 (2.20)
	Upper	24	n.d.	4.40	3.95	4.18 (0.32)
Second (24hr) incubation period						
3	Lower	3	1.70	0.64	0.58	0.97 (0.63)
		6	2.70	2.62	1.85	2.39 (0.47)
		12	4.18	4.08	5.14	4.47 (0.58)
		24	10.61	10.28	6.65	9.18 (2.20)
	Upper	24	n.d.	0.03‡	13.15	6.59 (9.28)
First (24hr) incubation period (sucrose solution + a root block)						
4	Lower	3	0.51	0.03‡	0.03‡	0.19 (0.28)
		6	0.69	0.03‡	0.03‡	0.25 (0.38)
		12	0.49	1.02	n.d.	0.76 (0.37)
		24	1.93	2.76	2.32	2.34 (0.42)
	Upper	24	3.06	4.20	3.35	3.54 (0.59)
5	Lower	3	0.03‡	0.03‡	0.03‡	0.03 (0.00)
		6	0.34	0.03‡	0.27	0.21 (0.16)
		12	0.49	0.34	n.d.	0.42 (0.11)
		24	1.32	0.32	0.54	0.73 (0.53)
	Upper	24	n.d.	1.49	1.12	1.31 (0.26)
Second (24hr) incubation period						
5	Lower	3	0.03‡	0.03‡	0.45	0.17 (0.24)
		6	0.03‡	0.47	0.47	0.32 (0.25)
		12	0.03‡	2.30	0.87	1.06 (1.14)
		24	1.32	7.45	3.59	4.12 (3.10)
	Upper	24	n.d.	8.13	4.36	6.24 (2.67)

† n.d. = not determined, insufficient sample volume

‡ Calcium ion concentration below minimum detection limit of assay; therefore a value of 10% of the minimum detection limit was selected for statistical analyses

incubation period ($p < 0.02$, Student's T-test). In contrast there were no significant differences between hydroxyapatite powder (unit **#3**) and root blocks (unit **#5**) in calcium release after the second incubation period ($p > 0.1$, Student's T-test). Furthermore there were no significant differences in calcium concentration between the upper and lower chambers of each unit ($p > 0.1$ in all cases, Student's T-test).

There were a number of samples in which the calcium ion concentration differed exceptionally from the replicates. For instance the calcium concentration at each time point in unit **#2** during the first repeat were three- to four-fold greater than in repeats 2 and 3. In addition 12 and 24hr samples from unit **#5** during the second incubation period of repeat 2 contained at least twice as much calcium as repeats 1 and 3. However these results did not contradict the overall trend, although they did make the data less robust statistically.

Actinomyces viscosus Table 5.6 shows the concentration of calcium ions liberated from either 5mg of hydroxyapatite powder (units **#2** and **#3**) or a root block (units **#4** and **#5**) plus 1ml 5% sucrose by the metabolic products of *A. viscosus* NCTC 10951.

No dissolved calcium was detected in the majority of samples aspirated from the negative controls; sucrose solution alone (units **#6** - **#8**), distilled water plus hydroxyapatite powder (unit **#9**) and distilled water plus a root block (unit **#10**). However, calcium ions were detected at a low level (0.28mM) in samples collected after the first day of incubation in unit **#10**. Furthermore any dissolved calcium was below the minimum threshold for the assay employed in the majority of units containing sucrose solution plus hydroxyapatite powder (units **#2** and **#3**) or sucrose solution plus root blocks (units **#4**

Table 5.6 **Calcium concentration (mM) in lower (centrifuge) tube and upper (filter) component during incubation of mineral with *A. viscosus* NCTC 10951**

Ultrafree-CL number	Sample source	Time of sampling	Calcium ion concentration (mM) during repeat 1	2	Mean $[Ca^{2+}]$ mM (± 1 S.D.)
First (24hr) incubation period (sucrose solution + hydroxyapatite powder)					
2	Lower	3	0.03‡	0.03‡	0.03 (0.00)
		6	0.03‡	0.51	0.27 (0.34)
		12	0.03‡	n.d.†	0.03 (/)
		24	0.03‡	3.72	1.88 (2.61)
	Upper	24	0.03‡	3.74	1.89 (2.62)
3	Lower	3	0.03‡	0.03‡	0.03 (0.00)
		6	0.03‡	0.03‡	0.03 (0.00)
		12	0.03‡	n.d.	0.03 (/)
		24	0.03‡	1.07	0.55 (0.74)
	Upper	24	0.03‡	1.84	0.94 (1.28)
Second (24hr) incubation period					
3	Lower	3	0.03‡	0.03‡	0.03 (0.00)
		6	0.29	0.03‡	0.16 (0.18)
		12	0.41	0.28	0.35 (0.09)
		24	0.89	0.45	0.67 (0.31)
	Upper	24	1.17	0.36	0.77 (0.57)
First (24hr) incubation period (sucrose solution + a root block)					
4	Lower	3	0.03‡	0.03‡	0.03 (0.00)
		6	0.03‡	0.26	0.15 (0.16)
		12	0.03‡	n.d.	0.03 (/)
		24	0.27	1.01	0.64 (0.52)
	Upper	24	n.d.	1.68	1.68 (/)
5	Lower	3	0.03‡	0.03‡	0.03 (0.00)
		6	0.03‡	0.31	0.17 (0.20)
		12	0.03‡	n.d.	0.03 (/)
		24	0.31	2.58	1.45 (1.61)
	Upper	24	0.34	2.14	1.24 (1.27)
Second (24hr) incubation period					
5	Lower	3	0.03‡	0.03‡	0.03 (0.00)
		6	0.03‡	0.03‡	0.03 (0.00)
		12	0.03‡	0.03‡	0.03 (0.00)
		24	0.03‡	0.03‡	0.03 (0.00)
	Upper	24	0.03‡	0.03‡	0.03 (0.00)

† n.d. = not determined, insufficient sample volume

‡ Calcium ion concentration below minimum detection limit of assay; therefore a value of 10% of the minimum detection limit was selected for statistical analyses

and #5). There did appear to be a minor trend towards increasing concentration with time in some units, however the lack of a third set of data meant that it was not possible to perform statistical analyses or draw firm conclusions regarding this trend.

Comparison of calcium release data The mean calcium ion concentration from *L. casei* films supplied with sucrose plus hydroxyapatite powder tended to be 2- to 3- fold greater than that released by *S. mutans* films and this difference was statistically significant ($p < 0.01$, Student's T-test). In contrast, there were no significant differences between the concentration of calcium liberated from root blocks by either species ($p > 0.1$, Student's T-test). The data for *A. viscosus* are equivocal and therefore difficult to compare with those for *S. mutans* or *L. casei*, although the trend suggests that *A. viscosus* was responsible for less demineralisation than the other two.

5.3.3 Total calcium released from either mineral source and Ca^{2+} released per cm^2 of root surface

Streptococcus mutans The total amount of calcium (μmol) released from either hydroxyapatite powder or a root block by *S. mutans* during each 1d of incubation with 5% sucrose is shown in table 5.7. This value was generated by totalling the amount of calcium released at each time point, which was itself calculated from the known calcium concentration and sample volume over time.

In general there was no dissolved calcium in samples from the negative controls, although a few contained trace amounts. For instance 0.14μmol of Ca^{2+} was found in samples from unit #10 (distilled water plus a root block) after the first day of incubation during repeat 2 of the experiment, whilst 0.01 and 0.12μmol were found in the same unit

Table 5.7 **Total calcium (µmol) - in the upper and lower chambers - released from hydroxyapatite and root blocks during the first and second 24hr incubation periods when exposed to *S. mutans* NCTC 10449**

Ultrafree-CL number	Mineral source	Total calcium released (µmol) from mineral during repeat 1	2	3	Mean $[Ca^{2+}]$ µmol (± 1 S.D.)
First (24hr) incubation period in sucrose solution					
2	HAp*	1.59	0.78	1.31	1.23 (0.41)
3	HAp	1.46	0.88	0.39	0.91 (0.54)
Second (24hr) incubation period					
3	HAp	2.09	0.79	1.63	1.50 (0.66)
First (24hr) incubation period in sucrose solution					
4	root block	0.98	0.49	1.58	1.01 (0.55)
5	root block	0.40	0.88	1.04	0.77 (0.33)
Second (24hr) incubation period					
5	root block	0.40	1.76	1.16	1.11 (0.68)

Table 5.8 **Total calcium (µmol/cm^2) - in the upper and lower chambers - released from root blocks during the first and second 24hr incubation periods when exposed to *S. mutans* NCTC 10449**

Ultrafree-CL number	Total calcium released (µmol/cm^2) from a root block during repeat 1	2	3	Mean calcium µmol/cm^2 (± 1 S.D.)
First (24hr) incubation period with sucrose solution				
4	6.11	3.05	9.88	6.35 (3.42)
5	2.46	5.47	6.49	4.81 (2.10)
Second (24hr) incubation period				
5	2.52	11.04	7.25	6.94 (4.27)
First (24hr) incubation period with water				
10	0.45	0.00	0.77	0.41 (0.39)
Second (24hr) incubation period				
10	0.00	0.89	0.00	0.30 (0.51)

* HAp = hydroxyapatite powder

after the second day of incubation during repeats 1 and 3 of the experiment. In contrast significantly more calcium was liberated from both hydroxyapatite powder and root blocks (units **#2** - **#5**) than in the controls (units **#9** and **#10**) during the first incubation period ($p = 0.008$, one-way analysis of variance). In addition significantly more calcium was released from either type of mineral in the experimental units (**#3** and **#5**) than in the control units (**#9** and **#10**) during the second incubation period ($p < 0.001$, one-way analysis of variance). However, when the mean total amount of calcium released from hydroxyapatite powder after the first incubation period was compared with that released from a root block, there were no significant differences ($p > 0.1$, Student's T-test) nor were there any when the amount of calcium released on the second day of incubation was compared ($p > 0.1$, Student's T-test).

The total amount of calcium released per cm^2 of exposed root surface during 24hr of incubation by *S. mutans* and either sucrose solution (unit **#4** and **#5**) or distilled water (unit **#10**) is shown in table 5.8. This data was derived by dividing the total amount of calcium released from root blocks by 16 (the area of exposed root in mm^2) and then multiplying by 100. Occasionally, trace amounts of calcium ions were detected in the distilled water negative control (unit **#10**). In contrast a significant amount of Ca^{2+} was released from root blocks exposed to *S. mutans* films and sucrose solution compared with the distilled water negative control after the first incubation period ($p < 0.02$, Student's T-test). However it was not possible to determine the significance of observed differences between the control and experimental calcium concentrations after the second incubation period due to variation between repeats of the experiment ($p > 0.1$, Student's T-test).

Lactobacillus casei The total amount of calcium released from either mineral source during 24hr of incubation with *L. casei* in the presence of either sucrose solution or distilled water is shown in table 5.9. There were trace amounts of Ca^{2+} in some distilled water negative controls (units **#9** and **#10**); e.g. 0.01µmol in unit **#9** (hydroxyapatite powder) repeat 1 during the first incubation period and 0.08µmol in unit **#10** (root block) repeat 2 during the second incubation period, although most were negative. In contrast a significant amount of calcium was found after both the first and second incubation periods in the experimental samples (units **#2** - **#5**) compared with the negative controls (units **#9** and **#10**)($p < 0.001$, first incubation period and $p = 0.001$, second incubation period, one-way analysis of variance). The mean total amount of calcium released, during the first day of incubation, from hydroxyapatite powder (units **#2** and **#3**) was significantly greater than from root blocks (units **#4** and **#5**)($p < 0.01$, Student's T-test). The total amount of calcium released from hydroxyapatite powder (unit **#3**) was also greater than from root blocks (unit **#5**) after the second day of incubation ($p < 0.05$, Student's T-test). Furthermore significantly more Ca^{2+} was released from hydroxyapatite powder after the second day of incubation than after the first (unit **#3**)($p < 0.01$, Student's T-test), whereas there were no significant differences between the first and second incubation periods in calcium release from root blocks (unit **#5**)($p > 0.1$, Student's T-test).

The total amount of calcium released per cm^2 of exposed root surface during 24hr of incubation with *L. casei* is shown in table 5.10. The table also shows the total amount of Ca^{2+} released per cm^2 from the (distilled water) negative control root block (unit **#10**). Generally no calcium was released from negative control root blocks (unit **#10**), except after the second exposure period when it was detected during repeat 2 at 0.53µmol/cm^2.

Table 5.9 **Total calcium (μmol) - in the upper and lower chambers - released from hydroxyapatite and root blocks during the first and second 24hr incubation periods when exposed to *L. casei* NCTC 6375**

Ultrafree-CL number	Mineral source	Total calcium released (μmol) from mineral during repeat 1	2	3	Mean $[Ca^{2+}]$ μmol (± 1 S.D.)
First (24hr) incubation period in sucrose solution					
2	HAp*	2.35	2.50	1.30	2.05 (0.65)
3	HAp	1.21	1.37	1.94	1.51 (0.38)
Second (24hr) incubation period					
3	HAp	4.00	2.63	4.97	3.87 (1.18)
First (24hr) incubation period in sucrose solution					
4	root	0.69	0.79	1.18	0.89 (0.26)
5	root	0.11	0.03	0.02	0.05 (0.05)
Second (24hr) incubation period					
5	root	0.12	2.20	0.47	0.93 (1.11)

Table 5.10 **Total calcium (μmol/cm^2) - in the upper and lower chambers - released from root blocks during the first and second 24hr incubation periods when exposed to *L. casei* NCTC 6375**

Ultrafree-CL number	Total calcium released (μmol/cm^2) from a root block during repeat 1	2	3	Mean calcium μmol/cm^2 (± 1 S.D.)
First (24hr) incubation period with sucrose solution				
4	4.32	4.48	7.61	5.47 (1.86)
5	0.68	0.16	0.10	0.31 (0.32)
Second (24hr) incubation period				
5 *L. casei*	0.72	13.70	2.92	5.78 (6.95)
First (24hr) incubation period with water				
10 *L. casei*	0.00	0.00	0.00	0.00 (0.00)
Second (24hr) incubation period				
10 *L. casei*	0.00	0.53	0.00	0.18 (0.30)

* HAp = hydroxyapatite powder

In contrast, significantly more calcium was released from root surfaces after the first incubation period with a bacterial film and sucrose solution (units **#4** and **#5**) than in the negative control (unit **#10**)($p = 0.001$, one-way analysis of variance), although no significance could be determined for the difference between unit **#5** compared with unit **#10** due to variation between repeats of the experiment ($p > 0.1$, Student's T-test).

Actinomyces viscosus The total amount of calcium released from each mineral source during 24hr of incubation with *A. viscosus* and either sucrose solution (units **#2** - **#5**) or distilled water (units **#9** and **#10**) is shown in table 5.11.

The amount of calcium released from mineral during incubation with *A. viscosus* was generally low, regardless of whether the bacteria were incubated with sucrose or water. Indeed calcium was usually undetectable, although the lack of third repeat prevented statistical analyses of the differences between experimental and control samples.

The total amount of Ca^{2+}/cm^2 of exposed root surface when incubated with *A. viscosus* and either sucrose solution (units **#4** and **#5**) or distilled water (unit **#10**) is shown in table 5.12. The mean data suggest that more calcium/cm^2 was liberated with 5% sucrose than distilled water after the first incubation period, although Ca^{2+} was not present in samples from units **#4** and **#5** during the first repeat whilst it was during the second which complicated matters. It was not possible to analyse the data statistically.

Comparison of total calcium release data for the three species A comparison of the mean calcium ion release data (μmol), from hydroxyapatite powder or root blocks, for each species of bacterium when incubated with sucrose solution (units **#2** - **#5**) is presented in

Table 5.11 **Total calcium (μmol) - in the upper and lower chambers - released from hydroxyapatite and root blocks during the first and second 24hr incubation periods when exposed to *A. viscosus* NCTC 10951**

Ultrafree-CL number	Mineral source	Total calcium released (μmol) from mineral during repeat 1	2	Mean $[Ca^{2+}]$ μmol (± 1 S.D.)
First (24hr) incubation period in sucrose solution				
2	HAp*	n.d.†	1.54	1.54 (/)
3	HAp	n.d.	1.24	1.24 (/)
Second (24hr) incubation period				
3	HAp	0.36	0.21	0.29 (0.11)
First (24hr) incubation period in sucrose solution				
4	root	0.01	0.77	0.39 (0.54)
5	root	0.14	0.80	0.47 (0.47)
Second (24hr) incubation period				
5	root	n.d.	n.d.	/

Table 5.12 **Total calcium (μmol/cm^2) - in the upper and lower chambers - released from root blocks during the first and second 24hr incubation periods when exposed to *A. viscosus* NCTC 10951**

Ultrafree-CL number		Total calcium released (μmol/cm^2) from a root block during repeat 1	2	Mean calcium μmol/cm^2 (± 1 S.D.)
First (24hr) incubation period with sucrose solution				
4	*A. viscosus*	0.04	4.86	2.45 (3.41)
5	NCTC 10951	0.85	5.02	2.94 (2.95)
Second (24hr) incubation period				
5	*A. viscosus*	0.00	0.00	0.00 (0.00)
First (24hr) incubation period with water				
10	*A. viscosus*	0.00	0.45	0.23 (0.32)
Second incubation period				
10	*A. viscosus*	0.00	0.00	0.00 (0.00)

* HAp = hydroxyapatite powder

† n.d. = not determined, Calcium concentration lower than threshold of assay (0.25mM)

Table 5.13 **Comparison of mean calcium (µmol) released from hydroxyapatite powder and root blocks when exposed to either *S. mutans* NCTC 10449, *L. casei* NCTC 6375 or *A. viscosus* NCTC 10951**

Organism	Mineral source	Mean calcium µmol (± 1 S.D.) for incubation period	
		First 24hr	Second 24hr
S. mutans NCTC 10449	HAp*	1.07 (0.46)	1.50 (0.66)
	root block	0.90 (0.43)	1.11 (0.68)
L. casei NCTC 6375	HAp	1.78 (0.56)	3.87 (1.18)
	root block	0.47 (0.49)	0.93 (1.11)
A. viscosus NCTC 10951	HAp	1.39 (0.21)	0.29 (0.11)
	root block	0.43 (0.41)	n.d.†

Table 5.14 **Comparison of mean calcium (µmol/cm^2) released from a root block when exposed to either *S. mutans* NCTC 10449, *L. casei* NCTC 6375 or *A. viscosus* NCTC 10951**

Organism	Mineral source	Mean calcium µmol/cm^2 (± 1 S.D.) for incubation period	
		First 24hr	Second 24hr
S. mutans NCTC 10449	root block	5.58 (2.67)	6.94 (4.27)
L. casei NCTC 6375	root block	2.89 (3.07)	5.78 (6.95)
A. viscosus NCTC 10951	root block	2.69 (2.62)	0.00 (0.00)

* HAp = hydroxyapatite powder
† n.d. = not determined, Calcium concentration lower than threshold of assay (0.25mM)

table 5.13. In general no calcium was released in the negative controls (units **#9** and **#10**, water plus hydroxyapatite powder or a root block respectively), although a few did contain trace Ca^{2+}. In contrast, a significant amount of calcium was released from both mineral sources by *S. mutans* or *L. casei* in experimental units (**#2 - #5**) compared with negative control units (**#9** and **#10**). Moreover, significantly more calcium was released from hydroxyapatite powder when incubated with *L. casei* and sucrose than with *S. mutans* and sucrose during either the first or second period of exposure ($p < 0.05$ in both cases, Student's T-test). In contrast there were no significant differences between the amount of calcium released from root blocks in the presence of sucrose during the first incubation period when *L. casei* was compared with *S. mutans*, nor were there any during the second incubation period ($p > 0.1$ in both instances, Student's T-test). It was not possible to perform statistical analyses upon the data for *A. viscosus*.

Comparison of calcium release data per cm^2 of exposed root for the three bacterial species Table 5.14 shows a comparison of the mean calcium release per cm^2 from root blocks when incubated with the three bacterial species and sucrose solution (units **#4** and **#5**). The calcium released per cm^2 exposed root during the first incubation period by *S. mutans* was significantly greater than that released by *L. casei* ($p = 0.041$, Student's T-test). However the same was not true for the calcium release data for the second incubation period when there were no significant differences ($p = 0.818$, Student's T-test). The lack of a third repeat for *A. viscosus* precluded statistical analysis of the data for this organism.

Table 5.15 **Total concentration of acid liberated by *S. mutans* NCTC 10449 during demineralisation experiments**

Unit #	Mineral source	Sample source	Total acid concentration (mM) in repeat number 1	2	3	Mean total acids (mM) (± 1 S.D.)
First (24hr) incubation period with sucrose solution						
6	None	Upper*	0	7.0	1.0	2.7 (3.8)
		Lower†	4.9	5.2	2.5	4.2 (1.5)
2	HAp‡	Upper	8.6	6.2	4.2	6.3 (2.2)
		Lower	7.8	7.7	3.7	6.4 (2.3)
4	root block	Upper	13.1	9.2	14.3	12.2 (2.7)
		Lower	14.7	11.0	13.3	13.0 (1.9)
8	None	Upper	n.d.+	4.4	0.6	1.6 (2.4)
		Lower	n.d.	1.2	0.4	0.5 (0.6)
3	HAp	Upper	13.3	7.2	4.6	8.4 (4.5)
		Lower	8.4	9.4	2.6	6.7 (3.6)
5	root block	Upper	4.8	n.d.	9.6	7.2 (3.4)
		Lower	4.9	6.7	11.3	7.6 (3.3)
Second (24hr) incubation period with sucrose solution						
8	None	Upper	4.9	12.9	8.1	8.6 (4.0)
		Lower	4.6	9.2	8.4	7.4 (2.4)
3	HAp	Upper	20.3	10.3	11.7	14.1 (5.4)
		Lower	29.8	n.d.	11.1	20.5 (13.2)
5	root block	Upper	11.3	31.6	27.1	23.3 (10.6)
		Lower	7.1	18.2	19.5	14.9 (6.8)
First (24hr) incubation period with distilled water						
9	HAp	Upper	1.1	0	0	0.4 (0.6)
		Lower	n.d.	0	0	0 (0)
10	root block	Upper	1.5	n.d.	0.6	1.1 (0.6)
		Lower	0	0	0	0 (0)
Second (24hr) incubation period with distilled water						
9	HAp	Upper	0	0	n.d.	0 (0)
		Lower	0	0	n.d.	0 (0)
10	root block	Upper	n.d.	0	0	0 (0)
		Lower	0	0	0	0 (0)

* Upper = from the upper (filter) chamber; above the bacterial film
† Lower = from the lower (centrifuge tube) chamber below the bacterial film
‡ HAp = hydroxyapatite powder
+ n.d. = not determined; insufficient sample volume

5.3.4 Acid anion concentrations

Streptococcus mutans; total acids The sum total acid anion concentrations in the spent culture fluid collected from each repeat of the experiment after 24hr incubation of *S. mutans* NCTC 10449 with 5% sucrose solution or distilled water in the presence or absence of mineral are shown in table 5.15. In addition the mean sum total acid concentration of each is supplied for illustrative purposes. The acid anions recorded were formate, pyruvate, lactate, succinate, acetate and propionate and the concentration of each was corrected using the bacteria-free negative controls.

On the whole there was no acid in the distilled water negative controls (units **#9** and **#10**), although low levels were detected in both units after repeat number 1 (first incubation period) and also unit **#10** after repeat number 3 (first incubation period). In contrast a significant amount of acid was detected when the bacterial films were supplied with sucrose solution alone (units **#6** and **#8**), sucrose solution plus hydroxyapatite powder (units **#2** and **#3**) or sucrose solution plus a root block (units **#4** and **#5**) compared with the distilled water negative controls (units **#9** and **#10**) after the first incubation period ($p < 0.001$, one-way analysis of variance). The acid concentration in units **#8**, **#3** and **#5** after the second incubation period was also significantly greater than in units **#9** and **#10** ($p < 0.001$, one-way analysis of variance).

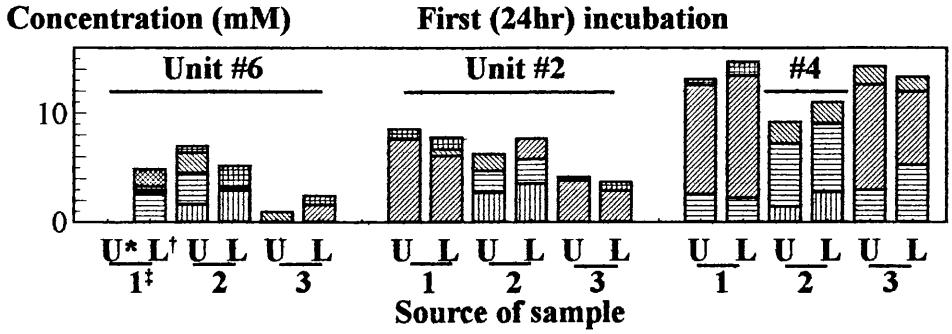
Moreover, significantly more acid was produced from sucrose when hydroxyapatite powder was present (units **#2** and **#3**) than when it was absent (units **#6** and **#8**)($p < 0.01$, Student's T-test) and significantly more was also produced when a root block was supplied along with the sucrose (units **#4** and **#5**) than when sucrose was supplied alone (units **#6** and **#8**)($p < 0.001$, Student's T-test) during the first incubation period. In

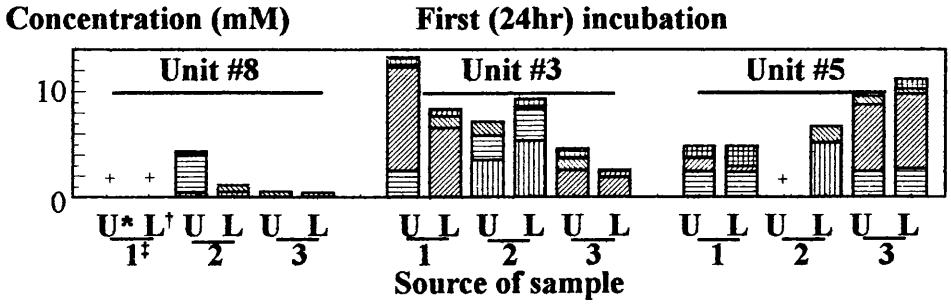
addition, significantly more acid was found when mineral was supplied along with sucrose (units **#3** and **#5**) than when sucrose was supplied alone (unit **#8**) during the second incubation period ($p < 0.02$ unit **#3** versus **#8** and $p < 0.05$ unit **#5** versus **#8**, Student's T-test). Furthermore the total acid concentrations were consistently and significantly greater after the second 24hr incubation period than after the first when the data for units **#8**, **#3** and **#5** were compared ($p < 0.001$, one-way analysis of variance).

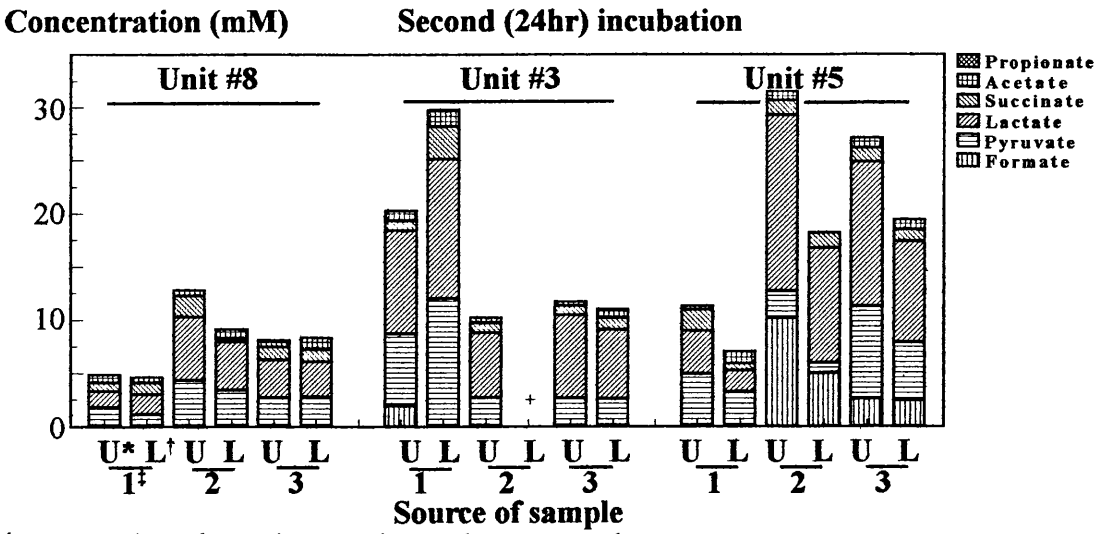
It is clear from the results that a greater concentration of acids was liberated by *S. mutans* in the presence of mineral than in its absence. However the results occasionally vary between some of the equivalent units within an experiment; for instance a greater total concentration of acids was detected in unit **#4** than in unit **#5** after the first and second repeats (both are sucrose plus a root block). There was also variation between repeats of the experiment; for example there was a higher concentration of acid in unit **#6** (first incubation period) and **#8** (first and second incubation periods) after repeat number 2 than repeats 1 and 3. Nevertheless, the total concentrations of acid detected in the upper (filter) chamber and lower (centrifuge tube) chamber of each unit were in general accord, although there were a number of notable exceptions (e.g. unit **#5** after the second 24hr incubation period in experimental repeats number 2 and 3).

Streptococcus mutans; individual acids The concentrations of individual acid anions detected after 24hr in the effluent reaction mixture from Ultrafree-CL units containing *S. mutans* NCTC 10449 when incubated with 5% sucrose solution in the presence or absence of mineral are shown in figure 5.5. Units **#6** and **#8** contained no mineral, units **#2** and **#3** contained hydroxyapatite powder and units **#4** and **#5** contained a root block.

Figure 5.6 **The concentrations of acids produced by *S. mutans* NCTC 10449 when incubated with sucrose solution plus no mineral (#6 & 8), hydroxyapatite powder (#2 & 3) or a root block (#4 & 5)**







‡ = 1, 2 and 3 refer to the experimental repeat number
* = from the upper (filter) chamber; above the bacterial film
† = from the lower (centrifuge tube) chamber; below the bacterial film
+ = not determined; insufficient sample volume

The results of the first and third repeats show one trend, whilst those of the second repeat show another; for example, in repeats number 1 and 3 (first incubation period) lactate and pyruvate formed a relatively low proportion of the total acids produced in the absence of mineral (units **#6** and **#8**), however when mineral was present in the units (**#2 - #5**) these two anions predominated. Lactate was the major acid when hydroxyapatite powder was present (units **#2** and **#3**), whilst there were also appreciable quantities of pyruvate when a root block was present (units **#4** and **#5**). In contrast there was very little lactate in samples from repeat number 2 (first incubation period) whilst pyruvate contributed to a large proportion of the total acids when mineral was present, although in the absence of mineral the other acids together formed the majority. When the concentrations of the different acid anions in units **#6**, **#8** and **#2 - #5** (sucrose solution alone, sucrose solution plus hydroxyapatite powder and sucrose solution and a root block) after the first incubation period were compared it was found that there were significant differences between them ($p < 0.001$, one-way analysis of variance).

After the second 24hr incubation period, the trends displayed by the three experimental repeats generally showed better agreement than after the first 24hr incubation period. Lactate usually predominated whether mineral was present or absent, whilst the other acids together rarely formed the majority (except for unit **#3** after the first repeat). When the concentrations of the different acid anions in samples from units **#8**, **#3** and **#5** (sucrose solution alone, sucrose solution plus hydroxyapatite powder and sucrose solution and a root block) after the second incubation period were compared it was found that there were statistically significant differences between them ($p < 0.001$, one-way analysis of variance).

Table 5.16 **Total concentration of acid liberated by *L. casei* NCTC 6375 during demineralisation experiments**

Unit #	Mineral source	Sample source	Total acid concentration (mM) in repeat number 1	2	3	Mean total acids (mM) (± 1 S.D.)
First (24hr) incubation period with sucrose solution						
6	None	Upper*	n.d.[+]	5.5	4.1	4.8 (1.0)
		Lower[†]	46.8	4.2	2.1	17.7 (25.2)
2	HAp[‡]	Upper	38.5	26.3	7.2	24.0 (15.8)
		Lower	0	45.6	5.4	17.0 (24.9)
4	root block	Upper	10.9	18.5	10.0	13.1 (4.7)
		Lower	26.2	8.1	8.4	14.2 (10.3)
8	None	Upper	n.d.	2.7	2.5	2.6 (0.1)
		Lower	14.7	5.6	0.4	6.9 (7.2)
3	HAp	Upper	n.d.	21.1	7.2	9.4 (10.7)
		Lower	43.1	26.9	3.0	24.3 (20.2)
5	root block	Upper	n.d.	6.5	1.6	4.1 (3.5)
		Lower	6.5	4.7	n.d.	5.6 (1.3)
Second (24hr) incubation period with sucrose solution						
8	None	Upper	13.0	18.7	19.0	16.9 (3.4)
		Lower	15.4	18.1	18.6	17.4 (1.7)
3	HAp	Upper	6.1	25.0	19.8	17.0 (9.8)
		Lower	21.8	27.8	10.0	19.9 (9.0)
5	root block	Upper	n.d.	28.3	2.9	15.6 (18.0)
		Lower	7.9	36.1	7.5	17.2 (16.4)
First (24hr) incubation period with distilled water						
9	HAp	Upper	0.9	0	0	0.3 (0.5)
		Lower	0	0	0	0 (0)
10	root block	Upper	0.5	0	n.d.	0.3 (0.3)
		Lower	0	0	n.d.	0 (0)
Second (24hr) incubation period with distilled water						
9	HAp	Upper	0	0	0	0 (0)?
		Lower	0	0	0	0 (0)?
10	root block	Upper	0	0	n.d.	0 (0)
		Lower	0	0	n.d.	0 (0)

* Upper = from the upper (filter) chamber, above the bacterial film
† Lower = from the lower (centrifuge tube) chamber, below the bacterial film
‡ HAp = hydroxyapatite powder
+ n.d. = not determined - insufficient sample volume

Lactobacillus casei; total acids The sum total acid anion concentration during repeats 1 - 3 in the effluent reaction mixture from each unit after 24hr incubation of *L. casei* NCTC 6375 with 5% sucrose solution or distilled water in the presence or absence of mineral are shown in table 5.16. In addition the mean sum total acid concentration of each is supplied for illustrative purposes. The acid anions recorded were formate, pyruvate, lactate, succinate, acetate and propionate and the concentration of each was corrected using the bacteria-free negative controls.

No acid was detected in the majority of the distilled water negative controls (units **#9** and **#10**). Acid was only detected twice in the negative controls at 0.5mM and 0.9mM in the fluid from units **#9** and **#10** respectively (both after the first incubation period during repeat number 1). Furthermore, as a result of the variation between repeats of the experiment, there were no significant differences in the acid concentrations detected after the first incubation period when *L. casei* films were incubated with sucrose solution alone (units **#6** and **#8**), sucrose solution with hydroxyapatite powder (units **#2** and **#3**) or with a root block (units **#4** and **#5**) compared with distilled water (units **#9** and **#10**) ($p = 0.063$, one-way analysis of variance). In contrast, after the second incubation period the acid concentration in units **#8**, **#3** and **#5** did prove to be significantly greater than in units **#9** and **#10** ($p < 0.001$, one-way analysis of variance).

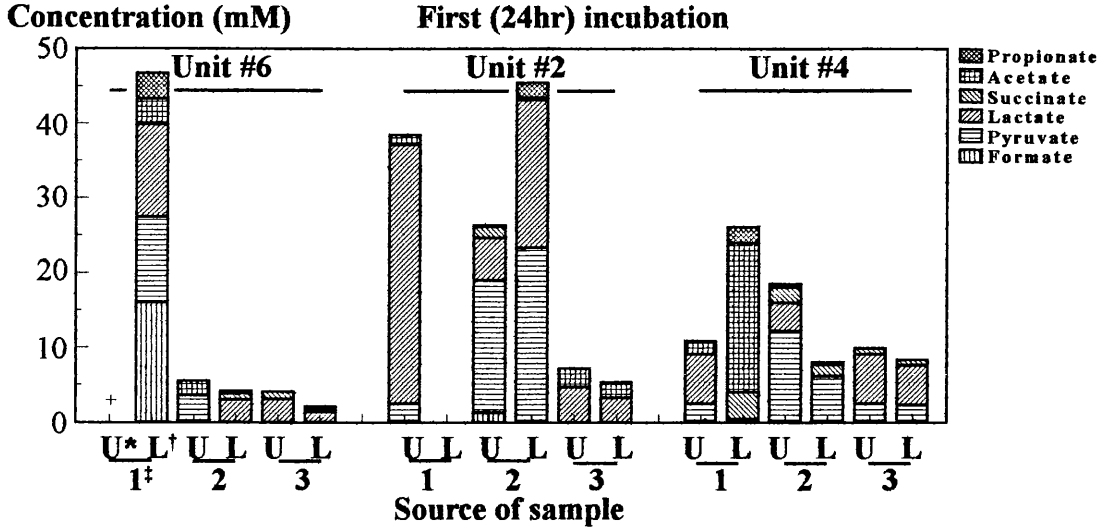
There did not appear to be any definite trends in the mean acid concentration data after the first incubation period when sucrose solution alone (units **#6** and **#8**), sucrose plus hydroxyapatite powder (units **#2** and **#3**) or sucrose plus a root block (units **#4** and **#5**) were supplied to the films. On closer examination there did appear to be a slight tendency towards higher acid concentrations in some of the individual repeats when

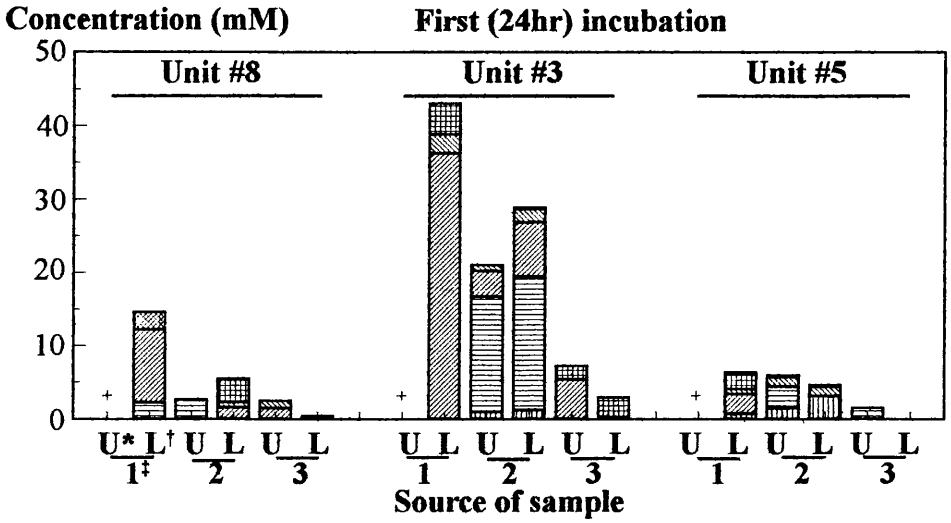
sucrose solution and mineral were supplied together rather than sucrose solution alone. However some data differed considerably from the other replicates and may therefore have obscured the trend overall. For instance the acid in the upper chamber of unit **#6** after repeat number 1 was more than 10 times greater than after repeats 2 and 3. Moreover this pattern was repeated erratically in many of the units during repeats 1 - 3, with one unit yielding substantially more acids than the replicate units. The variability of the data meant that it was not possible to establish any statistically significant trends in acid concentrations between the different treatments (sucrose solution with or without mineral)($p = 0.42$, one-way analysis of variance).

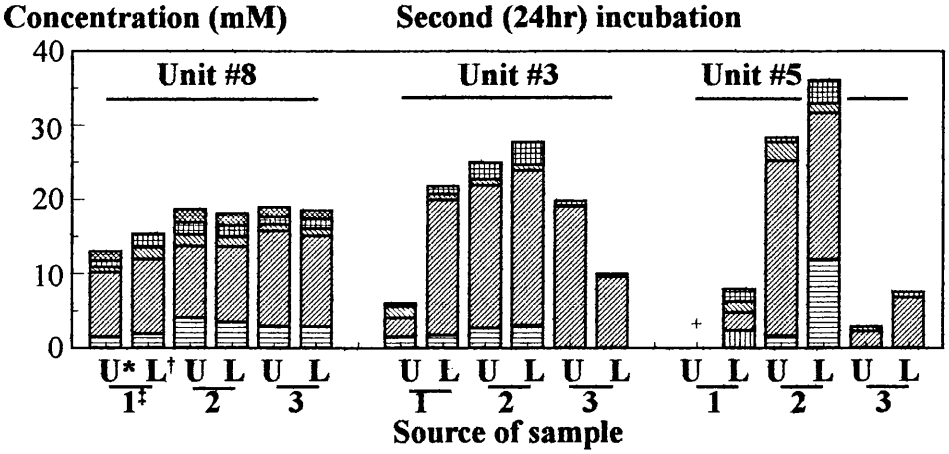
After the second incubation period there was no more of a clear trend to the mean acid concentration data than after the first. Furthermore the trends of the individual repeats of the experiment tended to conflict. For example in repeat 2 the total acid concentration was higher in the presence of mineral than in its absence, whilst in repeats 1 and 3 the total acid concentration (if anything) tended to be higher when mineral was not present. In contrast to the first incubation period, the data for acid concentration in the upper and lower chambers of units **#3**, **#5** and **#8** tended to agree more after the second incubation period, although there was no improvement in the variation between the three repeats. There were no significant differences between the acid concentration in unit **#3** (sucrose + hydroxyapatite powder) or unit **#5** (sucrose + root) compared with unit **#8** (sucrose alone) after the second incubation period ($p > 0.1$ in both cases, Student's T-test).

Lactobacillus casei; individual acids The concentrations of the different acid anions detected after 24hr in the spent culture fluid from Ultrafree-CL units containing *L. casei* NCTC 6375 when incubated with 5% sucrose solution in the presence or absence of

Figure 5.7 **The concentrations of acids produced by *L. casei* NCTC 6375 when incubated with sucrose solution plus no mineral (#6 & 8), hydroxyapatite powder (#2 & 3) or a root block (#4 & 5)**







‡ = 1, 2 and 3 refer to the experimental repeat number
* = from the upper (filter) chamber; above the bacterial film
† = from the lower (centrifuge tube) chamber; below the bacterial film
+ = not determined; insufficient sample volume

mineral are shown in figure 5.6. Units **#6** and **#8** contained no mineral, units **#2** and **#3** contained hydroxyapatite powder and units **#4** and **#5** contained a root block. During the first incubation period lactate and pyruvate tended to predominate whether mineral was present or not and if one predominated the other tended to be present at only low levels. However, acetate was often also present in appreciable proportions and in several cases even predominated (unit **#4** repeat 1, unit **#8** repeat 2 and unit **#3** repeat 3). The remaining acid anions (formate, succinate and propionate) were usually only present in trace amounts, although in one case formate did account for a substantial proportion of the acids present (unit **#6**, repeat number 1). The variability of the data between repeats after the first incubation period means that statistical analyses do not support convincing conclusions.

In contrast, after the second incubation period lactate almost always predominated and its proportions were very similar in both the upper and lower chambers (except for unit **#3** repeat 1 and unit **#5** repeat 1). However the other acid anions (formate, pyruvate, succinate, acetate and propionate) were generally only detected in trace amounts, except for substantial proportions of pyruvate in unit **#5** after repeat 2. There were significant differences between the acid anion concentrations in units **#3**, **#5** and **#8** after the three repeats of the experiment ($p < 0.001$, one-way analysis of variance).

Actinomyces viscosus; total acids The sum total acid anion concentrations detected after incubation of *A. viscosus* NCTC 10951 for 24hr with 5% sucrose solution or distilled water in the presence or absence of mineral are shown in table 5.17. In addition the mean sum total acid concentration of each is supplied for illustrative purposes. The acid anions

Table 5.17 **Total concentration of acid liberated by *A. viscosus* NCTC 10951 during demineralisation experiments**

Unit #	Mineral source	Sample source	Total acid concentration (mM) in repeat number 1	2	Mean total acids (mM) (± 1 S.D.)
First (24hr) incubation period with sucrose solution					
6	None	Upper*	n.d.[+]	1.7	1.7 (0)
		Lower[†]	4.2	1.8	3.0 (1.7)
2	HAp[‡]	Upper	6.4	2.0	4.2 (3.1)
		Lower	2.4	2.8	2.6 (0.3)
4	root block	Upper	n.d.	7.9	3.9 (5.6)
		Lower	n.d.	6.1	6.1 (0)
8	None	Upper	n.d.	2.7	1.3 (1.9)
		Lower	2.5	2.9	2.7 (0.3)
3	HAp	Upper	3.3	1.2	2.2 (1.5)
		Lower	2.1	3.2	2.6 (0.8)
5	root block	Upper	5.5	8.8	7.2 (2.3)
		Lower	5.6	8.8	7.2 (2.2)
Second (24hr) incubation period with sucrose solution					
8	None	Upper	5.8	11.5	8.7 (4.1)
		Lower	7.0	14.2	10.6 (5.1)
3	HAp	Upper	5.4	7.4	6.4 (1.4)
		Lower	0	8.4	4.2 (5.9)
5	root block	Upper	5.6	11.2	8.4 (4.0)
		Lower	5.3	12.2	8.7 (4.9)
First (24hr) incubation period with distilled water					
9	HAp	Upper	1.0	0.9	1.0 (0.1)
		Lower	0	0	0 (0)
10	root block	Upper	0.5	0	0.2 (0.3)
		Lower	1.3	0	0.6 (1.0)
Second (24hr) incubation period with distilled water					
9	HAp	Upper	n.d.	n.d.	n.d.
		Lower	0	n.d.	0 (0)
10	root block	Upper	n.d.	n.d.	n.d.
		Lower	0	n.d.	0 (0)

* Upper = from the upper (filter) chamber, above the bacterial film

† Lower = from the lower (centrifuge tube) chamber, below the bacterial film

‡ HAp = hydroxyapatite powder

+ n.d. = not determined - insufficient sample volume

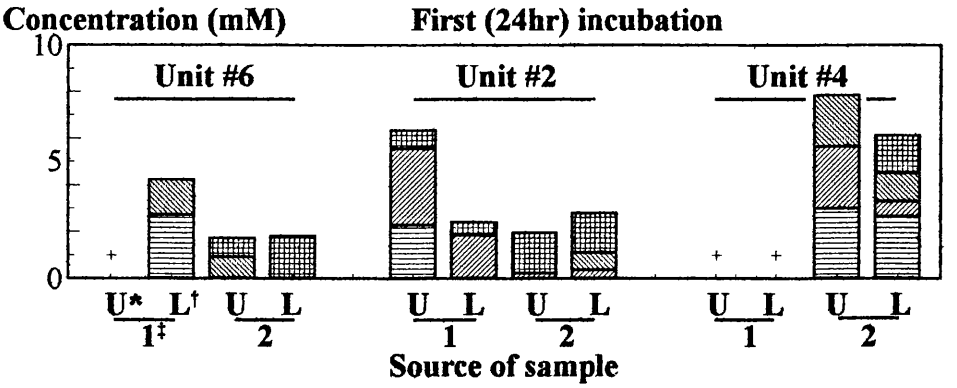
recorded were formate, pyruvate, lactate, succinate, acetate and propionate and the concentration of each was corrected using the bacteria-free negative controls. There are data for only two repeats of the experiment since there was insufficient time for a third.

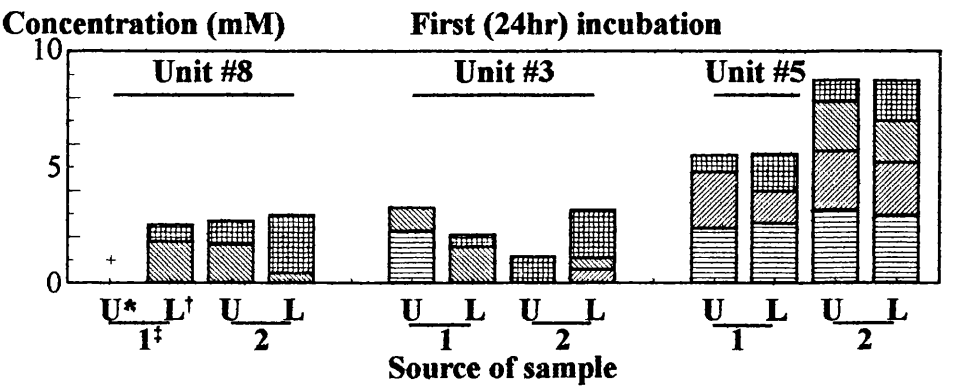
In general, the distilled water negative controls (units **#9** and **#10**) contained no detectable acids, although a number of samples did contain some acids (up to a maximum of 1.3mM). In contrast, during the first incubation period more acid was detected in the sucrose-fed units (**#2** - **#6** and **#8**) than the distilled water control units (**#9** and **#10**). Furthermore, there was a tendency for increased acid concentrations in units that contained sucrose solution and a root block (**#4** and **#5**), which was not observed when sucrose solution alone (units **#6** and **#8**) or sucrose solution plus hydroxyapatite powder (units **#2** and **#3**) were supplied to the bacterial films.

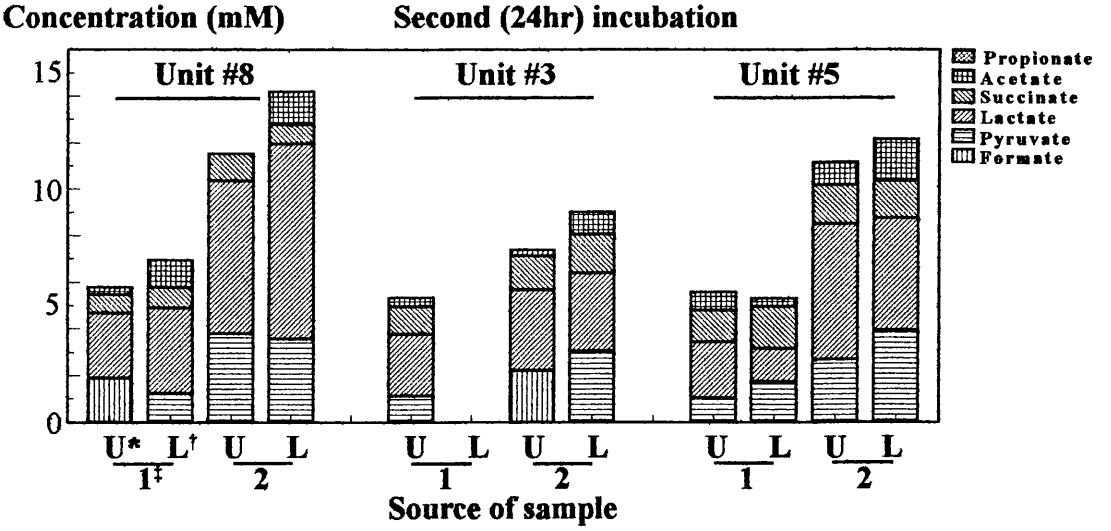
The total concentration of acid detected during the second incubation period was also greater in sucrose-fed units (**#3**, **#5** and **#8**) than in distilled water controls (units **#9** and **#10**). However, when sucrose solution alone (unit **#8**) or sucrose solution plus a root block (unit **#5**) were supplied to the *A. viscosus* films the acid concentrations were 30 - 50% greater than when sucrose solution plus hydroxyapatite powder (unit **#3**) were supplied.

Actinomyces viscosus; individual acids The concentration of each acid anion in the effluent reaction mixture from Ultrafree-CL units containing *A. viscosus* NCTC 10951 when incubated with 5% sucrose solution with or without mineral is shown in figure 5.7. Units **#6** and **#8** contained no mineral, units **#2** and **#3** hydroxyapatite powder and units **#4** and **#5** a root block.

Figure 5.8 **The concentration of acids produced by *A. viscosus* NCTC 10951 when incubated with sucrose solution plus no mineral (#6 & 8), hydroxyapatite powder (#2 & 3) or a root block (#4 & 5)**







‡ = 1, 2 and 3 refer to the experimental repeat number
* = from the upper (filter) chamber; above the bacterial film
† = from the lower (centrifuge tube) chamber; below the bacterial film
+ = not determined; insufficient sample volume

After the first (24hr) incubation period lactate, pyruvate and acetate were the predominant acid anions, whilst succinate was also present in substantial proportions in many of them. However, the presence of mineral had only a minimal impact upon the relative acid concentrations in units **#2** - **#5** compared with units **#6** and **#8**, although there appeared to be a minor trend towards increased pyruvate in units containing a root block (**#4** and **#5**). There was, on the whole, little reproducibility to the concentrations of each acid anion in units **#2** - **#6** and **#8** either between repeats or between the upper and lower chambers of each unit, except for unit **#5**. Statistical analyses were not possible since only two repeats of the experiment could be performed in the time available.

Lactate, pyruvate and acetate also predominated after the second (24hr) incubation period, whilst succinate also formed a considerable minority in many samples. However, there was no real difference between the acid anion concentrations whether mineral was supplied along with the sucrose or not. In contrast to the first incubation period, the degree of variation in the acid concentrations between repeats after the second incubation period was much less. Statistical analyses were not practical since the data were only duplicate.

Comparisons of sum total acid anion concentrations for the three species There was a tendency for *L. casei* to produce more acid than either *S. mutans* or *A. viscosus* during the first incubation period and this tendency was most pronounced when hydroxyapatite powder was present. In addition there were significant differences in the total acid concentration in units **#2** - **#6** and **#8** between *L. casei* and *S. mutans* ($p = 0.009$, one-way analysis of variance). Furthermore the amount of acid produced by *S. mutans* also tended to be greater than produced by *A. viscosus*, especially when either mineral source

was present. In contrast during the second incubation period *L. casei* only tended to produce more acid than *S. mutans* when incubated with sucrose solution alone (unit **#8**), whereas when either was incubated with sucrose plus mineral (units **#3** and **#5**) the amount of acid produced was comparable. Moreover there were no significant differences in the total acid concentration in units **#3**, **#5** and **#8** between *S. mutans* and *L. casei* ($p = 0.271$, one-way analysis of variance). In addition *L. casei* produced more acid during the second incubation period than *A. viscosus* whether mineral was present or not in units **#3**, **#5** and **#8**, whilst *S. mutans* only produced more when mineral was present (units **#3** and **#5**).

Comparison of individual acid anion concentrations for the three species When the individual acid anion concentrations for either *S. mutans*, *L. casei* or *A. viscosus* after the first (24hr) incubation period with sucrose in the presence or absence of mineral were compared there were no obvious trends. The pattern of acid anions produced by each organism tended to be erratic and unpredictable. Whilst lactate was usually the predominant acid anion produced by all three species, pyruvate often predominated when lactate did not. Occasionally formate, succinate and acetate formed a sizeable minority of the acids detected, especially in the case of *A. viscosus*, although there were no tangible trends linking mineral (or its absence) with the production of specific acid anions.

During the second (24hr) incubation period there was considerably more reproducibility between the acid anion concentration data for each experimental repeat than after the first. Lactate was generally present at a higher concentration than any of the other acid anions in units **#3**, **#5** and **#8** for all three species. Furthermore *L. casei* produced significantly more lactate than *S. mutans* when incubated with sucrose solution alone or

with sucrose solution plus hydroxyapatite powder ($p = 0.002$, one-way analysis of variance) and it also produced more lactate than *A. viscosus* when incubated with either of these. However when supplied with sucrose solution plus a root block there were no significant differences between the lactate concentration produced by *S. mutans* and that produced by *L. casei* ($p = 0.95$, Student's T-test).

The greater reproducibility in the data after the second 24hr incubation period meant that trends were more evident than after the first. For instance, the presence of mineral tended to be associated with more lactate production by *S. mutans* and *L. casei* than when it was absent, whilst the opposite was observed with *A. viscosus*. Formate, pyruvate, succinate and acetate were produced by the three species at lower concentrations than lactate, however the presence of mineral appeared to be directly related to increased concentrations of these anions with *S. mutans* but not *L. casei* or *A. viscosus*. Moreover, between them these acids often amounted to almost 50% of the total from *S. mutans* and *A. viscosus*, whereas they amounted to less than 33% from *L. casei*.

5.3.4 Correlation between acid and calcium ion concentrations

Streptococcus mutans When the total acid concentration was compared with the calcium ion concentration in units **#2 - #6** and **#8 - #10** containing *S. mutans* after the first 24hr period of incubation there was a strong positive correlation between them ($r = 0.761$). There was also a strong positive correlation between the total acid and calcium ion concentrations after the second 24hr incubation period ($r = 0.829$). Of the individual acid anions only lactate showed a strong positive correlation with calcium ion concentration during both 24hr incubation periods ($r = 0.884$ and 0.860, first and second incubation periods respectively), although succinate did show a positive correlation with calcium ion

concentration after the second incubation period (r = 0.763). Pyruvate, acetate and formate at best showed only a weak positive correlation with calcium ion concentration after the second incubation period (r = 0.630, 0.607 and 0.569 respectively) although their concentrations were very low and therefore not ideal for statistical analysis.

Lactobacillus casei There was a weak positive correlation between total acid and calcium ion concentrations in units **#2 - #6** and **#8 - #10** containing *L. casei* after the first incubation period (r = 0.531), however there was a much better positive correlation between the two after the second incubation period (r = 0.765). Of the individual acid anions, a good positive correlation between lactate and calcium ion concentration was found (r = 0.616) after the first incubation period. Moreover, there was a strong positive correlation between the lactate and calcium ion concentrations after the second incubation period (r = 0.827). However there was no correlation between any of the other acid anions and calcium ion concentration ($r < 0.5$).

Actinomyces viscosus As mentioned before, the lack of a third repeat due to time constraints meant that it was not possible to analyse statistically the data gathered with *A. viscosus* and therefore only trends could be discussed. To the eye it appeared that there may have been a tendency after the first incubation period for increased total acid production when a root block was present but not when hydroxyapatite powder or sucrose alone were present. In contrast there did not appear to be any obvious correlation between total acid and calcium ion concentrations after the second incubation period. Furthermore, there did not appear to be any obvious correlation between the individual acid anion and calcium ion concentrations after either the first or the second incubation periods.

5.4 Discussion and conclusions

5.4.1 Introduction

The results of preliminary experiments are described in this chapter which sought to study demineralisation of human tooth root surfaces by bacteria using the novel model system described in chapter 4. Hydroxyapatite powder was employed as a positive control in addition to human tooth root blocks since it has been used successfully by previous workers (Chestnutt, MacFarlane & Stephen, 1994). It was intended that the data provided by these preliminary experiments could then be used to suggest improvements to the model system.

During the current experiments the bacterial species were inoculated into the Ultrafree-CL units and then incubated for 1d prior to the commencement of the demineralisation part of the investigation. This was for two inter-related reasons; firstly past experience had indicated that bacterial films reached maximum density after about 1d of incubation in Ultrafree-CL units and secondly it was felt that this (pre-)incubation period would allow some degree of plaque 'maturation' so that the cells were in a state more akin to that present in dental plaque. However Todd Hewitt broth is a complex nutrient medium containing a variety of polypeptides, carbohydrates and salts, all of which could have interfered with the analytical techniques subsequently employed. The bacterial films thus needed to be rinsed in some way prior to the initiation of the demineralisation experiments and 135mM KCl was chosen as the diluent since Marsh *et al.* (1982) reported that this optimised acid production by *Streptococcus* species (including *S. mutans*).

During the demineralisation experiments the sucrose solution was overlaid onto the bacterial films, allowed to percolate through the membrane and then collected in the empty lower chamber. The alternative of adding sucrose to the lower chamber was not attempted because it was thought that the dilution effect of the relatively large volume of diluent on the small amount of acid produced (and calcium subsequently released) was unacceptable. The use of 1ml volumes of fluid applied to the upper chamber decreased such dilution effects. Three analyses were then selected to elucidate some of the events that occurred during the current experiments.

Firstly viable counts were determined to ensure that the bacterial films were behaving reproducibly between repeats and to allow comparison with previous experiments. Secondly the concentration of calcium ions present in the effluent reaction mixture was determined using a colorimetric calcium assay marketed by Sigma Diagnostics (Sigma, U.K.) to give an estimate of demineralisation. Atomic absorption spectroscopy (employed by Boonstra, ten Bosch & Arends, 1990) was precluded since the volume of sample required would have exceeded that available. Microradiography (employed by e.g. Clarkson *et al.*, 1987 and Kaufman, Pollock & Gwinnett, 1988; both *in vitro*) was also precluded on the grounds that each analysis would have required the system to be dismantled with the risk of contamination and disruption of the plaque/ mineral interface. In addition microradiography would have resulted in either fewer measurement time-points or a vast increase in the number of units since a separate Ultrafree-CL unit containing a root block would have been required for each time-point. The third analysis performed upon the effluent reaction mixture was isotachophoresis which rapidly provides quantitative data upon the concentration of selected acid anions present in fluid samples (Geddes and Weetman, 1981). Moreover, expertise in isotachophoresis was

readily available which made it both practical and logical to employ this particular technique. Due to time constraints it was decided to determine the concentration of acids in samples collected at 24hr, since this would have permitted maximum acid production by the bacteria within the experimental time-scale.

It seems reasonable to divide the discussion which follows along the lines of the analyses performed and therefore it will consist of sections on viable counts, calcium ion concentration (and amount of calcium liberated) and acid anion concentration. The inter-relationships between the three sets of data will also be explored.

5.4.2 Viable count data

Streptococcus mutans After 1 day of incubation with T.H.B. the mean number of *S. mutans* increased to 1.26×10^7 c.f.u./cm^2, which was comparable with the viable counts retrieved previously after 1d (section 4.2.2) and by extrapolation with the data of other workers, which were discussed in section 4.3.2. However, when *S. mutans* films were incubated with sucrose solution alone the viable counts were reduced markedly to a mean of 1.59×10^2 c.f.u./cm^2 (table 5.1) and in two out of three repeats a negative result was recorded, which corresponds with the observations in section 4.2.8 in spite of the shorter incubation period (1d here compared with 6d). Possible reasons for the reduced viable counts when *S. mutans* was incubated with sucrose were discussed in section 4.3 and included accumulation of acidic metabolic by-products and starvation. Nevertheless in the light of the current data it would appear that the number of retrievable c.f.u. actually decreased rapidly within the first day. The viable counts of films incubated with 5% sucrose for 1d and then returned to T.H.B. for a further 1d demonstrated a substantial increase (over 1,000-fold), which was also similar to the trend

observed in section 4.2.8. However the viable counts of the present experiments did not recover to baseline (i.e. day 1) levels when returned to T.H.B. (i.e. day 3) tending to contradict the results of section 4.2.8. Therefore it would appear that under the conditions employed, incubation for 24hr was not sufficient for the complete recovery of *S. mutans* viable counts after exposure to sucrose whereas a further day would probably have permitted this (as in section 4.2.8).

It was interesting to note that not only were the counts of *S. mutans* higher when incubated with mineral and sucrose solution after the first incubation period, which contrasts with the data for sucrose alone, but that they were further increased after a second incubation period with sucrose plus mineral. When Chestnutt (1992) incubated 40mg (wet weight) of a *S. mutans* NCTC 10449 cell slurry with 5% sucrose solution alone for a total of 5hr the viable counts were reduced by 17%, however when 10mg of hydroxyapatite was incorporated in the reaction mixtures the viable counts were reduced by only 8%. The observed 'protection' of viable counts by the presence of mineral in the current experiments corresponded with the data of Chestnutt, although the changes in viable counts retrieved from films incubated with and without mineral during the current experiments were more extreme (i.e. a reduction of > 99% or 100,000-fold without mineral compared with only 90% or 10-fold with mineral). There were differences in experimental design which could help to explain this discrepancy; for instance the initial number of bacteria employed by Chestnutt was approximately 10 $\log_{10}$ c.f.u. compared with approximately 7 $\log_{10}$ c.f.u. in the current experiment, whilst the incubation time was 5hr (Chestnutt) compared with 24hr (current experiments) and the reaction mixture volume was 4x greater in the current experiments. It is most likely that protection of

viable counts by mineral was related to the buffering capacity of dissolution products such as phosphate ions (discussed further in section 5.4.5).

When distilled water was employed instead of sucrose the picture was more confused since *S. mutans* films seeded with hydroxyapatite powder showed no real change in viable counts compared with baseline. Chestnutt (1992) also performed experiments with *S. mutans* and hydroxyapatite in which distilled water replaced the sucrose and recorded a reduction in viable counts of less than 5% compared with baseline, which agrees with the current data. In contrast there were no viable counts from two out of the three films incubated with distilled water and a root block during the current experiments. This was surprising since it represented a greater reduction than that caused by sucrose (in contradiction to the results of section 4.2.8). It is not clear why viable counts should be reduced so much with water and root since the results for water plus hydroxyapatite tend to rule out the likely involvement of starvation or osmotic shock.

Lactobacillus casei After 1d of incubation with T.H.B. the mean number of *L. casei* retrieved was 3.72×10^7 c.f.u./cm^2 which was comparable with the results reported in section 4.2.2 for 1 day-old films of *L. casei* and was therefore comparable with the majority of *in vitro* and *in situ* studies discussed in section 4.3. There were no statistically significant changes in the number of *L. casei* c.f.u. during the current experiments when transferred from T.H.B. to sucrose for 1d (with or without mineral) and back again. This contrasts with the 30-fold decrease in c.f.u. observed after 6d in section 4.2.8 and suggests that 1 day of incubation with sucrose and exposure to acidic fermentation products was insufficient to overcome the known acid tolerance of this organism (Stanier *et al.*, 1987). In contrast the viable counts of films supplied with water

plus hydroxyapatite powder or a root block were reduced 50-fold and 2,000-fold respectively which tends to correspond with the 1,000-fold reduction in viable counts that was reported in section 4.2.8 after 6d incubation with distilled water alone. These results tend to suggest that starvation or osmotic shock may have affected retrievable viable counts. There appear to be no directly comparable experiments in the available literature that enumerated the effect of sucrose or distilled water upon lactobacillus viable counts with which to compare the current results.

Actinomyces viscosus The number of retrievable *A. viscosus* c.f.u. increased substantially during the first 1d of incubation with T.H.B. to a mean of 1.26×10^7 c.f.u./cm^2 which was comparable with the mean 1d cell density reported in section 4.2.2 and was therefore also comparable with previous *in vitro* and *in situ* experiments (section 4.3). In spite of the lack of a third set of data, which made statistical analyses impracticable, there were still discernible trends in the viable counts.

After the first 1d of incubation with sucrose during the current experiments, the viable counts decreased to 7.94×10^5 c.f.u./cm^2 from baseline and further decreased to 1.00×10^5 c.f.u./cm^2 after the second 1d of incubation. The viable counts of *A. viscosus* incubated with sucrose solution alone in section 4.2.8 were reduced significantly (to 3.16×10^3 c.f.u./cm^2) during 6d of incubation which tends to agree with the trend here. However the magnitude of the decrease during the current experiments was less than in section 4.2.8 (only 20-fold here compared with 10,000-fold in section 4.2.8), presumably as a consequence of the shorter exposure time (1d compared with 6d respectively). Moreover, when returned to T.H.B. during the current experiments there was no recovery in viable counts, contradicting the results of section 4.2.8 which demonstrated

complete recovery when returned to T.H.B. Once again this is most likely a consequence of the different incubation periods of the two series of experiments (1d recovery in T.H.B. during the current experiments compared with 2d in section 4.2.8).

When the *A. viscosus* films were incubated with hydroxyapatite powder and sucrose the resultant viable counts were much higher than with sucrose alone, in fact after 1d with sucrose + hydroxyapatite the viable counts tended to be higher than the 1d baseline counts. This supports the idea suggested earlier that mineral helps to protect the bacteria against harmful alterations to the environment, possibly via buffering effects. However the number of c.f.u. retrieved after the second incubation period were generally lower than after the first in spite of a recovery period in fresh T.H.B., suggesting that recovery of stressed cells may have required more time than was allowed in the current experiment. Moreover root blocks did not provide the same degree of protection as hydroxyapatite powder when the bacterial films were incubated with sucrose. Furthermore the viable counts retrieved after the first and second incubation periods with sucrose plus a root block (units **#4** and **#5**) showed opposite trends between repeats 1 and 2 (i.e. they were higher after the second incubation period during repeat 1 but not repeat 2).

Hydroxyapatite powder also appeared to afford protection to *A. viscosus* films incubated with distilled water whereas root blocks did not. In section 4.2.8 the viable counts of *A. viscosus* were equally low after incubation with either sucrose or distilled water compared with T.H.B. Therefore protection of viable counts by hydroxyapatite but not root during the current incubation with water agrees with the above observations with sucrose. However, it is difficult to explain protection by hydroxyapatite powder and not

root when incubated with water since the obvious explanations of starvation or osmotic shock ought to have affected both sets of films equally.

The three species compared The mean viable counts of all three species retrieved on day 1 were similar to one another and were comparable with the results of section 4.2.2 and of other workers. However after day 1 when environmental conditions were changed by addition of sucrose solution or distilled water plus or minus mineral, the viable counts of the three species diverged, nor did they behave entirely as would have been predicted from the results of section 4.2.8. In summary both *S. mutans* and *A. viscosus* viable counts decreased as expected but failed to recover, whereas *L. casei* viable counts did not decrease as expected in the first place. When mineral was supplied along with sucrose the viable counts of *S. mutans* were protected to a large extent by both hydroxyapatite powder and root blocks, whereas the viable counts of *A. viscosus* appeared to be protected to a greater extent by the presence of hydroxyapatite powder. In contrast the viable counts of the relatively aciduric *L. casei* were not affected by the acids produced and therefore did not gain any benefit from the presence of mineral or its dissolution products.

The variability in the viable count data between repeats of the current experiment was within acceptable limits when the organisms were incubated with T.H.B., sucrose plus hydroxyapatite powder or water plus hydroxyapatite powder. However the data were not so reproducible between repeats when the films were incubated with sucrose plus a root block. Furthermore the variation in the data between repeats of the experiment were substantial when films were incubated with sucrose alone or water plus a root block. The level of variation experienced resulted from there being an absence of retrievable viable

counts during some repeats of the experiment but not others. The most similar experimental system to the one currently employed was that of Millward & Wilson (1989), however no data for reproducibility was supplied.

5.4.3 Calcium ion release

Streptococcus mutans The concentration of calcium ions liberated from either hydroxyapatite or root surface in the presence of *S. mutans* and sucrose solution showed a progressive increase with time. However there does not appear to be any directly comparable data available in the literature that shows an increase over time in calcium concentration released from mineral *in vitro* as a result of the activity of monospecies bacterial films (as shown by the current experiment), although a number of reports indicate that such a result would be expected. For instance Chestnutt (1992) found a steady increase in calcium release from hydroxyapatite powder (measured colorimetrically as in the current experiment) when it was incubated *in vitro* with a slurry of *S. mutans* and 146mM sucrose. Moreover the results of an *in situ* experiment in which root surface sections (with intact cementum) were exposed intra-orally in dental appliances also indicated increased release of calcium with time when the root sections were examined microradiographically (Øgaard, Rølla & Arends, 1988). In addition Herkströter, Witjes & Arends (1991) reported an increase in calcium release over time with an *in vitro* pH cycling device which employed organic acids and both human dentine and enamel. Boonstra, ten Bosch & Arends (1988 and 1990) also performed *in vitro* experiments to explore demineralisation of bovine dentine by 100mM acetic acid and found an increase in calcium release (measured using A.A.S.) with time. Thus it would appear that the mineral: *S. mutans* biofilm interaction in the Ultrafree-CL model system did not differ substantially from that observed previously both *in vitro* and *in situ*.

Calcium could be detected (albeit at low levels) in the spent culture fluid after as little as three hours in the current Ultrafree-CL model system, indicating rapid onset of mineral dissolution. Chestnutt (1992) using an *in vitro* system, as described above, was able to detect calcium after 30min when hydroxyapatite powder was incubated with *S. mutans* and 5% sucrose. In addition Margolis *et al.* (1993) using an *in situ* model detected an increase in the calcium ion concentration in plaque fluid overlying tooth roots within three minutes of a 1min sucrose rinse. The current data therefore suggests that the Ultrafree-CL model system does not substantially alter the dissolution characteristics of mineral exposed to bacteria in comparison with existing *in vitro* and *in situ* model systems. Furthermore the current data corresponds with existing data by indicating that changes in the environment can rapidly lead to perturbations in the equilibrium between solid and dissolved calcium (i.e. root solid and plaque fluid) in this case leading to nett dissolution.

The calcium concentration released from either mineral source during the current investigations tended to be lower than that reported by other studies. There are substantial differences between the design of the current and previous experiments that tends to limit the value of direct comparisons. For instance Chestnutt, MacFarlane & Stephen (1994) incubated hydroxyapatite powder with a slurry of *S. mutans* NCTC 10449 plus 5% sucrose and recorded a calcium concentration equivalent to 105M after 5hr, which is substantially greater than the 0.002 - 0.003M detected after 24hr currently. However Chestnutt, MacFarlane & Stephen employed a much higher number of bacterial cells to generate acid in a smaller volume of diluent with a subsequent increase in the acid concentration. Furthermore the hydroxyapatite powder was thoroughly mixed with the bacterial slurry by Chestnutt, MacFarlane & Stephen thus maximising the surface

area of hydroxyapatite powder in contact with bacteria whereas in the current model the contact area of bacteria with mineral was likely to be much less. When mixed plaques *in situ* were rinsed with sucrose by Margolis *et al.* (1993) 5mM calcium was detected in the plaque fluid after 3min compared with 2 - 3mM calcium after 24hr during the current experiments. However the plaques studied by Margolis *et al.* would almost certainly have consisted of a complex mixture of wild type bacterial strains rather than the laboratory mono-cultures as currently employed, which may possess altered phenotypic properties compared with wild-type strains (Vadenbocoeur & Trahan, 1983). Thus although the calcium released during the current experiments was less than that recorded in other model systems, this would appear to be largely due to inherent differences between the systems.

The total amount of calcium ions liberated from hydroxyapatite powder by *S. mutans* in the presence of sucrose solution was very similar to that released from root blocks. This is an interesting observation since the total surface area of 5mg of hydroxyapatite powder particles would have been many times greater than the $16mm^2$ of exposed root surface and therefore it might have been reasonable to anticipate greater calcium release from hydroxyapatite. Hydroxyapatite powder has very similar solubility properties to enamel (Driessens *et al.*, 1986) whilst root mineral begins to dissolve at a much higher pH than enamel (pH 6.7 for root compared with pH 5.4 for enamel)(Hoppenbrouwers, Driessens & Borggreven, 1987). This difference in acid solubility might help to explain the similarity in calcium concentration detected between root and hydroxyapatite by negating the effects of differences in exposed surface area. In addition it is possible that the amount of acid produced was sufficiently low as to limit the degree of dissolution of

either mineral source that occurred, by reaching an equilibrium before 24hr; the data (tables 5.15, 5.16 and 5.17) also tend to support this point of view.

Although there was variation in both calcium ion concentration and total amount of calcium detected between repeats of the present experiment, consecutive fluid samples from each unit showed the same trend (i.e. an increasing calcium ion concentration from 3 to 24hr). This tends to suggest that the trend itself can be viewed with confidence but that improvements need to be made to the system to make the data more robust. The variability in the calcium release data for *S. mutans* is discussed along with that for *L. casei* and for *A. viscosus* in the following section that compares the calcium release data for the three species, whilst possible modifications to help to reduce variation are discussed in chapter 6.

Lactobacillus casei The concentration of calcium ions liberated from both hydroxyapatite powder and root blocks increased over time when incubated with *L. casei* and 5% sucrose. As discussed in relation to the *S. mutans* data, an increase in calcium release with time tends to correspond with the observations of other workers in a variety of different model systems which include *in vitro* bacteria (Chestnutt, 1992), *in situ* bacteria (Øgaard, Rølla and Arends, 1988) and *in vitro* organic acids (Herkströter, Witjes & Arends, 1991 and Boonstra, ten Bosch & Arends, 1988 and 1990), although there are no directly comparable data since none employed monofilms of *L. casei* to generate demineralisation.

It seemed curious that *L. casei* demineralised root to a lesser extent than hydroxyapatite powder, since root mineral is more soluble than hydroxyapatite (compare

Hoppenbouwers, Driessens & Borggreven, 1987 with Driessens *et al.*, 1986) and *L. casei* has been reported to generate lesions in human tooth root sections (Kaufman, Pollock & Gwinnett, 1988). It is possible that the type strain used in the current experiments had become attenuated due to subculturing (Vadenboncoeur & Trahan, 1983) and so was less acidogenic than the strain employed by Kaufman, Pollock & Gwinnett (1988). However the total concentration of acid from hydroxyapatite-supplied films was also greater than from root block-supplied films. This poses various questions; was the acid production of *L. casei* in the root block-supplied films for some reason, leading to decreased dissolution or was the root mineral less soluble than hydroxyapatite (e.g. due to fluoride exposure; Mellberg & Sanchez, 1986) which led to decreased acid production due to decreased buffering by dissolution products?

Actinomyces viscosus As mentioned previously, the data for *A. viscosus* is incomplete since only two repeats of the experiment were performed with this organism in the time available and therefore it is difficult to draw any firm conclusions. It would appear that *A. viscosus* was responsible for increasing calcium ion release from either mineral source with time when incubated with sucrose, however the concentration of calcium detected was low, and the variation in values between repeats of the experiment was high. *Actinomyces viscosus* was responsible for virtually no demineralisation in one repeat whilst in the other there was a definite increase in dissolution with time to a maximum of almost 4mM.

A number of studies have found *A. viscosus* to be capable of demineralising human tooth root tissue even though they employed different methodologies to the current experiments. For example Kaufman, Pollock & Gwinnett (1988) exposed human tooth

root sections to *A. viscosus* for 8d in a medium supplemented with 2% dextrose and produced a lesion with a depth of 34μm as measured by microradiography. Furthermore Clarkson *et al.* (1987) exposed tooth root surfaces to *A. viscosus* for 21d in a defined medium supplemented with sucrose, starch or both and recorded lesion depths ranging from 88 to 178μm depending upon carbohydrate source. Firestone *et al.* (1993) also exposed human tooth roots to *A. viscosus* for 9d in broth supplemented with 0.25% sucrose and found lesion depths of between 246 and 251μm. Since the above demonstrated that *A. viscosus* was capable of demineralisation *in vitro*, even though they employed different incubation times and analytical techniques (microradiography instead of calcium ion detection) to the current experiments, it seems likely that the current relatively negative results should be interpreted with caution and that a further replicate of the experiment is required to assist with interpretation.

The three species compared The three species were not equal in their ability to demineralise hydroxyapatite powder or root blocks under the conditions employed; *S. mutans* and *L. casei* were more active in demineralisation than *A. viscosus* when incubated with sucrose. All of the *in vitro* studies which examined dissolution of tooth root tissue during incubation with bacteria (Kaufman, Pollock & Gwinnett, 1988 with *S. mutans*, *L. casei* and *A. viscosus* and Clarkson *et al.*, 1987 with *S. mutans* and *A. viscosus*) measured loss of mineral by means of altered density (microradiography) rather than loss of calcium ions (calcium assay or atomic absorption spectroscopy). Kaufman, Pollock & Gwinnett (1988) found a lesion depths of 230μm for *S. mutans*, 83μm for *L. casei* and 34μm for *A. viscosus*, whilst Clarkson *et al.* (1987) recorded lesion depths ranging from 0 to 247μm for *S. mutans* and 88 to 178μm for *A. viscosus* depending upon carbohydrate source. In spite of the different experimental procedures of

the above two studies compared with the current one (especially culture conditions and measurement techniques) it is still possible to compare and rank the demineralising activity of the test species (tables 5.13 and 5.14). The current data appear to concur with both Kaufman, Pollock & Gwinnett (1988) and Clarkson *et al.* (1987)(i.e. *S. mutans* ≈ *L. casei* > *A. viscosus*).

Although *A. viscosus* was not responsible for as much demineralisation as the other two species in this particular experiment it does not necessarily mean that this organism is actually less cariogenic *in vivo*, since in some circumstances it is capable of generating greater demineralisation than mutans streptococci. For instance Clarkson *et al.* (1987) found that mineral dissolution due to *A. viscosus* was greater than that due to *S. mutans* when supplied *in vitro* with starch and sucrose. Moreover Firestone *et al.* (1993) found that *A. viscosus* was responsible for greater *in vitro* lesion progression in tooth roots (with lesion depths of 246 - 251μm) than *S. sobrinus* (with a lesion depth of 219μm) which de Soet *et al.* (1991) reported was more cariogenic than *S. mutans* in laboratory rats.

Lactobacillus casei appeared to be responsible for greater calcium dissolution from hydroxyapatite powder than *S. mutans*, whereas it did not generate any greater demineralisation of root blocks than *S. mutans*. The latter results tend to agree with Kaufman, Pollock & Gwinnett (1988) who found that *L. casei* generated shallower root surface lesions *in vitro* than *S. mutans*. There was a tendency in the current studies for both species to cause greater demineralisation during the second incubation period than during the first, although *L. casei* was still responsible for greater demineralisation than *S. mutans*, possibly due both to its survival in greater numbers after the first incubation

period (tables 5.1 and 5.2) and also to the production of greater acid concentrations (tables 5.15 and 5.16).

It was also noted that all three species consistently generated very small but detectable levels of calcium from root sections when incubated with water rather than sucrose (unit **#10**). Calcium dissolution was not noted in bacteria-free control units (**#15**) and therefore it is possible that sufficient acid was produced via metabolism of endogenous carbohydrate stores to cause low levels of dissolution; Komiyama, Khandelwal & Duncan (1986) and Komiyama & Khandelwal (1992) both found *A. viscosus* to be capable of synthesising and utilising endogenous glycogen supplies. Denepitiya & Kleinberg (1984) reported that *S. mutans* 10449 produced intracellular polysaccharide stores whilst Wijeyweera & Kleinberg (1989) reported that strains of *S. mutans*, *L. casei* and *A. viscosus* generate similar large decreases in pH from internal carbon supplies. Since acids were detected at very low levels in units **#9** and **#10** on some occasions (tables 5.15, 5.16 and 5.17), metabolism of stored polysaccharide is the most likely explanation for demineralisation occurring with bacteria in distilled water.

In general there have been two approaches to modelling demineralisation of tooth material or a substitute calcium source. The first method exposed root sections to buffers of known pH (e.g. Almqvist *et al.*, 1988) whilst the second incubated root tissue or hydroxyapatite with bacterial cultures *in vitro* (e.g. Firestone *et al.*, 1993 and Chestnutt, MacFarlane & Stephen 1994) or *in situ* (e.g. Nyvad, ten Cate & Fejerskov, 1989). Some degree of variation in data can be expected when a biological experiment is repeated, but when a closely controlled *in vitro* system such as that of Almqvist *et al.* (1988) is employed then less variation is expected compared with an *in situ* system such as that of

Nyvad, ten Cate & Fejerskov (1989). Equally it might be reasonable to expect similar reproducibility in data from the current experiments compared with those of Firestone *et al.* (1993) or Chestnutt, MacFarlane & Stephen (1994), since both employed pure cultures of bacteria *in vitro*.

When hydroxyapatite powder was incubated with a dense slurry of *S. mutans* cells plus 146mM sucrose for 5hr by Chestnutt, MacFarlane & Stephen (1994) and then calcium dissolution measured using the same colorimetric assay as the current study the standard deviations amounted to, on average, 11% of the mean calcium concentration. Moreover, Firestone *et al.* (1993) incubated root surfaces for 9d *in vitro* with either *A. viscosus* or *S. sobrinus* in trypticase soy broth supplemented with sucrose and measured the resultant demineralisation by microradiography; if the standard deviations of the lesion depth data are expressed as a percentage of the corresponding means then the observed variation in the data amounted to 10%. The degree of variation in the calcium release data from the current experiments was generally greater than that recorded in these two studies. For instance at 24hr the variability in the dissolved calcium concentration, when either mineral source was incubated with *S. mutans* and 5% sucrose, amounted to 71.5% on average (standard deviation as a percentage of mean calcium ion concentration). Furthermore the average variation (standard deviation as a percentage of the mean) in the calcium concentration at 24hr with *L. casei* was 54%, whilst with *A. viscosus* it was 76%. In contrast to *S. mutans* the concentration of calcium detected from root blocks incubated with either *L. casei* or *A. viscosus* and sucrose tended to be more reproducible than from hydroxyapatite powder, although the variation between repeats was still substantial. However elements in the experimental design of both Chestnutt, MacFarlane & Stephen (1994) and Firestone *et al.* (1993) may have

helped to limit variation between repeats during these two studies. For example the number of bacteria employed by Chestnutt, MacFarlane & Stephen (1994) was very high and so would have been much more biased towards demineralisation than in the current study. Furthermore the experiments of Firestone *et al.* (1993) were performed over 9d which could have allowed the variation to 'average-out' to an extent that the current 1d incubation period may not have.

There are a number of possible reasons for the observed variation in the calcium data between repeats of these preliminary experiments. Most importantly, any variations in acid production will be reflected in the calcium release data (potential causes for variation acid concentrations are discussed in section 5.4.4), although variation in the human tooth roots employed might also have generated further divergence in the data. For instance fluoride has been correlated with increased resistance of tooth root tissue to demineralisation (e.g. Al-Joburi & Koulorides, 1984), which means that differences in the fluoride exposure of the donors would lead to variations in the acid sensitivity of the root blocks.

However the foregoing does not explain the variations in calcium release from hydroxyapatite powder, which is probably more strongly correlated with variability in the acids produced by the bacteria rather than in mineral sensitivity. Nevertheless it is clear that there are many factors that contribute to root surface caries that have inter-related effects and which can act together either to decrease or to increase the degree and rate of demineralisation that occurs (discussed further in section 5.4.5). If variation in the data between repeats for calcium release from the current preliminary experiments cannot be controlled then it would seem that the Ultrafree-CL model system would probably not

offer any advantages over the other model systems currently available. However there is a range of modifications to the Ultrafree-CL model system which can be implemented to control possible sources of variability which, if successful, would enhance its usefulness compared with other systems (see chapter 6).

5.4.4 Acid anion concentrations

Streptococcus mutans Significantly increased acid production from sucrose by *S. mutans* in the presence of mineral (compared with its absence) was noted during the current experiments and corresponds with the observation of Chestnutt (1992) that acid production by *S. mutans* NCTC 10449 was enhanced by hydroxyapatite powder. There were no significant quantitative differences in acid production between root and hydroxyapatite-supplied films in units **#3** and **#5** after the first or the second incubation period, which tends to agree with the calcium release data (table 5.4). In contrast there appeared to be a tendency towards more acid production in the presence of a root block than hydroxyapatite powder when units **#2** and **#4** were compared. Overall it would seem that the interaction of *S. mutans* with mineral led to enhanced acid production, perhaps mediated via buffering by dissolution products such as phosphate (a possibility suggested by Chestnutt, 1992). However Berry & Henry (1977) reported that enhanced acid production by *S. mutans* was not via buffering but surface-attachment. However, during the current studies the degree of *S. mutans* surface-attachment in different units is likely to have been similar and therefore not necessarily the most important factor.

There appeared to be greater acid production from sucrose by *S. mutans* after the second incubation period (table 5.15), whether mineral was present or not, which was not reflected in the calcium release data (table 5.4). However the variability in the acid

concentration data meant that this apparent increase between the first and second incubation periods could not be established statistically. Nevertheless it is possible that adaptation to environmental acids occurred, leading to a population with enhanced fermentation characteristics during the second incubation period.

The total concentration of acid anions produced by *S. mutans* was about 10x less in the current study compared to the *in vitro* experimental model of Chestnutt, MacFarlane & Stephen (1994). However there were differences between the two model systems which can to some extent explain this divergence of the data, these differences have been discussed previously but include surface attachment, viable counts, reaction mixture volume and incubation time. Nevertheless this tendency towards less acid production by *S. mutans* grown in the current model system compared with another *in vitro* one does suggest that there is room for improvement (discussed in chapter 6).

The degree of variation in the individual acid anion concentrations between repeats of the experiment made interpretation less easy (table 5.5). However, on the whole, lactate appeared to predominate in fermentation products during the current experiments which agrees with reports for pure cultures *in vitro* (e.g. Chestnutt, 1992 when *S. mutans* NCTC 10449 was incubated with 5% sucrose and Homer, Patel & Beighton, 1993 when glucose and N-acetylglucosamine metabolism by *S. mutans* was assayed) and from mixed plaques (e.g. Margolis *et al.*, 1993 when incubated with 5% sucrose *in vitro* and Geddes, 1975 after exposure to sucrose *in situ*). On a number of occasions pyruvate was found to predominate but this is a metabolic precursor of lactate from the Embden-Meyerhof pathway (Stanier *et al.*, 1987) and it is possible that the pyruvate could have diffused

from the cells prior to conversion to lactate if cell membrane integrity had been compromised.

If the standard deviation of the three replicate data sets for total acid concentration in each unit is expressed as a percentage of the corresponding mean (as for viable counts and calcium concentration) then the overall variability in the data between repeats amounted to the mean ±52%. However there was a tendency towards greater reproducibility between repeats when mineral was present during the current experiments which corresponds with the observations of Chestnutt (1992) during an *in vitro* assay of *S. mutans* NCTC 10449 cariogenic potential. Moreover the data for total (and individual) acid concentrations was less variable between repeats after the second incubation period which tends to suggest that either adaptation to acids had occurred or that the systems had become more stable for some reason. Possible reasons for the variability in acid production are discussed at the end of this section when the data for the three species employed are compared.

Lactobacillus casei There were no significant differences in the total acid concentrations produced from sucrose by *L. casei* films after the first or second incubation periods when mineral was present compared with when it was not (table 5.16). However there was variation between repeats of the experiment which affected the overall mean data. For instance the data indicate that in repeats 1 and 2 during the first incubation period, the total concentration of acids was greater in the presence of hydroxyapatite (units **#2** and **#3**) than in the presence of a root block (units **#4** and **#5**) or no mineral (units **#6** and **#8**). However the acid concentrations in units **#2** and **#3** after the third repeat were lower than after repeats 1 and 2 and also the acid concentration in unit **#6** was spuriously high

after the first repeat, both of which skewed the results. Since the concentration of calcium ions detected was also generally greater in units which contained hydroxyapatite powder than in units which contained root (table 5.5), the acid concentration data for the first incubation period tend to support the idea that buffering by mineral dissolution products enhanced acid production. In contrast the acid concentrations detected after the second incubation period did not show a relationship with mineral which suggests that adaptation to low pH may have enhanced the ability of cells to produce acid during the second incubation period and obscured any effects of buffering by dissolution products.

The individual acid anion data for *L. casei* from sucrose after the first 24hr incubation period varied between repeats of the experiment and also between comparable units (e.g. **#2** and **#3**) which made it less easy to interpret the data. However, after the first 24hr incubation period the predominant acid anion usually appeared to be lactate which is the major product of sugar fermentation by *Lactobacillus* species (Stanier *et al.*, 1987) although pyruvate predominated occasionally. As suggested previously the predominance of pyruvate in some samples could be related to the fact that it is a metabolic precursor of lactate in the Embden-Meyerhof pathway.

Historically when the role of bacterial acid production in root surface caries was considered, attention tended to focus upon *Actinomyces* species and more recently upon *Streptococcus* species (Bowden, 1990) since lactobacilli tend to be present in very low proportions in carious plaque (e.g. 1.8% of the total viable count according to van Houte, Lopman & Kent, 1994). This means that there are no data generated from *in vitro* model systems with which to make meaningful comparisons regarding the concentration of acids produced or the variability in the data between repeats of the

experiment. Nevertheless there was substantial variation between repeats in the acid concentrations detected and since variation was also observed in the acid concentrations produced by *S. mutans* and *A. viscosus* the possible reasons for this will be considered in the section which follows comparing the data for the three species.

Actinomyces viscosus Although the lack of a third repeat precluded statistical analyses of the acid concentration values collected from *A. viscosus* films it is still possible to discern trends. Higher concentrations of acid tended to be produced after incubation with a root block rather than hydroxyapatite or no mineral, although this was only a weak trend and in the absence of further replicate data it can not be verified. The total concentration of acids detected after the first incubation period, when *A. viscosus* was incubated with sucrose and either no mineral or hydroxyapatite powder, tended to be lower than after the second incubation period. In contrast the total concentration of acids detected when the films were incubated with root blocks did not appear to differ substantially between incubation periods. It is possible that sufficient dissolution products were liberated from root to potentiate acid production by buffering the pH during the first incubation period but that during the second incubation period adaptation of the bacteria to low pH had a greater effect than buffering upon their ability to ferment sucrose.

The acid concentrations produced by *A. viscosus* in this experiment were lower than those noted by Distler *et al.* (1992) *in vitro*, however a number of differences between the experimental designs help to account for this. Firstly Distler *et al.* (1992) employed a chemostat which would have resulted in more vigorous and controlled bacterial growth than the present batch culture experiment. Secondly glucose (as opposed to sucrose) in BM growth medium was employed by Distler *et al.* (1992) compared with 5% sucrose in

distilled water during the current experiments. Finally the strain of *A. viscosus* used was different from that employed in the current experiments and this could well explain differences in some properties (e.g. acidogenicity).

After the first incubation period in the current experiments, lactate was rarely the single-most predominant acid anion detected, often either pyruvate or acetate predominated. In contrast lactate predominated in all but one sample (from the lower chamber of unit #5) after the second incubation period. Distler *et al.* (1992) found lactic acid to be the predominant acid produced by *A. viscosus* (when grown for 65hr in a chemostat in the presence of glucose and CO_2) comprising 57% of the total acids whilst the remaining 43% was composed of approximately equal proportions of acetic, succinic and formic acids. However, in addition to the differences between the current experiments and those of Distler *et al.* (1992) described above, it is also possible that inefficient equilibration of gasses between the environment and the units affected the mix of acid anions produced. For instance all manipulations were performed aerobically on the bench whilst growth was in an anaerobic incubator (80% nitrogen, 15% carbon dioxide, 5% hydrogen) and Distler *et al.* (1992) observed increased proportions of lactate and decreased acetate, formate and succinate together with lower overall acid production when carbon dioxide was omitted from the environment. This might then help to explain the lack of reproducibility in the acid anion concentrations after the first incubation period and is obviously a potential drawback of the current model system that needs to be addressed (and is further discussed in chapter 6). Not only were the acid anion concentrations more reproducible after the second incubation period than after the first during the present experiments but they also tended to display closer agreement with those reported by

Distler *et al.* (1992), with lactate forming between 25% and 60% of the total whilst the minor anions comprised pyruvate, succinate and acetate (and in two samples, formate).

Comparison of the three species *Lactobacillus casei* appeared to produce more acid from sucrose than either *S. mutans* or *A. viscosus* during the first incubation period when either no mineral or a root block was present, whilst *S. mutans* tended to produce more acid than *A. viscosus* under these conditions. On the other hand, the total concentration of acids produced by either *S. mutans* or *L. casei* was similar in the presence of hydroxyapatite powder and both were greater than *A. viscosus*. In contrast, the total concentration of acids produced by *S. mutans* after the second incubation period, in the presence of both hydroxyapatite powder or a root block, was similar to *L. casei* and it was only *S. mutans* cultures not supplied with mineral that tended to produce less acid than *L. casei*. Moreover, both *S. mutans* and *L. casei* produced a greater total concentration of acid than *A. viscosus* when in the presence of either hydroxyapatite powder or a root block, whereas only *L. casei* appeared to produce more acid than *A. viscosus* when mineral was absent.

When Wijeyweera & Kleinberg (1989) incubated pure cultures of oral bacteria *in vitro* with 4.2mM glucose and then measured the pH, *L. casei* produced a greater 'pH response' (a function of the area between baseline (pH 7) and the pH curve over 4hr) than *S. mutans*, whilst both produced a greater 'pH response' than *A. viscosus* which agrees with the current data. Since the experiments of Wijeyweera & Kleinberg (1989) were performed *in vitro* with suspensions of bacteria rather than surface films and with glucose not sucrose as in the current experiments, there is a limit to the comparisons which can be made between the two studies. However it is encouraging that the

comparative acid-producing capabilities of the three species during the current series of preliminary experiments were similar to a previous *in vitro* study.

Chestnutt, MacFarlane & Stephen (1994) incubated *S. mutans* with 146mM (i.e. 5%) sucrose plus hydroxyapatite powder and showed a reduction in pH to barely 4.25 after 1hr whilst 112mM acids were detected after 5hr, which was greater than the concentration detected in the current experiments. Furthermore Distler *et al.* (1992) demonstrated that *A. viscosus* grown in a chemostat could produce over 200mM acids when incubated with 300mM glucose, which was also greater than the concentration of acids detected in the current experiments. The differences in experimental design between these two studies and the present one (such as viable counts, volume of diluent, use of glucose and bacteria present in a slurry or in a chemostat) can largely explain the detection of less acid during the current experiments. Nevertheless acid production in the current model system was lower than in others and there are a variety of modifications which could be made and which may help to address this problem (discussed further in chapter 6).

Berry & Henry (1977) found that the presence of hydroxyapatite crystals increased the acid producing potential of *S. mutans* which agrees with the current data. However, they reported that addition of phosphate buffer to the bacteria did not affect acid production and concluded that it was bacterial adhesion to a surface rather than buffering that was important. The *S. mutans* cells in the current experiment were very likely attached to a surface e.g. the membrane filter as well as the mineralised tissue which suggests that other factors came into play. In the present study it seems likely that phosphate and/ or other ions liberated from mineral may have affected buffering potential of the reaction

mixture and therefore the metabolism of *S. mutans*. Berry & Henry (1977) did not test either *L. casei* or *A. viscosus* in their system, although the current data for these species also support the view that buffering rather than simply bacterial attachment affected survival.

Stotzky & Rem (1966) suggested that the ion-exchange properties of an adsorbent surface can alter the metabolic rate of soil bacteria and so one possibility is that the filter membrane on which the bacterial films were grown had different electrochemical surface properties to mineral and did not affect cellular metabolism in quite the same way. For instance the membranes were composed of polyvinylidene difluoride which is a hydrophilic polymer, whilst hydroxyapatite is a hydrophilic salt. Although it is interesting to speculate about the tendency for increased acid production from sucrose when mineral was present than when it was absent, the experiment was not designed to demonstrate this but simply to determine whether demineralisation could be generated in this particular model system. Consequently it is not possible to draw definitive conclusions about this apparent phenomenon without designing and undertaking further experiments which contain the relevant controls.

Generally lactate appeared to be the predominant anion detected from all three species followed by pyruvate and very occasionally acetate was the major acid anion detected. The range of acid anions detected in the samples was in broad agreement with the data of others; e.g. Chestnutt, MacFarlane & Stephen (1994) recorded lactate as the predominant anion produced by *S. mutans* NCTC 10449 when incubated with sucrose and hydroxyapatite (whilst acetate was a minor acid). In addition Distler *et al.* (1992)

found lactate to be the predominant anion *in vitro* from glucose by *A. viscosus*, whilst acetate, formate and succinate were minor acids.

However there was variability in the total acid concentrations detected from all three species which requires to be reduced, since one of the prerequisites of a successful model system is that the data generated should be reproducible within rather narrow confines. Therefore the sources of variation in the current system need to be identified and then either controlled or eliminated if it is to find future applications. There are a number of possible reasons for the variations in acid concentrations detected and these are discussed below.

Firstly there was variation in the viable counts retrieved after incubation with the various reaction mixtures that could have had an impact upon the ability of each film to produce acid. For instance a rapid decrease in the number of metabolically active or viable bacterial cells in a film could have led to reduced acid production compared with a film in which the cells remained metabolically active or viable for a longer period. Experience indicates that the culture densities would have been reproducible up to the time that the sucrose solution/ distilled water/ mineral combinations were supplied to the bacterial films. However no data was gathered regarding the rate at which the films lost viability after the addition of the various reaction mixtures.

Secondly, as mentioned earlier inefficient equilibration of gasses between the external environment and the Ultrafree-CL unit may have led to variation in the acid production profiles of the bacteria. Finally, it was suggested by Chestnutt (1992) that the presence of chloride ions in samples to be analysed affected the way in which the various acid anions

traversed the isotachophoresis capillary. It is possible therefore that Cl^- residues from the KCl used to wash the bacterial films in the current study could have contaminated the effluent reaction mixture samples subsequently collected. If the concentration of Cl^- in the samples varied between repeats of the experiment this could have affected the reproducibility of the acid anion data. Although the above is a possibility it should not itself have had a substantial impact upon acid concentrations detected since the wash buffer, containing KCl, was aspirated from the units prior to commencing the experiment. However it is possible that it was one small factor which had a cumulative effect along with the other possibilities suggested above.

5.4.5 Relationship between viable counts, acid concentrations and calcium ion concentration liberated

It seems logical that the viable counts of bacteria in a film, the concentrations of acids produced and the concentration of calcium ions liberated from the tooth root mineral are inter-related. For instance it would be reasonable to expect a large population of bacteria to produce acid more rapidly than a small population of bacteria, although the total concentration of acids produced would be regulated by the equilibrium between availability of fermentable carbohydrate, the aciduric properties of the bacterial population and any environmental factors which may remove acids from the equilibrium. However during the current experiments population size should not have had a substantial role in acid production since the viable count data gathered in chapter 4 all indicated that the population density of 'mature' films in Ultrafree-CL units was reproducible. Indeed this was substantiated by the day 1 viable count data reported in this chapter since they were highly reproducible between repeats.

Nevertheless as the concentration of acidic fermentation products in the environment increases it might be expected that the rate of acid production would decrease as a result of end-product inhibition. For instance Assinder (1995) demonstrated that the rate of acid production by *S. mutans* from glucose *in vitro* in a pH-stat decreased in direct proportion to the pH of the medium. Furthermore both Assinder (1995) and Dashper & Reynolds (1996), who also measured *S. mutans* glycolytic activity *in vitro* in a pH-stat, demonstrated that the external lactic acid acted to reduce the rate of acid production and excretion. In other words there would also appear to be an equilibrium between acid production and environmental acid concentrations as well as between viable counts, carbohydrate and 'acid-sinks'.

It is accepted that environmental pH and tooth root mineral dissolution are directly proportional until equilibrium is achieved between dissolution and reprecipitation of mineral (i.e. between environmental pH and degree of saturation with respect to mineral components)(Hoppenbrouwers, Driessens & Borggreven, 1987). However the dissolution products of mineral will tend to buffer the acids produced by fermentation; for example Chestnutt (1992) observed a higher pH in slurries of *S. mutans* incubated with sucrose and hydroxyapatite powder compared with sucrose alone. The buffering effect observed would tend to favour dissociation of lactic acid to lactate which cannot permeate the cell membrane (Assinder, 1995) and so the internal pH of the cell would be stabilised sufficiently to permit further fermentation since some glycolytic enzymes are sensitive to low pH (Bunick & Kashket, 1981).

The current data demonstrate a link between the concentration of acids detected, the presence of mineral and the viability of the bacterial population. In many cases during the

current experiments viable counts were reduced substantially and even significantly when incubated with 5% sucrose alone, yet when hydroxyapatite powder or a root block were introduced into the system the viable counts were often similar to baseline. This corresponds with the findings of Denepitiya & Kleinberg (1984) that there was a substantial decrease in the viability of *S. mutans* 10449 when the culture pH was reduced from 5.5 to 5.0 and tends to support the idea that buffering by mineral dissolution products assists both survival and activity of the bacterial cells. The current data in conjunction with the available literature make it possible to propose this kind of inter-relationship between viable counts, acid production and mineral dissolution for *S. mutans*. However the data for both *L. casei* and *A. viscosus* were much more equivocal; there were no significant differences in viable counts or acid concentrations between *L. casei* films supplied with sucrose alone and films supplied with sucrose and mineral whilst on the other hand the *A. viscosus* viable counts tended to decline in spite of relatively low acid and calcium concentrations.

5.4.6 Characteristics of the Ultrafree-CL model system compared with previously available model systems

The results of the experiments described in chapter 4 indicated that the test bacterial species could be cultured reproducibly in Ultrafree-CL units. In addition the bacterial cells were affected by changes such as addition of sucrose or water to their environment, although for *S. mutans* and *L. casei* this could be reversed by T.H.B. Furthermore it was considered that the current model system offered advantages over previous ones in that; firstly Ultrafree-CL units are likely to be easier to handle than complex 'model mouth' systems such as that described by Hudson, Donoghue & Perrons (1986) or Sorvari, Spets-Happonen & Luoma (1994) since there are fewer parts to assemble and maintain

sterile, secondly the bacterial films are not subject to shear forces from medium flow as they are in the two 'model mouths' mentioned (therefore cell density is likely to be more reproducible within the film) and finally the nutrients are separate from the bacterial film which should make sample collection and analysis simpler with no bacteria present to interfere.

The experiment performed in chapter 5 confirmed the view that the Ultrafree-CL was relatively simple to prepare and inoculate. Furthermore the exchange of one nutrient supply with another and the addition of mineral were also straightforward. It was felt that in addition to the advantages described, the present model system offered a number of other advantages. For instance the ability to interchange nutrient supplies and to keep the bacteria separate from the fluid meant that a series of samples could be collected and analysed over a period of time. This flexibility should provide more detailed data regarding the dissolution rates of tooth root material than an *in vitro* model system such as that of Clarkson *et al.* (1987) which provided information on mineral density in a lesion only at the end of the incubation. Furthermore the current model system allowed the demineralising potential of the three test species to be studied at comparable cell densities which may not have been possible with a 'model mouth' system such as that of Hudson, Donoghue & Perrons (1986) since cell density could have been affected by the interaction of nutrient flow with bacterial adhesion to the tooth surface if an organism such as *L. casei* had been employed. Moreover the current system potentially offers greater control over both external variables and sample collection than occurs in *in situ* model systems such as that of Nyvad, ten Cate & Fejerskov (1989) or Margolis *et al.* (1993), although this is means that some of the complex interactions that occur in the oral environment are lost.

However it is apparent from the preceding discussion sections that the concentrations of acid and calcium ion detected during the current experiments were not as great as in some previous reports (e.g. Chestnutt, MacFarlane & Stephen, 1994 and Margolis *et al.*, 1993) which means that there is a greater risk of natural variation between the data obscuring the underlying trends and making it less easy to interpret the data. Nevertheless there are a variety of measures which could be taken to increase the reproducibility of the data generated in a novel *in vitro* model system such as this which are discussed in chapter 6. If these remedial measures were successful (simplicity, ease of handling and sampling, ability to take a series of measurements over time, ability to transfer the same film between different nutrient sources and the range of analytical techniques that could be employed) then the model system would prove very useful.

5.4.7 Conclusions

In this chapter preliminary experiments were described which aimed to generate demineralisation of human tooth root blocks and hydroxyapatite powder *in vitro* by means of bacterial films. The experiment was partially successful since the bacterial films of three different species produced detectable demineralisation. Furthermore the extent of acid production and of demineralisation were correlated and were directly proportional to the length of exposure time. However, the degree of variation in both the acid anion and calcium ion concentrations made it difficult to interpret the data. Nevertheless, in general terms it appeared that *S. mutans* was responsible for more mineral dissolution than *L. casei* which in turn generated more demineralisation than *A. viscosus* under the same experimental conditions. A number of potential sources for the observed variation in the data have been identified, and if these can be controlled then the model system could be used to investigate the role of bacteria in root surface caries.

Chapter 6 Concluding discussion

6.1 Introduction

Throughout the previous chapters of this thesis an attempt has been made to develop a novel *in vitro* system to model human tooth root surface caries. In chapter 3 the model system employed Millicell-HA tissue culture inserts as bacterial growth vessels. However, although Millicell-HA units had many of the required characteristics for a model system, the test bacteria were able to penetrate and contaminate the nutrient medium. In chapter 4 a similar series of exploratory experiments was performed in which Ultrafiltration units were employed in place of Millicell-HA units. It was concluded that Ultrafree-CL ultrafiltration units were indeed satisfactory for the growth of stable reproducible bacterial biofilms and so a preliminary demineralisation experiment was performed. The methodology and results of this preliminary experiment were described in chapter 5 and suggested that, although demineralisation generated by bacterial metabolism can occur, the experimental system as presently described is not ideal and requires some modification. Potential problem areas and faults of the demineralisation experiment in the Ultrafree-CL model system were identified in chapter 5 and suggestions for possible remedial action to improve the model are outlined in this chapter.

6.2 Possible improvements

In retrospect it is possible that the experiment in chapter 5 was too complex, which meant that there were too many variables to control effectively and these affected the ease of interpreting the data. Therefore in any future experiments the number of variables examined should be reduced until the different elements of the model yield reproducible results. Since all three species managed to generate some degree of demineralisation it

would be better to concentrate activity on one, probably *S. mutans* since it tended to cause most dissolution of mineral. Secondly since the object of the current experiments was to develop a model system to demineralise human tooth roots it might be more appropriate to concentrate upon this particular mineral source and thus reduce the complexity of the experiments. It is recognised that the chemical composition of root surfaces can vary (Selvig & Selvig, 1962) and that one method of reducing this variation is to physically remove the cementum layer, thus making demineralisation products (e.g. Ca^{2+} and PO_4^{3-}) more reproducible (Al-Joburi & Koulourides, 1984). Alternatively it may be more effective to powder the root tissue to a homogeneous mixture so that inter-individual variations are evened-out.

There were also variations in the amount and nature of the acid anions produced by the bacteria. Indeed it could be argued that much of the variability in the calcium release data was a direct consequence of the variations in acid production between the different repeats of the experiment. It is also possible that, in this model, some of the variation in acid production could have been due to alterations in the buffering potential of the culture fluid as a result of mineral dissolution. There are therefore a number of modifications that could be made to the model to reduce variations; firstly it is possible that there was inefficient equilibration of environmental gases with those inside the Ultrafree-CL units due to blockage of the channels in the rim of the cap by condensation or by poor fit of the cap into the filter component of the unit. As a consequence of this it would be preferable to perform all manipulations of the units inside the anaerobic incubator. However this is not the easiest of tasks to carry out and it would take too much time to deal with all of the units adequately, therefore the next best option would be to perform the manipulations on the open bench and then either plug the units with

cotton wool or leave the caps loose for 10 - 15min to allow the aerobic gases to diffuse out of the units and be replaced with the anaerobic mixture.

It was noted in chapter 5 that when the bacteria were incubated with sucrose solution alone and then returned to T.H.B. to recover, the viable counts did not increase to the extent anticipated from the results of section 4.2.7 and it was felt that this was a result of only allowing one day for recovery to take place instead of two. Therefore it would be logical to set the recovery period of further demineralisation experiments to two days since this should allow the bacteria to recover maximally. In addition the acid and calcium ion concentrations were much lower than those with *S. mutans* NCTC 10449 noted by Chestnutt, MacFarlane & Stephen (1994). It is possible that the acidogenic and aciduric properties of the laboratory strains employed was reduced due to extensive subculturing (Vadenboncoeur & Trahan, 1983) and so in future experiments a wild-type strain of *Streptococcus mutans*, *Lactobacillus casei* and *Actinomyces viscosus* should be employed. Furthermore there could be benefits in reducing the volume of sucrose solution present during the demineralisation experiment so as to increase the ratio of bacterial cells to culture fluid to a level more akin to that used by Chestnutt, MacFarlane & Stephen (1994) and therefore boost the concentration of acid, although the reduced volume of fluid would restrict the number of different analyses that could be performed.

Moreover Chestnutt (1992) suggested that chloride ions in the KCl wash buffer employed can interfere with the isotachophoretic detection of bacterial acids by increasing the length of the leading electrolyte zone and so extending the time taken for the acid anions to reach equilibrium. It is possible that KCl residues may have been present in the bacterial film, the filter structure and the centrifuge tubes during the

current experiments. Therefore a brief rinse with distilled water after the KCl washes and the use of fresh clean centrifuge tubes to collect the effluent reaction mixture could help to remove chloride residues and so make detection of acid anions more reproducible.

From the above it is clear that there are a number of modifications which could be made to both the structure of the model system and to any demineralisation experiments performed with it. Modifications to the model system include improvements to atmospheric equilibration, the reduction of chloride ion contamination and a decrease in the volume of the demineralising culture fluid. Alterations to the experimental design include a reduction in complexity through the inclusion of fewer variables (i.e. one bacterial species and one mineral source), the use of more standardised root tissue and the inclusion of more acidogenic/ aciduric strains of the test species. If the modifications were unsuccessful then the model system is probably less suited to the study of human tooth root surface caries as some of the alternative systems available, although it may have other applications in biofilm research. If on the other hand these modifications did indeed prove successful, then there is a range of experiments that would be interesting to perform and these are discussed in section 6.3.2.

6.3 Possible future work

6.3.1 Assuming the modifications are not successful

Hopefully the modifications suggested in section 6.2 will prove to be successful in eliminating a large proportion of the variability observed in the data presented in chapter 5. Since it is not possible to accurately predict the true results of any experiment, then the suggested improvements may prove to have little or no effect. In this scenario it would have to be concluded that the Ultrafree-CL model system was probably not

suitable to model human tooth root surface caries. Nevertheless since the current model system has proven to yield reproducible biofilms which remain stable over a period of at least 3 weeks, it might be possible to apply it to other areas of biofilm research; for instance to test the effects of antibacterial or antiplaque agents against bacteria in biofilms.

6.3.2 Assuming the modifications are successful

Provided that the observed variations in the demineralisation data can be controlled, there is a wide range of experiments which could be performed. First of all there are the other analyses that could be performed upon the effluent reaction mixture recovered from demineralisation experiments. For instance the effluent could be analysed for other mineral dissolution products such as phosphate, magnesium and fluoride. Moreover, the rate of accumulation of these ions in the effluent could be determined, which should demonstrate whether there is preferential loss of any of these during lesion development. Moreover if the pH could be monitored continuously, either by introducing a micro-electrode into the effluent stream or by implanting one into the bacterial film, it might be possible to monitor the rate of change of pH in comparison with the rate of dissolution of ions from the mineral and relate this to the effect of introducing antibacterial or anticariogenic agents.

In chapter 4 it was demonstrated that the three test species (*S. mutans*, *L. casei* and *A. viscosus*) could be cultured together in this model system and therefore, in addition to examining the abilities of the individual species to generate demineralisation, it would be of interest to explore the effect of mixing the bacterial cultures. For instance, do two or three species mixtures have an overall negative, additive or neutral effect, upon the

degree of demineralisation compared to single species films. Both *A. viscosus* and *S. mutans* can prolong the period of acidogenesis by metabolising stored carbohydrate (Komiyama, Khandelwal & Heinrich, 1988 and Wijeyweera & Kleinberg, 1989). Moreover it would also be of interest to explore the effect, upon a defined plaque, of other organisms such as *Veillonella* species which have been demonstrated to metabolise lactate (Donoghue & Perrons, 1988) and are present in carious plaques (van Houte, Lopman & Kent, 1994) since it has been suggested that a reduction in lactate concentration may actually stimulate acid production by *Streptococcus* species (Hamilton & Ng, 1983). Furthermore *Porphyromonas gingivalis* is known to synthesise a collagenase (Robertson *et al.*, 1982) and, although it has not been found to any extent in root surface carious plaques, it would be interesting to know if the inclusion of organisms capable of collagen degradation had a nett effect on demineralisation. However no serious attempts have been made, as yet, to culture any species other than the three that were employed throughout this thesis and so it is not certain that other organisms will grow reproducibly in the Ultrafree-CL model system.

In addition to employing other organisms on an individual basis, it would be interesting to collect natural plaque samples from volunteers and to determine what happens to the viable counts of the various member species of the plaque. An exploratory experiment was performed once to determine whether it was possible to culture wild-type plaque collected from a volunteer's teeth in the Ultrafree-CL model system. In this experiment a sample of supragingival plaque was dispersed into T.H.B. by sonication and then 100µl volumes inoculated into Ultrafree-CL units which were subsequently supplied with broth and incubated as described in chapter 4. The bacteria were retrieved from the units as previously described and the viable counts determined at baseline and after 1, 2, 6 and

14d by inoculation onto C.B.A., M.S.B. and Rogosa agar. *Streptococcus mutans*, which was present at 3 x 10^2 c.f.u./ml at baseline, was undetectable in samples collected on day 1 and in all subsequent samples. In contrast the number of c.f.u. capable of growth on Rogosa agar at baseline was 4 x 10^3 per ml, increased 10-fold by day 1 and then stabilised until day 6 when they were found to have increased to about 10^5 c.f.u./ml which remained relatively stable up to day 14. The total anaerobic count increased from 10^4 per unit at baseline to 2 x 10^8 per unit by day 1 and then showed a slow increase to 10^9 per unit by day 14. Furthermore the anaerobic flora was composed of a range of genera including species of *Streptococcus* and *Actinomyces* as well as veillonellae and lactobacilli which appeared to show some degree of succession since the counts of different species appeared to peak at different times and then either stabilise or decrease. The full results of this 'one-off' experiment are summarised in Appendix 11. Marsh (1991) recommended the control of dental plaque rather than its elimination and experiments of the type described above could be used to screen potential strategies for encouraging weakly cariogenic organisms to out-compete and replace strongly cariogenic ones. This information could then be tested in a more detailed way in a chemostat system.

It would also be interesting to determine the effect of environmental alterations upon the cariogenic activity of selected organisms in the current model system. For instance *Actinomyces viscosus* has been demonstrated by Clarkson *et al.* (1987) to be more cariogenic than *Streptococcus mutans* when supplied with starch rather than sucrose *in vitro*. Furthermore the presence of bicarbonate ion has been shown to enhance the rate of acid production of *A. viscosus in vitro* (Takahashi & Yamada, 1992) whilst a CO_2-rich atmosphere leads to the production by *A. viscosus* of a different mix of acids *in vitro*

compared with a CO_2-deficient atmosphere (Distler *et al.*, 1992). The results of experiments of this type might help to clarify the involvement of a range of organisms which are either currently considered to be non-cariogenic or about which there is little data available.

Over-and-above examining the abilities of different organisms to demineralise root tissue in conditions representative of those found *in vivo*, it would also be interesting to attempt to quantify the loss of the protein matrix into the effluent reaction mixture. It has been shown that a period of demineralisation is required before proteolytic activity can then degrade the collagenous matrix (Klont & ten Cate, 1991). Nevertheless there is little information concerning the comparative rates at which the different components of the organic matrix are lost during lesion formation nor indeed about the cause of their loss from the root structure. One report that did determine the release of various protein components of bovine tooth roots during demineralisation with 100mM acetic acid for 1 week found that no collagen was released compared with almost 50% of the non-collagenous proteins, which suggests that reduced pH will solubilise some components of the root structure in preference to others (Boonstra, ten Bosch & Arends, 1990). However it is not clear what effect the presence of bacteria will have on the release of collagen and non-collagenous proteins.

The lack of data in this area is due in part to the low concentrations of protein that are released during demineralisation; for example no collagen was released per gram of mineral solubilised (Boonstra, ten Bosch & Arends, 1990). Moreover, any proteins released would be extremely difficult to discern against the background noise of polypeptides derived from normal bacteriological media. Furthermore the period of time

involved in the development of a cavitated carious lesion could have contributed to the lack of data since it may take time for measurable levels of material to be released. It may be possible to perform an experiment to examine the release of components of the organic tooth root matrix in the Ultrafree-CL model system since it is possible to culture bacteria for an extended period of time. However the model system as currently employed would probably need further modification to allow a successful qualitative and quantitative assessment of the organic tissue breakdown products from root surface caries. For example the concentration of polypeptides released in the early stages of demineralisation could be obscured by traces of T.H.B. Furthermore the volume of culture fluid currently employed would probably dilute the organic material released to a level beyond the sensitivity of detection systems. For example Boonstra, ten Bosch & Arends (1990) reported that 10mg of n.c.p. were released per 1g of root mineral solubilised, by extrapolation this suggests that an absolute maximum of 78µg of n.c.p. would have been released during the most vigorous demineralisation that occurred in chapter 5 (*L. casei* unit **#2** repeat 1) whilst the average would have been closer to 20µg. It is possible to concentrate the small level of proteins released from root tissue to give detectable amounts, although this would introduce a further step into the collection and detection process which may well increase variability. On balance it would seem preferable to reduce the volume of medium initially employed, although this would limit the number of analyses that could be performed.

If an experiment could be designed which measured successfully the organic as well as the inorganic matrix components released into the spent culture fluid then this could provide information which might be of use in the development of treatment strategies. For instance it might be possible to predict the period of time during which a lesion is

potentially reversible since it has been suggested that the presence of collagen fibres (as nucleation points) is required for the reprecipitation of hydroxyapatite crystals (Klont & ten Cate, 1991).

In addition to experiments designed to elucidate the interactions of various plaque bacteria with developing human tooth root surface caries lesions, the *in vitro* model system based upon Ultrafree-CL ultrafiltration units could also have other applications in the study of bacterial plaques. It could be employed to investigate some of the aspects of bacterial invasion of tooth root structure, and help to resolve the questions as to whether bacteria grow actively into cementum or dentine towards a nutrient source or if successive cell divisions force the daughter cells physically further and further into the cementum/ dentine structure?

It would also be necessary to return to the findings in section 4.2.8 and also chapter 5 that *S. mutans*, *L. casei* and *A. viscosus* interacted differently with sucrose solution and distilled water since it raises a number of questions. For instance, if the three test species are grown as a mixed culture will they react in the same way to the addition of sucrose solution, distilled water or nutrient broth as when grown in pure culture or will there be an intermediate response? In addition what effects do other dietary components such as starch, proteins and fats have upon viable counts? Furthermore if a wider range of species is grown as a plaque in the model system, does the application of (for example) sucrose alter the balance of organisms and if so what happens to the population when the sucrose is replaced with another nutrient source? This could provide information on the population dynamics of a multi-species plaque that might be expected *in situ*. Thus there

is a range of potential areas for study to which the Ultrafree-CL model system could be applied - if it could be successfully modified.

The current system was designed to model the development of root surface caries in the presence of oral bacteria and since the major nutrient source for intra-oral bacterial plaques is saliva it could be argued that future experiments should incorporate this secretion. However collecting the large volume of human saliva likely to be required and then sterilising and storing it for use in experiments in a form similar to that which occurs *in vivo* is likely to be difficult. Therefore it might be necessary to employ an artificial saliva which incorporates either bovine submandibular glycoprotein (Shellis, 1978) or porcine gastric mucin as its nutrient source (Glenister *et al.*, 1988). However, it is not clear whether human, animal or artificial saliva would be compatible with the model system since large molecules such as mucin might not readily diffuse through the membrane. Furthermore saliva alone is unlikely to support the growth of axenic cultures since the vast majority of bacterial species require to act synergistically in the degradation of salivary glycoproteins to gain carbon and nitrogen (Marsh & Martin, 1992). Therefore the T.H.B. employed in the present study may well be a suitable compromise to support both pure cultures and the limited mixtures of oral bacteria investigated within the Ultrafree-CL model system.

Appendix 1 Composition and preparation of fastidious anaerobe broth (F.A.B.)

Composition	per litre
Peptone mixture	15.0g
Yeast extract	3.0g
Sodium thioglycollate	0.5g
Sodium chloride	2.5g
Agar #1	13.0g
L-cysteine HCl	0.5g
Resazurin	0.001g
Sodium bicarbonate	0.4g
Haemin	0.005g
Vitamin K	0.0005g

Final pH 7.2 ± 0.2

Preparation

(i) Weigh out 29.7g of medium per litre required.

(ii) Soak for 10min.

(iii) Dissolve the powder with distilled water in a Koch steamer, mixing regularly.

(iv) Dispense to containers leaving minimal headspace

(v) Autoclave at 121°C for 15min.

(vi) Tighten caps after autoclaving.

Appendix 2 Composition and preparation of Columbia blood agar (C.B.A.)

Composition	per litre
Peptone 140 (Pancreatic digest of casein)	13.0g
Peptone 100 (Peptic digest of animal tissue)	6.0g
Yeast extract	3.0g
Beef extract	3.0g
Starch	1.0g
Sodium chloride	5.0g
Agar	13.0g

Defibrinated horse blood

Vitamin K and Haemin solution

Final pH 7.3

Preparation

(i) Weigh out 44g of medium per litre required.

(ii) Dissolve the powder with distilled water in a Koch steamer, mixing regularly.

(iii) Autoclave at 121°C for 15min.

(iv) After cooling to 50°C add sterile defibrinated horse blood to a final concentration of 7.5%. Also add vitamin K and haemin solution to give 0.5mg/l vitamin K and 5mg/l haemin.

(v) Mix (avoid generating bubbles) and pour plates.

Appendix 3 Composition and preparation of mitis salivarius bacitracin agar (M.S.B.)

Composition	per litre
Bacto tryptose	10.0000g
Proteose peptone No. 3, Difco	5.0000g
Proteose peptone, Difco	5.0000g
Bacto dextrose	1.0000g
Bacto saccharose	50.0000g
Dipotassium phosphate	4.0000g
Trypan blue	0.0750g
Bacto crystal violet	0.0008g
Bacto agar	15.0000g

(May be adjusted to meet performance specifications).

Final pH 7.0

Preparation

(i) Weigh out 90g of medium and 20g of sucrose (BDH chemicals Ltd., U.K.) per litre required.

(ii) Dissolve the powder with distilled water in a Koch steamer, mixing regularly.

(iii) Autoclave at 121°C for 15min.

(iv) After cooling to 50°C add 1ml of 0.1% Chapman tellurite and 1ml of 20U/ml Bacitracin (Sigma Chemical Co., U.K.) per 100ml of agar.

(v) Mix (avoid generating bubbles) and pour plates.

Appendix 4 Composition and preparation of Rogosa agar

Composition	per litre
Bacto tryptone	10.00g
Yeast extract	5.00g
Bacto dextrose	10.00g
Bacto arabinose	5.00g
Bacto saccharose	5.00g
Sodium acetate	15.00g
Ammonium citrate	2.00g
Monopotassium phosphate	6.00g
Magnesium sulphate	0.57g
Manganese sulphate	0.12g
Ferrous sulphate	0.03g
Sorbitan monooleate	1.00g
Bacto agar	15.00g

Final pH 5.4

Preparation

(i) Weigh out 75g of powder per litre required.

(ii) Dissolve with distilled water in a Koch steamer mixing regularly.

(iii) Remove from steamer add 1.32ml of glacial acetic acid (BDH Chemicals Ltd., U.K.) per litre.

(iv) Mix and boil for a further 3min.

(v) Cool to 50°C, mix (avoid generating bubbles) and pour plates.

Appendix 5 Materials and methods for Gram's stain

Materials

Crystal violet concentrate (Pro-Lab diagnostics, U.K.) diluted 1:10 with distilled water to give working solution.

Iodine concentrate (Southern GRP Laboratory, U.K.) diluted 1:10 with distilled water to give working solution.

Acetone (BDH Chemicals Ltd., U.K.)

Carbol fuchsin (Pro-Lab diagnostics, U.K.) diluted 1:10 with distilled water to give working solution.

Methodology

(i) Emulsify a single 24hr-old bacterial colony in a drop of sterile distilled water on a glass microscope slide (Chance-Proper Ltd., U.K.).

(ii) Allow the smear to dry and then fix the bacteria to the slide by heating briefly in a bunsen flame.

(iii) Once the slide has cooled, flood with crystal violet and stain for 1min.

(iv) Rinse the slide gently under a stream of water and then flood with Lugol's Iodine for 1min.

(v) Rinse the slide gently under a stream of water and then destain for 5s with a gentle stream of acetone.

(vi) Rinse the slide gently under a stream of water and then flood with carbol fuchsin for 30s.

(vii) Rinse the slide gently under a stream of water and then dry before examining.

Appendix 6 Substrates for api 20 STREP identification system

Substrate	Reaction/ enzyme
Pyruvate	Acetoin production
Hippurate	Hydrolysis
Esculin	β-glucosidase
Pyrrolidonyl 2 naphthylamide	Pyrrolidonylarylamidase
6-Bromo-2napthyl α-D-Galactopyranoside	α-galactosidase
Naphthol AS-BI β-D-glucuronate	β-glucuronidase
2-naphthyl-β-D galactopyranoside	β-galactosidase
2-naphthyl phosphate	Alkaline phosphatase
L-leucine-2-naphthyl-amide	Leucine arylamidase
Arginine	Arginine dihydrolase
Ribose	Acidification
L-Arabinose	Acidification
Mannitol	Acidification
Sorbitol	Acidification
Lactose	Acidification
Trehalose	Acidification
Inulin	Acidification
Raffinose	Acidification
Starch	Acidification
Glycogen	Acidification

Appendix 7 Substrates/ reactions for Minitek Anearobe II identification system

Substrate or reaction

Esculin

Nitrate reduction

Indole

Dextrose

Arabinose

Glycerol

Lactose

Maltose

Mannitol

Rhamnose

Salicin

Sucrose

Xylitol

Catalase

Cellobiose

Mannose

Raffinose

Sorbitol

Urease

Appendix 8 Composition and preparation of anaerobic blood broth (A.B.B.)

Composition	per litre
Pancreatic casein digest	12.000g
Beef extract	4.000g
Yeast extract	5.000g
Sodium carbonate	0.106g
Sodium bicarbonate	0.900g
Dithiothreitol	0.100g
L-cysteine	1.000g
Liver digest	3.000g
Glucose	5.000g
Sodium chloride	3.840g
Vitamin K	0.005g
Haemin (Bovine)	0.005g

Final pH 7.4 $\pm$ 0.2

Preparation

(i) Weigh out 32g of powder per litre required.

(ii) Dissolve in distilled water.

(iii) Divide into 20ml volumes.

(iv) Autoclave at 121°C for 15min.

Appendix 9 Composition and preparation of Todd Hewitt broth (T.H.B.)

Composition	per litre
Infusion from 450g fat-free minced beef	10.0g
Tryptone	20.0g
Dextrose	2.0g
Sodium bicarbonate	2.0g
Sodium chloride	2.0g
Disodium phosphate, anhydrous	0.4g

Final pH 7.8 (approximately)

Preparation

(i) Weigh out 36.4g per litre required.

(ii) Dissolve with distilled water in a Koch steamer, mixing regularly.

(iii) Autoclave at 121°C for 15min.

Appendix 10 Preparation of acids for isotachophoresis

A standard acid solution containing formate, pyruvate, phosphate, lactate, succinate, acetate and propionate (BDH, U.K.) was prepared using the following formula;

$$\frac{\text{W.W. x 5 x 100}}{\text{SG x \% purity}}$$

Where;

$$\frac{\text{M.W. = molecular weight,}}{\text{S.G. = specific gravity and}}$$

% purity = manufacturer's declared purity of reagent.

Appendix 11 **Results of a one-off experiment to culture wild-type plaque in Ultrafree-CL ultrafiltration units for 14 days**

Organism	Viable counts c.f.u./ml on day 0	1	2	6	14
Total anaerobic count	1.0×10^6	2.1×10^8	3.2×10^8	4.8×10^8	1.2×10^9
Streptococcus mutans	4.0×10^4	n.d.*	n.d.	n.d.	n.d.
Streptococus sanguis	4.0×10^4	n.d.	8.0×10^6	n.d.	n.d.
Streptococcus mitis	1.0×10^5	1.6×10^7	4.0×10^7	n.d.	n.d.
Streptococcus constellatus	2.0×10^4	n.d.	8.0×10^6	n.d.	2.0×10^6
Streptococcus gordonii	2.0×10^4	2.6×10^7	1.6×10^8	n.d.	n.d.
Streptococcus oralis	n.d.	6.0×10^7	1.6×10^7	n.d.	2.0×10^6
Neisseria species	n.d.	2.0×10^6	n.d.	n.d.	n.d.
Anaerobic streptococci	2.0×10^5	3.8×10^7	3.2×10^7	2.2×10^8	3.8×10^8
Actinomyces viscosus	1.6×10^5	4.0×10^6	n.d.	n.d.	n.d.
Actinomyces naeslundii	2.0×10^4	4.0×10^6	n.d.	1.6×10^7	2.0×10^6
Actinomyces odontolyticus	2.0×10^4	n.d.	n.d.	n.d.	n.d.
Actinomyces israelii	2.0×10^4	n.d.	8.0×10^6	n.d.	n.d.
Lactobacillus fermentum	2.0×10^4	n.d.	n.d.	n.d.	n.d.
Veillonella species	8.0×10^4	6.0×10^7	4.0×10^7	3.2×10^7	7.4×10^8
Anaerobic gram negative rods	1.2×10^5	8.0×10^6	n.d.	1.6×10^7	n.d.

* = not determined

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