THE EFFECT OF CHLORHEXIDINE AND OTHER ANTIMICROBIAL AGENTS ON THE FORMATION AND VIABILITY OF ORAL BACTERIAL BIOFILMS

Thesis submitted by
Jonathan Pratten BSc (Hons)
for the degree of
DOCTOR OF PHILOSOPHY
in the
Faculty of Medicine
University of London

Department of Microbiology
Eastman Dental Institute
for Oral Health Care Sciences
256 Gray’s Inn Road
London, WC1X 8LD
and
SmithKline Beecham
Oral Care Research
St. George’s Avenue
Weybridge, Surrey
KT13 ODE

-1998-
Abstract

Caries and periodontal diseases have been shown to stem from an imbalance in the normal oral microflora present on tooth surfaces as biofilms (plaque). There is considerable interest in developing chemical agents to supplement mechanical means (i.e. toothbrushing) of controlling these diseases. Although many studies have shown that planktonic oral bacteria can be easily killed by a range of available agents, such studies are largely irrelevant to the situation in vivo as the sessile (biofilm) forms of these bacteria are less susceptible to the action of such agents. The aims of this study, therefore, were to develop and test a laboratory model suitable for evaluating the effectiveness of antimicrobial agents against oral bacterial biofilms. A constant depth film fermentor was used to grow the biofilms. In order to mimic the situation in vivo, the biofilms were grown in an aerobic environment on a substratum similar to human enamel, with nutrients supplied by an artificial saliva.

Initial studies were carried out using a mono-species (Streptococcus sanguis) biofilm grown on a variety of substrata and these were found to influence the susceptibility of the biofilms to various agents including chlorhexidine (CH), cetylpyridinium chloride and triclosan. The effectiveness of mouthwashes (containing different antimicrobial agents) against S. sanguis biofilms was then determined. The effect of CH on multi-species biofilms and microcosm plaques was also investigated. The reproducibility of the multi-species biofilms was poor, with varying proportions of species present in each run. The microcosm plaques were far more reproducible and the proportions of the predominant species were comparable with those
found in supra-gingival plaque. The effects of a number of treatment regimes (involving CH) against these biofilms were investigated. When challenged with CH pulses there was an initial $2 \log_{10}$ reduction in the number of viable organisms, however, although pulsing continued, the biofilms recovered in terms of both the viability and relative proportions of the constituent species. Cryosectioning of the microcosm plaques was carried out to determine the viability and proportions of species present at various depths throughout the biofilm.

The CDFF proved to be a good method for reproducibly generating large numbers of bacterial biofilms comparable to supra-gingival plaque and enabled the evaluation of the activity of antimicrobial agents in a model which mimicked the situation \textit{in vivo}. 
Declaration

I hereby certify that the work embodied in this thesis is the result of my own investigations, except where otherwise stated. The electron microscopy sample preparation was carried out by Mrs Nikki Morgan of the Electron Microscopy unit at the Eastman Dental Institute. The scanning confocal laser microscopy work was carried out in collaboration with Dr. John Warrack, Microscopy and Flow Cytometry Analytical Sciences Department, SmithKline Beecham, Harlow, Essex, U.K.
Acknowledgements

I would like to sincerely thank my supervisor Prof. Mike Wilson and my industrial sponsors Dr. Paul Barnett and Dr. Andy Smith for their support and guidance throughout this project.

I would also like to thank all of my colleagues at the Eastman Dental Institute for all their help and encouragement throughout this thesis. I would particularly like to thank Dr. Tracy Burns, Dr. Dave Spratt, Ms. Zoey Jackson, Mr. Alun Kirby, Mr. Warwick May and Ms. Julie Fletcher.

I am very thankful to my Mum and Dad for their continued support over my student years, and to my brother, David, for his friendship and assistance, especially in computing matters.
For Susie
Index of contents

Title........................................................................................................... 1  
Abstract..................................................................................................... 2  
Declaration.............................................................................................. 4  
Acknowledgements............................................................................. 5  

...  
Dedication............................................................................................... 6  
Index of contents..................................................................................... 7  
List of figures........................................................................................... 12  
List of tables............................................................................................ 16  
Abbreviations.......................................................................................... 17  
Publications as a result of this thesis.................................................. 18  

Chapter One 19  
Introduction...................................................................................................  
1.1. The State vs. Copley Pharmaceuticals............................................. 20  
1.2. Biofilms............................................................................................. 21  
1.2.1. Biofilm structure......................................................................... 23  
1.2.2. Extracellular Matrix..................................................................... 27  
1.2.3. Bacterial Adherence..................................................................... 28  
1.2.4. Bacterial Accumulation............................................................. 30  
1.2.5. Biofilm Nutrition......................................................................... 32  
1.2.6. Biofilm Resistance....................................................................... 33  
1.3. Dental Plaque..................................................................................... 36  
1.3.1. The Development of Dental Plaque........................................... 38  
1.4. Caries.............................................................................................. 41  
1.4.1. Microbiology of Caries............................................................... 44  
1.4.2. Oral Streptococci........................................................................ 45  
1.5. Control and Prevention of Caries.................................................. 47  
1.5.1. Saliva......................................................................................... 49
Chapter Two

Materials and Methods .......................................................... 66

2.1. Bacteriological media .......................................................... 67
  2.1.1. Cadmium fluoride acriflavin tellurite agar ........................ 67
  2.1.2. Artificial saliva ............................................................. 67

2.2. The Constant Depth Film Fermentor .................................... 68

2.3. Organisms ............................................................................ 73

2.4. Inoculation of the fermentor .................................................. 73
  2.4.1. Inoculation of single species ............................................ 74
  2.4.2. Inoculation of multi-species ............................................ 74
  2.4.3. Inoculation of microcosm plaque .................................... 75

2.5. Continuous flow ..................................................................... 76

2.6. Culture methods .................................................................... 78

2.7. Hydroxyapatite and bovine enamel preservation methods ...... 79
  2.7.1. Mineralising solution ...................................................... 79
  2.7.2. Cleaning discs which have been exposed to agents ......... 79

2.8. Viability staining assays ......................................................... 80
  2.8.1. Live / dead staining ........................................................ 80
Chapter Three
Effect of mouthwashes on the formation and growth of *Streptococcus sanguis* biofilms on various substrata

3.1. Aims
3.2. Materials and Methods
   3.2.1. Growth of Biofilms
   3.2.2. Effect of mouthwashes on the viability of *Streptococcus sanguis* biofilms
   3.2.3. Effect of mouthwashes on biofilm formation
3.3. Results
3.4. Discussion

Chapter Four
The susceptibility of *Streptococcus sanguis* biofilms grown on bovine enamel to chlorhexidine

4.1. Aims
4.2. Materials and Methods
4.3. Results
4.4. Discussion
Chapter Five
Growth and susceptibility of a 6 membered biofilm community to chlorhexidine ................................................... 111
5.1. Aims .................................................................................................. 112
5.2. Materials and Methods ................................................................... 112
5.3. Results ............................................................................................. 113
5.4. Discussion ....................................................................................... 125

Chapter Six
Growth and structure of a microcosm community biofilm and susceptibility to chlorhexidine .................................... 132
6.1. Aims .................................................................................................. 133
6.2. Materials and Methods ................................................................... 133
  6.2.1. Inoculation and sampling .................................................. 133
  6.2.2. Chlorhexidine pulsing ........................................................ 133
  6.2.3. Chlorhexidine penetration through the biofilm ............... 134
  6.2.4. Susceptibility of varying thicknesses of microcosm plaque.................................................................................. 134
  6.2.5. Scanning confocal laser microscopy ................................... 134
6.3. Results ............................................................................................. 136
  6.3.1. Growth ................................................................................. 136
  6.3.2. Susceptibility ....................................................................... 136
  6.3.3. Structure ............................................................................. 139
6.4. Discussion ...................................................................................... 155
Chapter Seven

Growth and susceptibility of microcosm plaques grown in the presence of sucrose ........................................................ 166

7.1. Aims .................................................................................................. 167
7.2. Materials and Methods ................................................................... 167
  7.2.1. Growth of sucrose supplemented biofilms ...................... 167
  7.2.2. Determination of pH ........................................................... 167
7.3. Results ............................................................................................. 168
7.4. Discussion ........................................................................................ 175

Chapter Eight

Summary and Conclusions ........................................................................ 182

References ............................................................................................... 191

...
List of figures

1.1 The archetypal biofilm model 24
1.2 Heterogeneous mosaic biofilm model according to Keevil et al. 25
1.3 Water channel model according to Costerton et al. 26
1.4 Chronic plaque build-up on the teeth 36
1.5 A diagrammatic representation of the attachment of bacteria from saliva to pellicle-coated enamel 39
1.6 Tooth structure and sites of carious infections 42
1.7 Structure of Chlorhexidine 56
1.8 The modified Robbins device showing the sample ports and the stud design 59
2.1 Constant Depth Film Fermentor 69
2.2a Schematic vertical section through the fermentor 71
2.2b Schematic horizontal section through the fermentor 72
2.3a Schematic diagram of fermentor showing recirculation of inoculum 74
2.3b Schematic diagram of fermentor with through-flow of medium 77
2.4 Structures of fluorescent probes 80/81
2.5 Diagrammatic cross-section of embedded sample for cryosectioning 82
3.1 Scanning electron micrograph of Streptococcus sanguis biofilm at 24 h 90
3.2 The growth of Streptococcus sanguis biofilms on various substrata 91
3.3 Effect of mouthwashes on the viability of Streptococcus sanguis biofilms grown on hydroxyapatite discs 92
3.4 Effect of mouthwashes on the viability of S. sanguis biofilms grown on bovine enamel discs 92
3.5 Formation of Streptococcus sanguis biofilms on
hydroxyapatite discs treated with mouthwashes

3.6 Formation of S. sanguis biofilms on bovine enamel discs treated with mouthwashes

4.1 Scanning electron micrograph of Streptococcus sanguis biofilm at 120 h.

4.2 Effect of pulsing with 0.1 % chlorhexidine on the viability of S. sanguis biofilms

4.3 Effect of pulsing with 0.2 % chlorhexidine on the viability of S. sanguis biofilms

4.4 Live / dead staining results following pulsing of S. sanguis biofilms with 0.1 % chlorhexidine

4.5 Live / dead staining results following pulsing of S. sanguis biofilms with 0.2 % chlorhexidine

5.1 Growth of individual species comprising a multi-species biofilm formed on bovine enamel discs with artificial saliva as the sole nutrient source

5.2 Viable counts of constituent bacteria in 30 μm thick sections of a 300 μm thick biofilm

5.3 Transmission electron micrograph of a transverse section of biofilm (216 h) at the biofilm/air interface

5.4 Transmission electron micrograph of a transverse section of biofilm (216 h) at the biofilm/substratum interface

5.5 The relative proportions of live and dead bacteria in 30 μm thick sections of a 216 h old, 300 μm thick biofilm

5.6a/b Response of individual species comprising multi-species biofilms to various periods of exposure to 0.2 % chlorhexidine

5.7 Growth of 6 membered biofilm community inoculated with S. sanguis for 24 h prior to inoculation with the remaining 5 species

5.8 Composition of nine-membered community biofilms - from Kinniment et al (1996).


6.1 Section and plan schematic diagrams of sample holder for
6.2 Percentage proportions of species comprising pooled saliva from 5 separate inocula

6.3a The total aerobic viable counts (120 h) from 5 pans of the same run

6.3b The total aerobic viable counts (120 h) from 5 pans of the same run

6.3c The total Actinomyces species viable count (120 h) from 5 pans of the same run

6.3d The total Streptococcus species viable count (120 h) from 5 pans of the same run

6.3e The total Veillonella species viable counts from 5 pans of the same run

6.4 Graph showing the anaerobic counts from 3 separate runs over 192 h

6.5 Effect of pulsing 0.2 % chlorhexidine on the viability of microcosm plaques (168 h)

6.6 Growth of a microcosm plaque community on bovine enamel discs treated with 0.2 % chlorhexidine and then pulsed with chlorhexidine after 8 h

6.7a Susceptibility of 100 μm thick microcosm plaques to cetylpyridinium chloride and chlorhexidine for 1 and 5 mins.

6.7b Susceptibility of 300 μm thick microcosm plaques to cetylpyridinium chloride and chlorhexidine for 1 and 5 mins.

6.8a Absorbance readings of constituents used in cryosectioning microcosm plaque biofilms over a range of wavelengths

6.8b Absorbance readings taken from sections through a microcosm plaque community

6.9 Viable counts from 30 μm thick sections through a microcosm plaque

6.10 Viable counts from 30 μm thick sections through a microcosm plaque which had been exposed to 0.2 % chlorhexidine for 1 min.
6.11a Confocal laser scanning microscope image of a microcosm plaque grown on bovine enamel viewed with live/dead stain

6.11b Confocal laser scanning microscope image of a microcosm plaque grown on bovine enamel exposed to 0.2 % chlorhexidine for 1 h viewed with live/dead stain

6.12 Transmission electron micrograph of a section through a microcosm plaque (120 h) grown in the CDFF

6.13 Transmission electron micrograph of in vivo dental plaque

7.1 Growth of various groups of bacteria comprising a microcosm plaque community pulsed thrice daily with sucrose

7.2 Viable counts of sucrose pulsed microcosm plaque additionally pulsed twice daily with 0.2 % chlorhexidine at 120 h

7.3 Comparisons between the pH of three different types of biofilms

7.4 Viable counts from 30 µm thick sections through microcosm plaque grown in the presence of sucrose

7.5 Viable counts of 30 µm thick sections through microcosm plaque grown in the presence of sucrose following exposure to 0.2 % chlorhexidine for 1 min.
List of tables

1.1 Currently recognised S. mutans species ......................................................... 46
1.2 Specific and non-specific host defences in the mouth ...................................... 48
1.3 Composition of synthetic saliva from Shellis (1978) ....................................... 50
2.1 CFAT agar composition (g/L in dH2O) ............................................................. 67
2.2 Composition of artificial saliva in g/L ............................................................. 68
2.3 Composition of suspending medium ............................................................... 75
2.4 Composition of mineralising solution ............................................................ 79
5.1 Total viable counts and percentage proportions of each species comprising 216 h biofilms formed during three separate runs ........................................... 118
6.1 Comparison of the bacterial composition of approximal dental plaque (data from Newman and Nisengard, 1988), microcosm plaques and the pooled human saliva used as the inoculum 141
7.1 Percentage of genera (relative to the total anaerobic count) comprising sucrose-pulsed microcosm plaque ................................................................. 170
Abbreviations

02  Aerobic
0C  degrees Celsius
An02  Anaerobic
CDFF  constant depth film fermentor
Cfu  colony forming units
CH  Chlorhexidine digluconate
CPC  Cetyl pyridinium chloride
CLSM  confocal laser scanning microscopy
h  Hours
L  Litre
Log  Logarithmic
μg  Microgram
MBC  minimum bactericidal concentration
MIC  minimum inhibitory concentration
ml  Millilitre
MRD  Modified Robbins device
PBF  perfused biofilm fermentor
PTFE  Polytetrafluoroethylene
SEM  scanning electron microscopy
T  Triclosan
TEM  transmission electron microscopy
Publications resulting from this thesis


Chapter 1 - Introduction
1.1. The State vs. Copley Pharmaceuticals

100 people died in late 1993 and early 1994 due to a mysterious bacterial infection that struck hundreds of asthmatics throughout the United States. The antibiotics administered to the patients in hospital apparently failed to subdue the virulent infection.

All the patients had been using a generic albuterol inhalant, produced by Copley Pharmaceuticals, to treat their asthma. The infection was traced back to the manufacturer's albuterol processing tank. Nothing illegal had been carried out, the tank had been treated with chemical disinfectants, the standard treatment according to the health and safety laws.

Many court cases arose from the incident, one in Cheyenne, Wyoming in the summer of 1995. The lawyer for the plaintiffs called to the stand a microbiologist, Dr. Costerton, who examined the records submitted to the court by the Food and Drug Administration. He noted the presence of a particular species of bacteria, *Pseudomonas aeruginosa*, floating freely in the tank. Not only does this species cause pneumonia, but *P. aeruginosa* is notorious for forming biofilms, a matt of bacteria covered in 'slime' which forms on surfaces. These biofilms are resistant to chemical disinfectants, antibiotics, and the immune system.

It was not the first time that biofilms had played a role in high-profile cases, Costerton has testified in court about the presence of biofilms on intrauterine devices, but the asthmatics tragic experience highlighted a gap in the way the microbial world was generally viewed.
1.2. Biofilms

The consequences, both beneficial and harmful, of the association between microbes and surfaces have long been recognised. As far back as the 14th Century, Guy de Chauliac, a French surgeon, recorded the relationship between foreign bodies and delayed wound healing (Voorhees, 1985), while just over a century ago the symbiosis between *Rhizobium* and the roots of leguminous plants was first recorded (Beijerinck, 1888). Although the first detailed description of microbial attachment to surfaces appeared more than 50 years ago (Zobell, 1943), it was not until the late 1970's that the term ‘biofilm’ made its first appearance in the scientific literature.

The almost universal association between micro-organisms and surfaces is now widely accepted. Mature biofilms may contain as many as $10^{10}$ cells/ml, considerably more than usually arise in suspension. Indeed many regard surfaces as the preferred site for microbial growth.

Costerton et al (1995) have now observed that adhesion of a bacterium to a surface triggers the expression of a sigma factor that depresses a large number of genes so that biofilm cells are clearly phenotypically distinct from their planktonic counterparts. Each biofilm bacterium lives in a customised microniche in a complex microbial community that has primitive homeostasis, a primitive circulatory system, and co-operates metabolically, and each of these sessile cells reacts to its special environment so that it differs fundamentally from a planktonic cell of the same species.

Biofilms usually become thick enough that certain solutes, in particular oxygen, become exhausted before the base of the biofilm is reached. This region is therefore suitable for the proliferation of anaerobic species, as long
as nutrients are available. The close association between aerobic and anaerobic species causes many interactions to take place. For example, where anaerobic corrosion, due to sulphate-reducing bacteria takes place, sulphide formed in the anaerobic regions can be reoxidised in the aerobic surface layers by sulphide-oxidising bacteria (Hamilton, 1985).

Microbial films have an important economic role besides their obvious interest ecologically. Examples of the problems that biofilms can cause include the colonisation of boat hulls, leading to fouling by larger organisms; films causing the erosion of marine steel or concrete installations; growth in water pipes causing reduced flow rates and infection. Moreover, considerable morbidity and mortality results from the microbial biofilms that may arise on implanted medical devices, for example, catheters and artificial heart valves.

The effects of biofilms are not always adverse, most effluent treatment plants encourage the growth of microbial films, for example, on aerobic or anaerobic filter systems and on rotating disc aerators which are all used to recycle organic pollutants (Characklis, 1980).

Biofilms are also associated with numerous other surfaces. Most solid-liquid interfaces can become coated with microbes that tend to attach to a thin layer of adsorbed macromolecules, which quickly bind to any ‘clean’ surface immersed in natural aquatic systems. Films are found on the gastric mucosa and internal epithelial linings of many animals. While many oral organisms attach to dental enamel, others ‘prefer’ the cheek and tongue epithelial cells. Films are formed on most surfaces immersed in any of the natural water systems. These surfaces include not only mineral or wooden structures, but
also the surfaces of plants and aquatic animals. Microbes develop on terrestrial surfaces, too. The phylloplane is a habitat on the surface of leaves, which shows a succession of organisms throughout the growing season. Sometimes these proliferate enough to form a coherent film. A corresponding region around plant roots, the rhizoplane, leads to a cylindrical film-like proliferation of microbes using root exudates as nutrients. There is even a region around germinating seeds called the spermosphere that has some of the characteristics of a biofilm.

Research that has been carried out on biofilms has tended to focus on two main aspects. Firstly, the elimination of the biofilm from an infected area which would involve both killing and removal of the film, and secondly, the control of their formation and activity where they perform some useful function. Microbiologists have become increasingly interested not so much in the film itself but in the mechanisms involved in the attachment of microbes to surfaces. Very little is known about the structure and physiological functioning of biofilms. In nature a biofilm is rarely composed of just a single species, but a group of different genotypes each having some part to play in the overall behaviour of the community.

1.2.1. Biofilm structure.

The use of new microscopic and computer-aided techniques has been instrumental in the development of models which assist in explaining the complex structures of biofilm communities.

Scanning and transmission electron microscopy have been used for several years to study the structure and morphology of dental plaque. Plaque grows
as a dense biofilm which is complex in structure and is associated with a large number of different bacteria. Observations from several studies (Marsh, 1995; Listgarten, 1976) have shown that there is structural organisation present in these dense biofilms. These structures include microcolonies of similar-shaped bacteria, parallel orientated bacteria and specific associations between bacteria, described as 'corn cobs'. A generalised biofilm structure is shown diagrammatically below (Fig. 1.1), it consists of a base film attached to the conditioning film with bulk liquid and gas space above.

Figure 1.1. The archetypal biofilm model.

Differential interference contrast microscopes have been used by Keevil et al to study biofilms in water distribution systems (Walker et al., 1995). Using this technique the structure of the biofilm as a whole can be examined. According to their observations, such biofilms consist of a thin film (approx.
5\mu m) of attached cells over the surface with microcolonies attaching to the substratum forming 'stacks'. These 'stacks' are well separated allowing a flow of nutrients through the biofilm (Fig. 1.2).

Figure 1.2. Heterogeneous mosaic biofilm model according to Keevil et al.

The use of confocal scanning laser microscopes (CSLM) has now enabled the examination of living, fully hydrated, biofilms and the use of this technique has provided valuable structural information. Costerton et al. (1994) have studied many pure cultures and natural bacterial populations using CSLM, the studies concluded that biofilm bacteria grow predominantly in microcolonies of similar morphotypes and that these colonies are interspersed between water channels (Lawrence et al., 1991). The channels contained few bacterial cells and appeared to contain a more permeable matrix material. The 'idealised' structure is shown in Figure 1.3 (Costerton et al., 1994).
The cellular automaton model (Wimpenny and Colasanti, 1997) suggests that the structure of a biofilm depends on the substrate concentration. The model indicates that the highest concentration of substrates lead to the formation of dense films while, contrary to this, the lowest concentrations form stacked structures. The model also implies a layer of individual cells at the surface in a low nutrient environment from which stacks develop. The dense biofilms of which dental plaque is an example can also be simulated and, using slightly lower substrate concentrations, channels which are often observed using electron microscopy can be demonstrated.

1.2.2. Extracellular Matrix.
One of the most notable features of biofilms is their high content (50-90 %) of exopolysaccharide or EPS (Characklis and Cooksey, 1983). The terminology for the extracellular material associated with cell aggregates or biofilms varies in the literature, being referred to as slime, capsule, sheath, EPS and glycocalyx. Zobell (1943) suggested the involvement of extracellular 'cementing' substances in the adhesion of cells to the substratum. The last stage of cell attachment to a surface, involving specific interactions, is associated with the production of adhesive materials such as exopolysaccharides (Lappin-Scott and Costerton, 1989). Corpe (1970) demonstrated the involvement of acidic polysaccharides in bacterial adhesion, and Fletcher and Floodgate (1973) observed this by means of electron microscopy.

The matrix is usually assumed to be constructed of long-chain or polymeric materials (lipids, proteins, polysaccharides, polyphenols, nucleic acids) although the proportions of these materials are not known (Palenik, 1989). The majority, however, are polysaccharides. Common sugars such as glucose, galactose, mannose, fructose, rhamnose, N-acetylglucosamine, glucuronic acid, galacturonic acid, mannuronic acid and guluronic acid, are all typical constituents of bacterial polysaccharides (Christensen, 1989).

*Pseudomonas aeruginosa* synthesises an exopolysaccharide called alginate in response to environmental conditions (Boyd and Chakrabarty, 1995). Alginate serves to protect the bacteria from adversity in its surroundings and also enhances adhesion to solid surfaces. Transcription of the alginate genes is induced upon attachment to the substratum and this leads to increased alginate production. As a result, biofilms develop which are
advantageous to the survival and growth of the bacteria. In certain circumstances, \textit{P. aeruginosa} produces an alginate lyase which cleaves the polymer into short oligosaccharides. This negates the anchoring properties of the alginate and results in increased detachment of the bacteria from the surface, allowing them to spread and colonise new sites. Thus, both alginate biosynthetic and degradative enzymes are important for the development, maintenance and spread of \textit{P. aeruginosa} biofilms.

The extracellular matrix may also contain particulate materials: clays, organic debris, phages, lysed cells and precipitated minerals. Understanding the physical and chemical characteristics of the matrix and its relationship to the resident organisms may influence the understanding of the structure and function of biofilms.

1.2.3. Bacterial Adherence.

Several stages are involved in the adhesion of a microbe to a surface whether inanimate or another living cell. If we imagine a microbe approaching a surface then at a distance of tens of nanometres the two objects are influenced by two types of forces - van der Waals and electrostatic. At a distance of >50 nm Van der Waals interactions occur and these are the result of the mutual induction of dipoles in the two objects resulting in their mutual attraction. As the distance between the objects decreases (10-20 nm), electrostatic forces become significant and, as most microbes and surfaces have a net negative charge, the net effect is repulsion. However, these repulsive forces decrease with increasing ionic strength and in many natural environments the ionic strength is sufficient to
reduce or overcome this repulsion.

As the bacterium approaches more closely, intervening water molecules will act as a barrier to attachment. However, hydrophobic molecules on the surface of either the bacterium or host cell (or both) can exclude these other molecules. Hydrophobic interactions between the bacterium and the host cell can then result in adhesion or can enable a close enough approach (<1.0 nm) for other adhesive interactions to occur. The latter include hydrogen bonding, cation bridging and receptor-ligand interactions i.e. the specific binding of a molecule (ligand) on the bacterial surface to a complementary substrate molecule (receptor) on the host cell surface (Fletcher, 1996).

Bacterial adhesion to host cells is thought to be mediated primarily by hydrophobic interactions, cation bridging and receptor-ligand binding. While hydrophobic interactions are recognised as being important in the adhesion of bacteria to host cells and to inanimate substrata, more is known about the role of receptor-ligand interactions. The specific molecules on the bacterial surface responsible for adhesion are known as adhesins.

Collectively, bacteria elaborate a number of structures which may be involved in adhesion to cell surfaces. These include fimbriae and proteinaceous fibrils whose primary function appears to be that of adhesion, as well as capsules and flagella which have other functional roles namely protection and locomotion respectively. All of these structures contain adhesins although the chemical identity of many of these has not yet been determined. As well as these structures, the cell walls of many species contain macromolecules which function as adhesins. A particular species may be able to produce a whole series of adhesives structures (or adhesins)
either concurrently or consecutively. In the case of the latter, this may enable the organism to adhere to the different cell types it encounters during the course of the infectious process in which it partakes.

1.2.4. Biofilm Accumulation.

The initial events of biofilm accumulation or colonisation at a substratum are the net result of transport, adsorption and desorption and growth processes. Diffusive or advective transport processes carry the cell to a point adjacent to the substratum. Colonisation processes can be expressed in terms of two variables: colony forming units (CFU) and cells. This distinction is important as cells can adsorb in groups or as single cells. Thus a single cell is a CFU, but an aggregate of five cells is also a CFU. It must also be taken into account that not all cells accumulate at the substratum through transport, but cells also form at the substratum through growth. The following four processes have been distinguished by Escher (1986).

1. Diffusive or advective transport carries the cfu to a point adjacent to the substratum. In laminar flow, only diffusive transport is involved. In turbulent flow, advective transport generally dominates.

2. Adsorption is the linking of the cfu with the substratum. The cell is adsorbed to the substratum if it has a linkage to it and hence becomes immobilised for a certain time period.

3. Desorption is the breaking of the substratum-cfu linkage and the complete removal of the cfu from the substratum, and is therefore the opposite of adsorption.
4. Cfu separation, although not related to adsorption or desorption, contributes to the accumulation of cfu at the substratum by changing the number of cfu adsorbed. A cfu with more than one cell can separate into two independent cfu as a result of a fluid shear or even cell motility. Cfu separation does not influence cell numbers on the substratum.

The processes can be described in terms of cells by determining the number of cells in each cfu. In addition to advective transport, adsorption, and desorption, two additional processes need to be considered when the concentration of cells at the surface is the variable:

1. *Multiplication* is related to cellular growth. In contrast to growth, which includes the entire growth cycle of the cell, multiplication represents the singular event of cell division. Cells within a cfu multiply, and the number of cells within this cfu increases. This does not change the accumulated number of cfu, but does change the accumulated number of cells.

2. *Cfu erosion* arises from the fact that cells within a cfu can detach and hence reduce the cell number of the cfu. This process is the reverse of multiplication in the sense that it is a non-selective ‘death’ rate in the cfu. Erosion is distinct from desorption, which is the detachment of an entire cfu from the substratum.

There are therefore many factors which contribute to the accumulation of bacteria to form a biofilm over time and its subsequent detachment to colonise other surfaces.
1.2.5. Biofilm Nutrition.

Naturally-occurring biofilms generally exist under conditions of extremely low nutrient concentration (Peters and Wimpenny, 1988) and growth is generally substrate limited (Walsh, 1989). In fact adhesion of bacteria to solid surfaces is said to encourage growth of bacteria when the organic nutrient concentration is very low (Fletcher and Floodgate, 1973). It has been suggested that the exopolysaccharide in a biofilm may bind nutrients that are essential for growth, thereby creating a nutrient-rich micro-environment in an otherwise nutrient-poor micro-environment (Allison, 1993). Bacterial polysaccharides also have the ability to bind cations, with ion-uptake being selectively influenced by the level of exopolysaccharide acetylation. Hence, the glycocalyx matrix modifies the environment of the adherent cells by concentrating nutrients (Prosser et al., 1987). In fact, the glycocalyx performs a homeostatic function and minimises the consequences of fluctuations in the macro-environment. In this way, sessile biofilm populations have many important properties which are distinct from their planktonic counterparts and which contribute towards their survival (Brown et al., 1988).

Within a biofilm, microbial cell-cell interactions between primary colonisers and other micro-organisms with different nutritional requirements can occur (Allison, 1993). Micro-colonies of cells capable of primary production of nutrients are often surrounded by heterotrophic organisms that are stimulated by the exudate to grow and to produce adjacent colonies. Death and cell lysis of primary producers stimulates biofilm growth because the
biofilm traps and recycles cellular components. The formation of biofilms on surfaces can be regarded as a universal bacterial strategy for survival and for optimum positioning with regard to available nutrients. Even so, bacteria in biofilms grow extremely slowly due to the depletion of organic nutrients, inorganic ions and oxygen (Nichols, 1991).

1.2.6. Biofilm Resistance.

It is well documented that when cells grow in the form of a biofilm they are less susceptible to attack by antibiotic or biocide treatments than are freely suspended cells of the same strains (Costerton et al., 1994). The biofilm structure can also provide protection against host defence systems in the case of pathogenic bacteria (Sheth et al., 1983).

An antibacterial molecule must interact with the surface components of the bacterial cell to gain access to its targets located inside the cell. The interactions of the cells with each other and their interaction with the antibiotic molecules obviously play important roles in this resistance. Protection may arise from the numbers of cells present and their close proximity to each other, thus preventing passage of antibacterial molecules through to the core mass of cells. Biofilms vary greatly and although some may only comprise a monolayer of bacteria other cells are enmeshed within a thick matrix of fibrous glycocalyx. Exopolysaccharide slimes could make up to 90% of the dry weight of a biofilm with microbial cells comprising just a small percentage, it may be these other components of the biofilm which cause the resistance by protecting the cells from direct contact or altering the physiology of the cells.

Although physical exclusion may result in increased resistance to
antibacterial agents, changes in the physiology of the cells in response to their different environment within the biofilm also contribute to this recalcitrance. Any changes in the surface composition of the bacterium may cause dramatic alterations in the ability of antibacterial molecules to cross the cell envelope. Studies from a number of laboratories have concluded that the surface composition of micro-organisms is remarkably flexible and is regulated by the nature of the environment (Gibbons and Etherden, 1983; Busscher et al., 1992). The protein, phospholipid and divalent metal cation components of the outer membrane have been shown to be influenced by the conditions used in the cultivation of micro-organisms (Marshall, 1994). It is of immense importance for the cell to maintain a certain degree of plasticity in the composition of its envelope to respond to the frequent changes in its growth environment.

Some studies suggest that growth rate plays a role in mediating resistance of biofilms to antibiotics. Several classes of antibiotics were assessed by (Ashby et al., 1994) for activity against non-growing Escherichia coli and cells grown as a biofilm. Antibiotics which had activity against non-growing cells also showed some activity against biofilms. Cephamycins were more active than other cephalosporins, but the most effective antibiotics were imipenem and ciprofloxacin, which were also active against steady state biofilms. However, none of the antibiotics studied was capable of completely eradicating a biofilm. The effect of growth rate on the antimicrobial susceptibility of Staphylococcus epidermidis has been determined using a culture device developed by Duguid et al. (1992). Changes in biofilm susceptibility were compared with those for suspended
populations grown in a chemostat, and also for newly-formed daughter cells shed from the biofilm during its growth and development. Susceptibility increased for intact and resuspended biofilms, and also for planktonic cultures, with increases in growth rate. The dependence of susceptibility upon growth rate was greatest for slow-growing cells. At any particular growth rate, biofilms appeared more susceptible than their planktonic counterparts. Newly-formed daughter cells were relatively tolerant to the agent at all rates of growth. Lack of growth rate dependency for the newly-formed cells suggested a role for the cell-division cycle in determining resistance. This was confirmed by examining the susceptibility of *S. epidermidis* throughout batch cultures with cell division synchronized. Perfusion of various steady-state biofilms with ciprofloxacin demonstrated killing of the adherent population even at much reduced rates of growth.

The retention of antibiotic-inactivating enzymes (such as β-lactamases) by the matrix, resulting in high local concentrations has also been suggested as a reason for reduced susceptibility by Lambert *et al* (1994). They showed that *P. aeruginosa*, when in a biofilm is highly resistant to piperacillin and imipinem but is susceptible to these agents when the biofilms were disrupted. High levels of β-lactamase were detected in the matrix of the intact biofilm.

As already mentioned, the induction or repression of gene expression in organisms constituting a biofilm could result in a phenotypic change that results in a reduced susceptibility to an antimicrobial agent.
1.3. Dental plaque

Dental plaque is the diverse microbial community found on the tooth surface embedded in a matrix of polymers of bacterial and salivary origin (Fig. 1.4).

Figure 1.4 Chronic plaque build-up on the teeth (A) causing inflammation of the gums (B).

Once a tooth surface is cleaned, a conditioning film of proteins and glycoproteins is adsorbed rapidly to the tooth surface. Plaque formation involves the interaction between early bacterial colonisers and this film (the acquired enamel pellicle). To facilitate colonisation of the tooth surface, some receptors on salivary molecules are only exposed to bacteria once the molecule is adsorbed to a surface. Subsequently, secondary colonisers adhere to the already-attached early colonisers (co-aggregation) through specific molecular interactions. These can involve protein-protein or carbohydrate-protein (lectin) interactions, and this process contributes to determining the pattern of bacterial succession. As the biofilm develops, gradients in biologically-significant factors (e.g. oxygen levels, redox
potential, pH) develop, and these permit the co-existence of species that would be incompatible with each other in a homogeneous environment (Marsh and Martin, 1992).

Dental plaque is found on healthy enamel but when the balance of this community becomes altered it is implicated in the aetiology of two of the most prevalent diseases in industrial societies: caries and periodontal disease (McKee et al., 1985). Plaque is a heterogeneous system which is composed of a liquid phase containing salivary components, bacteria and their products: principally polysaccharides which retain the acidic products arising from the fermentation of carbohydrates adjacent to the enamel and cause its demineralisation (resulting in caries) (Glenister et al., 1988). The bacterial composition of plaque varies widely depending on its location within the oral cavity but the largest differences are between plaques formed above (supra-gingival) and below (sub-gingival) the gingival margin (Marsh and Martin, 1992).

Supra-gingival plaque exists in an aerobic atmosphere and receives its nutrients mainly from saliva. Sub-gingival plaque, however, is bathed in gingival crevicular fluid and is in an anaerobic environment.

1.3.1. The Development of Dental Plaque.

Bacteria rarely come into contact with clean enamel. As soon as the tooth surface is cleaned, salivary glycoproteins are adsorbed, forming the acquired enamel pellicle (Marsh, 1995).

Bacteria attach to teeth and oral mucosal surfaces in a surprisingly selective manner, and attachment is the first step in the colonisation process.
Attachment is thought to involve lectin-like and/or hydrophobic ligands, called adhesins, often present on bacterial surface appendages which interact with receptors on oral tissues. A variety of factors can influence bacterial attachment, and therefore have the potential to affect host-parasite interactions in the mouth (Gibbons, 1984).

The pioneer species include *Neisseria* and streptococci, predominantly *Streptococcus sanguis* (McBride and Gislow, 1977 and Shibata *et al.*, 1980). These pioneer populations multiply, forming micro-colonies and secrete the extracellular matrix. Salivary polymers will continue to be adsorbed on to bacteria already on the tooth surface and so contribute to the extracellular matrix (Fig. 1.5).

Coaggregation is one of the most important mechanisms by which plaque is built-up and encourages species diversity (Kolenbrander and London, 1992). For example, bacterial accumulation will be accelerated by intrageneric coaggregation among streptococci and among actinomyces as well as intergeneric coaggregation between these. The subsequent development of dental plaque will involve intergeneric coaggregation between other genera and the primary colonisers.

Several salivary components have been shown to aggregate microorganisms. This aggregating ability has been taken to support a role of certain salivary components in microbial adhesion to the pellicle-covered tooth surface and to confer specificity to the adhesive process of the early colonisers (Ericson and Magnusson, 1976). For instance, salivary oligosaccharide-containing glycoproteins may serve as receptors for oral streptococci in the salivary pellicle (Gibbons and Qureshi, 1978), and the
salivary proline-rich protein 1 and statherin have been implicated as receptors for type 1 fimbriae of A. viscosus (Gibbons et al., 1988).

Figure 1.5. A diagrammatic representation of the attachment of bacteria from saliva to pellicle-coated enamel. From Marsh and Martin (1992). The pattern of attachment is influenced by the composition of the pellicle (ligands) and the surface components of the bacteria (adhesins). The diagram illustrates the role of specific molecular interactions both between the enamel pellicle and cells A and D, and in the co-aggregation of cell B₁ to cell A. Salivary components can both promote plaque formation by, for example, facilitating adhesion between cells B₂ and B₁, and also prevent plaque attachment by saturating receptors on the bacterial surface, as with cell C. Salivary components can also prevent adhesion by causing aggregation; large aggregates of cells are more easily lost from the mouth by swallowing.

Eventually, growth of individual colonies results in the formation of a
confluent film. As the plaque develops, the metabolism of the pioneer species creates an environment suitable for further colonisation by bacteria with different atmospheric and nutritional requirements. Oxygen is consumed by the aerobic and facultatively anaerobic species and replaced with carbon dioxide. This allows the colonisation of anaerobic rods and filaments, which if the plaque is allowed to accumulate, can dominate the climax community. Metabolic end-products of primary colonisers can serve as nutrients for other organisms e.g. some strains of *S. mutans* require p-aminobenzoic acid for growth, and this could be supplied by *S. sanguis* (Marsh and Martin, 1992). This process of plaque formation leads to an increase in the diversity of the microflora. Variations occur in the plaque microflora at the same site both between and within mouths; variations can also occur at the same site over relatively small distances (Marsh, 1995). The balance of this microflora remains stable unless influenced by an environmental stress, this is therefore able to prevent colonisation by exogenous species. The stability or homeostasis is due, in part, to a dynamic balance of microbial interactions, including synergism and antagonism. Synergistic interactions include coaggregation, the development of food-chains, and the degradation of complex host and bacterial polymers (Grenier and Mayrand, 1986). Antagonism can be due to the production of enzymes, organic acids and low pH (Perrons and Donoghue, 1990). The heterogeneity of a biofilm such as plaque can lead to the co-existence of species that would be incompatible with one another in a homogeneous environment (Marsh, 1995).
1.4. Caries

Dental caries, or tooth decay, can be defined as a pathological process involving localised destruction of the tissues of the teeth by micro-organisms (Bowden, 1996). For caries to occur there must be a susceptible host, a cariogenic oral micro-flora and a suitable metabolisable substrate which must be present for an adequate length of time.

In industrialised societies, enamel caries affects the majority of individuals, particularly up to the age of 20 years, after which time its incidence is reduced (Brunelle et al., 1982 and Easley, 1995). Root-surface caries is becoming a problem in the elderly due to gingival recession exposing the vulnerable cementum to destruction by bacterial action (Powell et al., 1981 and Sheiham et al., 1979).
Cavities begin as small demineralised areas on the surface of the enamel, and can progress through the dentine and into the pulp. Demineralisation of the enamel is caused by acids, and in particular lactic acid, produced from the microbial fermentation of dietary carbohydrates (Hazen, 1973). Lesion formation involves the dissolution of the enamel and the transport of the calcium and phosphate ions into the surrounding environment. This initial stage is reversible and remineralisation can occur, particularly in the presence of fluoride (Sognnaes, 1965).

In the last century, Leber and Rottenstein in 1867 and Miller in 1890 deduced the fundamental principles involved in dental caries. Miller suggested the Chemico-Parasitic Theory which stated that oral bacteria converted dietary carbohydrates into acid which solubilised the calcium
phosphate of the enamel to produce a caries lesion. Clarke (1924) isolated an organism (which he called *Streptococcus mutans*) from a human caries lesion, however, proof of the causative role of bacteria came only in the 1950s and 1960s following experiments with germ-free animals (Ellen, 1961).

Pioneer experiments showed that germ-free rats developed caries when infected with bacteria described as enterococci. Evidence for the transmissibility of caries came from studies on hamsters. Caries-inactive animals had no caries even when fed a sucrose-rich diet. Caries only developed in these animals when they were caged with or ate the faecal pellets of a group of caries-active hamsters. Further proof came when streptococci, isolated from caries lesions in rodents, caused rampant decay when inoculated into the oral cavity of previously caries-inactive hamsters (Ellen, 1961).

The importance of diet became apparent when the colonisation and production of caries by most streptococcal populations occurred only in the presence of sucrose (Holbrook *et al.*, 1995). Subsequent research has shown that some oral streptococci not only produce acid from sucrose (i.e. they are acidogenic), but also they can tolerate the low pH produced, and synthesise extracellular polysaccharides that are important in oral colonisation and plaque development (Marsh, 1995).

*Mutans* streptococci can cause caries of smooth surfaces (as well as in pits and fissures) in hamsters, gerbils, rats and monkeys fed on cariogenic diets, and are the most cariogenic group of bacteria found (Thott *et al.*, 1974). Other bacteria, including members of the mitis, anginosus and salivarius
groups, *E. faecalis*, *A. naeslundii*, *A. viscosus* and lactobacilli can also produce caries under conducive conditions in some animals, although the lesions are usually restricted to fissures. Evidence for the significance of the role of mutans streptococci in dental caries has also come from vaccination studies. Immunisation of rodents or monkeys with whole cells or specific antigens of *S. mutans* and *S. sobrinus* leads to a reduction in the number of these organisms in plaque and a decrease in the number of caries lesions (Caldwell and Lehner *et al.*, 1982). 

Unlike animal studies, any relationship between particular oral bacteria and caries in humans must be derived by indirect means. Evidence for bacterial involvement has come from several sources. Patients on long-term broad-spectrum antibiotic therapy frequently exhibit a reduced caries experience. Similar results are found with experimental animals kept on diets supplemented with antibiotics active against Gram-positive species. A variety of epidemiological surveys of different human populations have found a strong association between mutans streptococci and caries (Leverett, 1982). Much research effort over the past two decades has been focused on determining the precise bacterial aetiology of caries so that effective preventive measures can be devised.

1.4.1. Microbiology of Caries.

Superimposed on the problems of study design are those associated with the microbiological analysis of plaque. The plaque microflora is diverse, and disease is not due to exogenous species, which would be relatively easy to identify, but to changes in the relative proportions of members of the resident microflora, so that when data is accumulated from numerous individuals,
clear associations between bacteria and disease can be obscured. Likewise, once mutans streptococci had been strongly implicated in human caries, many follow-up studies only looked to confirm this association. In such studies, the role of any other species could not be determined. Although it is generally accepted that two or three organisms are likely to be responsible for caries and other opportunistic infections, 300 species of bacteria have been isolated from the oral cavity making identification of further causative organisms difficult (Marsh and Martin, 1992).

1.4.2. Oral Streptococci.

Regardless of the age of plaque and diet, the predominant organisms are Gram-positive cocci of the genus *Streptococcus* (Ritz, 1967). Streptococci have been isolated from all sites in the mouth and comprise a large proportion of the resident oral microflora. The majority are alpha-haemolytic on blood agar although many oral species contain strains showing all three types of haemolysis, alpha, beta and gamma.

More is known about the mutans streptococci than any other group because of their purported role in the aetiology of dental caries. Six distinct species of mutans streptococci have now been described (Kawamura *et al.*, 1995) (Table 1.1).
mutans group

<table>
<thead>
<tr>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. mutans</em></td>
</tr>
<tr>
<td><em>S. sobrinus</em></td>
</tr>
<tr>
<td><em>S. cricetus</em></td>
</tr>
<tr>
<td><em>S. rattus</em></td>
</tr>
<tr>
<td><em>S. macacae</em></td>
</tr>
<tr>
<td><em>S. downei</em></td>
</tr>
</tbody>
</table>

Table 1.1. Currently recognised species comprising the mutans group.

*S. mutans* is now limited to human isolates and is the most commonly isolated species of mutans streptococci from plaque. Mutans streptococci are capable of making both the soluble and insoluble extracellular polysaccharides from sucrose which are associated with plaque formation. They produce acids at a rapid rate from fermentable carbohydrates and are capable of growing and surviving in acid conditions (Berry and Henry, 1977).

Three other main groups of oral streptococci are recognised; salivarius, mitis and anginosus.

Within the *S. salivarius* group are the species *S. salivarius* and *S. vestibularis*. *S. salivarius* can be isolated from all areas of the mouth but is isolated only occasionally from diseased sites and is not considered a significant opportunistic pathogen. *S. vestibularis* is isolated mainly from the mucosa of the mouth. *S. salivarius* produces a polymer of fructose from sucrose which may be metabolised in the mouth by other oral microorganisms while *S. vestibularis* strains are unable to produce extracellular polysaccharides from sucrose but do produce urease and hydrogen peroxide.

Species from the anginosus group are known to be opportunistic pathogens.
and are isolated from both dental plaque and mucosal surfaces. No strains from this group are, however, capable of making extracellular polysaccharides from sucrose. However, *S. intermedius*, isolated mainly from liver and brain abscesses, has been shown to produce a range of glycosidase enzymes which are thought to play a role in facilitating their growth *in vivo* (Homer et al., 1994).

The mitis group contains opportunistic pathogens associated with infective endocarditis which are presumed to have originated from the mouth. *S. gordonii* can bind to α-amylase to break down starch (Scannapieco et al., 1995) and *S. oralis* is capable of producing neuraminidase. *S. mitis* produces IgA protease, especially in strains found in early plaque formation or those colonising the buccal mucosa. *S. parasanguis* can bind salivary α-amylase but cannot produce extracellular polysaccharides from sucrose. *S. sanguis* is also classified in this group and is known to produce extracellular polymers of glucose from sucrose and is involved in the development of plaque.

### 1.5. Control and prevention of caries

The health of the mouth is dependent on the integrity of the mucosa and enamel which acts as a physical barrier to prevent penetration by microorganisms or macromolecular antigens. The host has a number of additional defence mechanisms which play an important role in maintaining the integrity of the oral surfaces (Table 1.2). These defences are divided into specific or immune components and non-specific, or innate, factors. The latter, unlike antibodies, do not require prior exposure to an organism or antigen for activity and so provide a continuous, broad spectrum of
protection.

<table>
<thead>
<tr>
<th>Defence Factor</th>
<th>Main Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Non-specific</strong></td>
<td></td>
</tr>
<tr>
<td>Saliva flow</td>
<td>Physical removal of micro-organisms</td>
</tr>
<tr>
<td>Mucin/agglutinins</td>
<td>Physical removal of micro-organisms</td>
</tr>
<tr>
<td>Lysozyme-protease-anion system</td>
<td>Cell lysis</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>Iron sequestration</td>
</tr>
<tr>
<td>Apo-lactoferrin</td>
<td>Cell killing</td>
</tr>
<tr>
<td>Sialoperoxidase</td>
<td>Hypothiocyanite production (neutral pH)</td>
</tr>
<tr>
<td></td>
<td>Hypocyanous acid production (low pH)</td>
</tr>
<tr>
<td>Histidine-rich peptides</td>
<td>Antibacterial and antifungal activity</td>
</tr>
<tr>
<td><strong>Specific</strong></td>
<td></td>
</tr>
<tr>
<td>Intra-epithelial lymphocytes</td>
<td>Cellular barrier to penetrating bacteria and/or antigens</td>
</tr>
<tr>
<td>Langerhans cells</td>
<td>Prevents microbial adhesion and Slga metabolism</td>
</tr>
<tr>
<td>IgG, IgA, IgM</td>
<td>Prevents microbial adhesion; opsonins; complement activators</td>
</tr>
<tr>
<td>Complement</td>
<td>Activates neutrophils</td>
</tr>
<tr>
<td>Neutrophils/macrophages</td>
<td>Phagocytosis</td>
</tr>
</tbody>
</table>

**Table 1.2.** Specific and non-specific host defences in the mouth
1.5.1. *Saliva.*

Several antibacterial factors are present in saliva which are important in controlling bacterial and fungal colonisation of the mouth, and these include lysozyme, lactoferrin and the sialoperoxidase system (Marsh and Martin, 1992).

Saliva significantly influences the carious process as was shown by animal experiments in which the salivary glands were surgically removed (Finn *et al.*, 1955). The specific properties of saliva which might be involved in protecting against, or limiting, caries attack have remained undetermined. The mechanical washing away of food debris, bacteria and their products by saliva will certainly play a role. Some investigators claim a relationship exists between caries prevalence and salivary amylase, urea, ammonia, calcium phosphate, pH etc. while others find no such relationship. It is the presence of the calcium and phosphate ions which are thought to be responsible for remineralisation of early carious lesions (Edgar *et al.*, 1994).

Saliva is composed of 95 % water; the remaining 5 % is composed of potassium, sodium, chloride, carbonate, hypophosphate ions mucopolysaccharides and glycoproteins (Sonju and Glantz, 1975). Whole saliva also contains cellular elements including bacteria ($2 \times 10^8$ bacteria/ml), peripheral mononuclear (PMN) cells and epithelial cells.

Saliva enters the oral cavity via ducts from the major paired parotid, submandibular and sublingual glands as well as from the minor glands of the oral mucosa where it is produced. The chemical composition of saliva from each of the glands is different but once in the oral cavity is termed ‘whole saliva’.
Shellis (1978) carried out an extensive literature search to establish the composition of saliva in order to derive a synthetic saliva. Although this medium is too defined for many purposes, it provides a useful guideline as to levels of components for a complex artificial saliva.

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Conc. (mg/l)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium chloride</td>
<td>233</td>
<td>Jacobson, 1950</td>
</tr>
<tr>
<td>Calcium chloride, dihydrate</td>
<td>210</td>
<td>Becks, 1943</td>
</tr>
<tr>
<td>Magnesium chloride, hexahydrate</td>
<td>43</td>
<td>Gow, 1965</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>1162</td>
<td>Shannon, 1958</td>
</tr>
<tr>
<td>Potassium di-hydrogen orthophosphate</td>
<td>354</td>
<td>Shannon, 1958</td>
</tr>
<tr>
<td>Potassium thiocyanate</td>
<td>222</td>
<td>Shannon, 1958</td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>13</td>
<td>Shannon, 1958</td>
</tr>
<tr>
<td>Sodium hydrogen carbonate</td>
<td>535</td>
<td>Shannon, 1958</td>
</tr>
<tr>
<td>di-sodium hydrogen orthophosphate</td>
<td>375</td>
<td>Shannon, 1958</td>
</tr>
<tr>
<td>Glycoprotein</td>
<td>2500</td>
<td>Inouye, 1930</td>
</tr>
<tr>
<td>Albumin</td>
<td>25</td>
<td>Ferguson, 1975</td>
</tr>
<tr>
<td>Urea</td>
<td>173</td>
<td>Nikiforuk et al., 1956</td>
</tr>
<tr>
<td>Uric acid</td>
<td>10.5</td>
<td>Altman and Dittner, 1968</td>
</tr>
<tr>
<td>Creatinine</td>
<td>0.1</td>
<td>Afonsky, 1961</td>
</tr>
<tr>
<td>Choline</td>
<td>13</td>
<td>Eagle, 1941</td>
</tr>
<tr>
<td>Mixture of amino acids</td>
<td>41</td>
<td>Battistone and Burnett, 1961</td>
</tr>
<tr>
<td>Mixture of vitamins</td>
<td>0.8</td>
<td>Glavind et al., 1948</td>
</tr>
<tr>
<td>Alpha amylase</td>
<td>3.1 x 10^5</td>
<td>Battistone and Burnett, 1961</td>
</tr>
<tr>
<td></td>
<td>units/l</td>
<td>Somogyi 1961</td>
</tr>
</tbody>
</table>

Table 1.3. Composition of synthetic saliva from Shellis (1978).

Previous attempts to model the ecology of oral diseases by utilising
continuous culture techniques have used conventional complex media derived from animal sources with little regard to the actual composition of human saliva (Glenister et al., 1988).

1.5.2. Mechanical Methods.

Addy et al., (1992) reviewed this area and stated the following ‘Toothbrushing with toothpaste is arguably the most commonly practised method of cleaning teeth in developed countries (Frandsen, 1986). On a per capita basis, however, it is probable that more of the world's population use wood sticks rather than toothbrushes to clean their teeth (Khoory, 1983). A number of beneficial qualities have been ascribed to wood sticks, and there is evidence from in vitro studies of antimicrobial compounds within the wood (El-Said et al., 1971). A toothpaste containing an extract, Salvadora persica, of a Middle Eastern 'chewing stick' had some effects in reducing plaque (Gazi et al., 1987).

Time, effort and money have all been expended on developing aids and techniques for mechanical cleaning. Additionally, the pros and cons of these have been extensively researched with, in most cases, an uncertain outcome (Frandsen, 1986).

The major factor which determines the success of mechanical cleaning seems to be the compliance and dexterity of its use. As with most hygiene habits, numerous variables influence how well they are performed. Psychosocial factors appear particularly important in relation to oral hygiene and dental health with large differences in caries predominance amongst the social classes and their gender sub-groups (Addy et al., 1990).’
1.5.3. Fluoride.

Dental caries is a complex disease involving the interplay of several factors. Therefore, it is unlikely that any one approach will lead to its control and prevention. Mechanical removal of plaque by oral hygiene can prevent caries, especially when combined with a reduction in the intake of sugars. However, alternative measures are required as long term eating habits are difficult to change and effective oral hygiene requires a considerable degree of motivation and dexterity.

Fluoride therapy continues to be the cornerstone of any caries preventative program. Fluoride exerts its main anti-caries effect following its incorporation into enamel but it may also have an antimicrobial effect. Following a wide range of studies on the effect of fluoride (Beighton and McDougal, 1977; Petersson, 1975; Margolis et al., 1975), the use of fluoridated water over 40 years has reduced the incidence of caries by 50 % (Easley, 1995). Fluoride has also been incorporated into toothpastes and mouthwashes and has more recently been incorporated into films used to coat the teeth. Incorporation of fluoride results in the formation of crystalline apatite in enamel containing high levels of fluoride which is more stable and resists acid dissolution to a greater extent. Fluoride also has antibacterial activity by inhibiting a number of bacterial processes including glycolysis (Beighton, 1980), sugar transport and membrane permeability (Sturr and Marquis, 1990; Hamilton, 1990). Concern has been expressed as to whether bacteria become resistant to fluoride (Bowden, 1990; Treasure, 1986) but laboratory studies suggest that there would be no increase in caries risk if the bacteria were fluoride resistant (van Loveren et al., 1989).
1.5.4. Antimicrobials.

Plaque control plays a central role in the therapeutic arsenal directed against gingivitis and periodontitis (Christersson et al., 1991). The choice of any anti-plaque agent used depends upon the form in which it is to be administered as incorporation into dentifrices presents formulation problems with some compounds. There are many classes of antimicrobial and anti-plaque agents: antibiotics, bisanguanides, enzymes, 'essential oils', fluoride, metal ions, 'plant extracts', phenols, quaternary ammonium compounds and surfactants (Marsh, 1991). Their principal mechanisms of action are either reduction of plaque formation by direct or sustained antimicrobial activity, inhibition of bacterial adsorption to the tooth surface or interference with the metabolic processes of dental plaque bacteria. Antibiotics are inappropriate for routine use as their spectrum of activity is too broad and resistant organisms may emerge. Furthermore they can induce the overgrowth of opportunistic pathogens, such as Candida albicans, due to the suppression of the resident microflora (Scheie, 1994).

Antimicrobial properties do not necessarily correlate with anti-plaque activity. An antimicrobial agent could be of considerable clinical value even if it had marginal effects on the plaque mass if it suppressed the selection of potentially pathogenic bacteria under conditions where they would otherwise flourish. An agent which interferes with the rate of accumulation or metabolism of supra-gingival plaque would inhibit the production of inflammatory compounds and therefore reduce the risk of gingivitis developing.

Dental plaque contains a diverse mixture of micro-organisms that co-exist in
relative stability due to the dynamic balance of synergistic and antagonistic interactions among the constituent species. Hence an antimicrobial agent can inhibit an organism by both direct and indirect means. A micro-organism that was dependent for growth on the provision of essential nutrients or co-factors by 'directly-inhibited' species, would be 'indirectly-inhibited'.

An infection can respond to antimicrobial chemotherapy only if the agent reaches the site at which it is required in adequate, active concentrations (Phillips, 1991). The minimum inhibitory concentration (MIC) is defined as the lowest concentration of antimicrobial that will inhibit visible growth of a micro-organism after appropriate incubation. Minimum bactericidal concentration (MBC) is defined as the lowest concentration of antimicrobial that prevents growth after subculture in an antibiotic-free medium. Although MIC data may be available for a particular compound showing that compound has antimicrobial activity, it is difficult to extrapolate from such studies and predict the effectiveness and behaviour of the compound in vivo.

The MIC test is concerned with the inhibition of growth of the organism, no attempt is made to distinguish between inhibition and death. In systemic infections a bacteriostatic effect would be sufficient to allow the host immune system to clear the invader. Host defence mechanisms are less effective in the oral cavity and hence a bactericidal effect is desirable (Wilson, 1993).

An effective antimicrobial, for use against oral diseases, should be bactericidal, effective over short time periods, effective in the presence of non-target organisms and effective against the target organisms when they are present in the biofilm. Ideally the development of resistant populations should be slow (Wilson, 1993). Anti-plaque agents should have high intrinsic
safety, oral substantivity, no adverse reactions e.g. staining, impaired taste, irritation and good chemical stability (van der Ouderaa, 1991).

Scheie (1975) concluded that the ideal agent was not yet available. Any chemical agent which affects microbial cells may be expected to have some adverse affects against host cells, unless the target structure, or metabolic pathway, is unique to the microbial cell.

There is a lack of relevant in vitro methods for determining the susceptibility of suspected pathogens to topically applied antimicrobial agents (Caufield et al., 1987). Traditional susceptibility tests emulate systemic infections involving $10^5$ to $10^7$ micro-organisms per ml of body fluids (Lorian and Atkinson, 1982). In contrast, the bacteria associated with dental microbial infections colonise in dense aggregates approaching $10^{11}$ per g of wet plaque (Gibbons and Nygaard, 1970). As bacterial susceptibility tests are bacterial density-dependent, conventional methods with comparatively small numbers of bacteria would be expected to under-estimate by several orders of magnitude the concentration of antimicrobial agent that would be efficacious in the oral cavity. Traditional in vitro methods simulate systemic infections which are exposed to antibiotics over the course of days, whereas topically-applied disinfectants are in contact with the plaque bacteria for only a few minutes. Penetrability is therefore likely to be one of the parameters in selecting an antimicrobial agent for use against oral infections (Caufield and Navia, 1980).

1.5.4.1. Chlorhexidine.

Chlorhexidine gluconate is a cationic bis-biguanide (Fig. 1.7), with a very
broad antimicrobial spectrum although the activity in vitro is not outstanding. The first report of its anti-plaque activity was by Loe and Schiott (1970). It is now used as a positive control in many clinical trials of new mouthrinse formulations. Chlorhexidine is a safe material, with low toxicity when used correctly. The most conspicuous side-effects are the development of yellow-brown stains on the teeth, tongue, and at the margins of anterior restorations, and an alteration in taste sensation (Addy, 1986). To overcome the problem of staining, lower concentrations have been formulated (Addy et al., 1989).

\[
\text{Figure 1.7. Structure of Chlorhexidine.}
\]

*Streptococcus mutans* seems to be particularly sensitive, whereas *S. sanguis*, for example, exhibits a greater variation in susceptibility between and among various strains. In relatively high concentrations, chlorhexidine is bactericidal, but low concentrations may be bacteriostatic to susceptible bacteria (Hennessey, 1973).

The positively-charged molecules of chlorhexidine bind readily to the negatively-charged cell wall, mainly to phosphate groups in lipopolysaccharides and carboxyl groups in proteins (Hugo and Longworth, 1966) and interfere with membrane transport initiating a leakage of low-molecular-weight substances (Rye and Wiseman, 1966). In high concentrations, the agent penetrates the cell wall and causes precipitation of
the cytoplasm (Hennessey, 1973). This mechanism seems to explain the bactericidal action of bis-biguanides in general (Davies and Field, 1969). After the first report of complete inhibition of plaque formation by chlorhexidine (Loe and Schiott, 1970), it soon became evident that the effect could not be explained by a general suppression of the oral flora during mouthrinsing alone. Gjermo et al (1970) showed that several other antibacterial agents with equal or stronger in vitro activity against salivary bacteria were not able to prevent plaque formation in vivo. At the same time Rolla et al (1970) showed that chlorhexidine had a strong affinity to salivary mucins in vitro and suggested that this could be an important mechanism for explaining the in vivo effect of the drug. They hypothesised that chlorhexidine molecules might be retained on oral surfaces by reversible electrostatic binding, then subsequently slowly released from the retention sites as the concentration in saliva decreased and the relative concentration of competing salivary calcium increased. This would indicate that the type of binding between the drug and the oral surfaces is of paramount importance in order for an optimal balance between retention and the rate of release to be established.

Thus, oral surfaces can act as a reservoir, releasing molecules of the agent over a prolonged period of time in amounts sufficient to maintain a bacteriostatic environment in the oral cavity and inhibit the normal metabolic activities of bacteria necessary for multiplication and adherence.
1.5.4.2. Cetyl pyridinium chloride.

Cetyl pyridinium chloride is one of a number of quaternary ammonium compounds that have been tested for their anti-plaque efficacy. Quaternary ammonium compounds are cationic surface-active agents. In this respect, they have some similarities to chlorhexidine. The molecules possess both hydrophilic and hydrophobic groups, allowing for ionic and hydrophobic interactions. It is assumed that interaction with bacteria occurs via cationic binding to phosphate groups of cell wall teichoic acid in Gram-positive bacteria, and to phosphate groups in the lipopolysaccharides of Gram-negative bacteria (Heptinstall et al., 1970). The membrane integrity may subsequently be disrupted by interactions with the lipophilic portion of the molecule, causing disturbance of membrane function and leakage of cytoplasmic material.

The antimicrobial activity of cetyl pyridinium chloride is equal to, or better than, chlorhexidine (Gjermo et al., 1970), whereas its plaque-inhibitory property is inferior to that of chlorhexidine (Bonesvoll and Gjermo, 1978; Gjermo et al., 1970). The difference in anti-plaque efficacy is probably related to the retention time of the agents in the oral cavity. Initial retention of cetyl pyridinium chloride is higher than for chlorhexidine. Cetyl pyridinium chloride is however, cleared from the oral cavity more rapidly than chlorhexidine (Bonesvoll and Gjermo, 1978). Also, quaternary ammonium compounds lose part of their antibacterial activity upon adsorption to surfaces (Moran and Addy, 1984).
1.5.4.3. Triclosan.

Triclosan is a chlorinated bis-phenol antiseptic, with low toxicity and a broad spectrum of antimicrobial activity (Scheie, 1989). It is effective against Gram-positive and most Gram-negative bacteria but with variable or poor activity against *Pseudomonas* spp. In low concentrations, triclosan exerts a bacteriostatic effect *in vitro*. In recent years, a considerable amount of clinical data has been amassed on the ability of oral products containing triclosan to reduce plaque, gingivitis and calculus, and to maintain gingival health (Lindhe, 1990; Gjermo and Saxton, 1991; Saxton *et al.*, 1987). The early studies indicated that, despite its antibacterial properties, the anti-plaque effect was moderate, most probably because of its rapid release from oral binding sites (Gilbert, 1987). However, the mechanisms of the antimicrobial action are not entirely clear. Due to its hydrophobic and lipophilic nature, triclosan absorbs to the lipid portion of the bacterial cell membrane and in low concentrations interferes vital transport mechanisms (Meincke *et al.*, 1980).

Studies on the retention of triclosan in the oral cavity indicate that triclosan possesses a certain degree of substantivity. Approximately 25 % of the applied amount was retained in the oral cavity after a mouthrinse with a
dentifrice slurry, and triclosan activity was still present 8 hours after a single dose (Gilbert and Williams, 1987).

1.6. Caries models

Models of dental caries (laboratory, animal, and human in situ models) vary markedly in their microbiological complexity. Laboratory models range from mono-cultures of cariogenic species providing an acidic challenge to enamel, to the development of diverse mixed cultures growing on a habitat-simulating medium in an artificial mouth or chemostat (Marsh and Martin, 1992 and Marsh, 1995). The latter systems are of value in determining either mechanisms of action or cause-and-effect relationships, for example, between dietary components or antimicrobial agents and the microflora. Laboratory models have also shown that the sensitivity of oral bacteria to inhibitors is markedly reduced when growing in biofilms such as dental plaque (Coombe et al., 1981). Animal models have proved unequivocally that caries is an infectious and transmissible disease. Their use has enabled comparisons to be made of (a) the cariogenic potential of different bacterial species, (b) the role of the diet, and (c) the effects of potential anti-caries agents (Characklis, 1989). It has been claimed that no caries-protective agent currently in use has failed a rodent test. In situ human models have been designed to permit the development of 'natural' plaque on standardised enamel surfaces freely exposed to the human oral environment (Mckee et al., 1985). The microflora that develops on unadulterated surfaces is similar in composition to that found at comparable sites on vital teeth. Demineralisation can be accelerated by the inoculation of additional
cariogenic bacteria coupled with oral sucrose rinses. The increased realism associated with the transition from laboratory to human *in situ* models is countered by a reduced ability to control or manipulate the system for experimental purposes.

McKee *et al* (1985) reported on the problems with caries models:

I. Repeated samples of plaque for bacteriological or metabolic studies cannot be taken without disturbing the integrity of the film.

II. The precise environmental conditions within the film cannot be determined.

III. The known variability of the bacterial composition of dental plaque samples from different sites, or even from the same site at different times imposes a limitation on the repeated use of plaque as an inoculum.

The development of model growth systems can help in the investigation of microbial growth from natural environments. The growth of the microbial film usually goes through an irregular life-cycle, finally leading to large masses of biofilm removal, often due to the destabilising effects of cell death and lysis and anaerobic fermentation reactions at the base of the film (McKee, 1985). The process is akin to growth of a bacterial batch culture, but is much less predictable. There is, therefore, a case for devising a biofilm growth system in which film formation is controlled and reproducible, and is at the same time easy to sample under controlled conditions of nutrient and gas supply.

There are a number of laboratory systems that go some way to satisfy these demands. These include the Modified Robbins device, perfused biofilm fermentor, and the constant depth film fermenter.

The Modified Robbins device (MRD) is a good system for a range of biofilms that form in flow systems these can range from water conduits to indwelling catheters (McCoy et al., 1981). The MRD is constructed from a long Perspex block with a rectangular lumen containing evenly-spaced sample ports. Polypropylene connectors at either end attach the tubing from the culture vessel (Fig. 1.8). The sample studs, also made from Perspex are inserted into the sample ports. The stud design includes a bevelled upper section for easy removal from the MRD. The lower section of the stud is designed to fit tightly into the MRD and this is assisted by a rubber O-ring which seals the port to prevent leakage. The bottom surface of the sample stud has a rim and the surface to be colonised is cut into a disc and placed inside this rim, together with a backing disc. Biofilms grow both on the inner wall of the tube and on the surface disc. The latter may be removed and replaced during the operation of the system. The film thickness cannot, however, be controlled accurately and changes in the flow rate of recirculating medium can lead to the removal of biofilm surface layers by shear forces acting upon them. One theoretical problem with the device is that, at slow flow rates, there is likely to be a longitudinal nutrient gradient which could lead to position-dependent changes in biofilm properties (Wimpeny et al., 1993).
1.6.2. The Perfused Biofilm Fermentor (PBF).

This is an *in vitro* model, designed by Gilbert *et al* (1989), which, through application of appropriate nutrient limitations, enables growth rate control of adherent bacterial populations. Exponentially-growing cells are collected by pressure filtration onto cellulose acetate membranes. Following inversion into the bases of modified fermentors, membranes and bacteria are perfused with fresh medium. Newly-formed and loosely-attached cells are eluted with spent medium. Steady-state conditions (dependent upon the medium flow rate) at which the adherent bacterial biomass is constant and proportional to the limiting nutrient concentrations are rapidly achieved, and within limits, the growth rate is proportional to the medium flow rate.
PBF can generate biofilms as thin as 10 μm and these, if dehydrated, contain sufficient cells to generate a mono-layer covering the substratum. It would therefore be appropriate for in vivo infections of soft tissues and intravascular devices.

1.6.3. Constant Depth Film Fermentor.
The constant depth film fermentor (discussed in detail in Chap. 2) was developed by Wimpenny (1981). The apparatus consists of a rotating turntable which holds fifteen polytetrafluoroethylene (PTFE) pans located flush around its rim. Each pan contains 5 or 6 cylindrical holes containing PTFE plugs. These plugs can be recessed to a range of depths (usually 300μm) and the biofilms form in the recess. Various discs, notably bovine enamel, hydroxyapatite and amalgam, can be placed on the PTFE plugs and recessed in the same manner. Once the biofilm reaches the required depth any further expansion is prevented by scraper blades, so the depth of the biofilm is kept constant. The model is particularly suited to the growth of dense biofilms, such as dental plaque, although initial colonisation can also be investigated.

1.6.4. Continuous culture chemostat.
The use of continuous culture chemostats was one of the first methods employed to mimic natural ecosystems more closely and move away from batch culture techniques. They are better-able to simulate what might occur in nature than batch culture because growth is maintained by the continual replenishment of nutrients, thus communities may remain in the log phase of growth. Communities comprising several bacterial species can also be
maintained using this system. Bradshaw et al (1996a/b) have developed a two-stage chemostat in order to investigate communities of oral bacteria. This method has been used to investigate biofilms by inserting removable hydroxyapatite discs into the chemostat to allow biofilm formation. This allows comparisons between planktonic and biofilm communities within the same vessel.

1.7. Aims of the study

The purpose of this *in vitro* investigation was to develop a reproducible method of testing the susceptibility of oral microbial biofilms to antimicrobial and anti-plaque agents using the constant depth film fermentor. The initial stages of the study employed a simple, mono-species biofilm to determine the effect of a number of antimicrobial agents on biofilm formation and viability. Using multi-species and microcosm biofilms then increased the complexity of the model. The distribution of the constituent species throughout the biofilms was determined and the effect of repeated exposure to chlorhexidine was investigated.
Chapter 2 - Materials and Methods
2.1 Bacteriological media

Most of the bacteriological media were purchased from Unipath Limited, Basingstoke, Hampshire, U.K with the exception of mitis salivarius agar, Veillonella agar and d/e neutralising broth which were supplied by Difco Limited, U.K. The artificial saliva and cadmium fluoride acriflavin tellurite (CFAT) agar were prepared as detailed below. The chemicals were purchased from Sigma Chemical Company Limited, Poole, U.K. or Merck Limited, Poole U.K. unless otherwise stated.

2.1.1 Cadmium fluoride acriflavin tellurite (CFAT) agar.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone soya broth powder</td>
<td>30 g</td>
</tr>
<tr>
<td>Agar technical powder</td>
<td>15 g</td>
</tr>
<tr>
<td>Glucose</td>
<td>5 g</td>
</tr>
<tr>
<td>Sheep blood</td>
<td>50 ml</td>
</tr>
<tr>
<td>Cadmium sulphate</td>
<td>13 mg</td>
</tr>
<tr>
<td>Sodium fluoride</td>
<td>80 mg</td>
</tr>
<tr>
<td>Neutral acriflavin</td>
<td>1.2 mg</td>
</tr>
<tr>
<td>Potassium tellurite</td>
<td>2.5 mg</td>
</tr>
<tr>
<td>Basic fuschin</td>
<td>0.25 mg</td>
</tr>
</tbody>
</table>

Table 2.1. Composition of CFAT agar (g/L in dH₂O)

2.1.2 Artificial saliva

The nutrient source in all experiments was an artificial saliva, the composition of which was based on the work by Russell and Coulter (1975) and Shellis (1978).
Table 2.2. Composition of artificial saliva (g/L)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>'Lab-lemco' powder</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>2.0 g</td>
</tr>
<tr>
<td>Proteose Peptone</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Mucin Type III: Partially Purified from Porcine Stomach*</td>
<td>2.5 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>0.35 g</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>0.2 g</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>0.2 g</td>
</tr>
</tbody>
</table>

After autoclaving 12.5ml of 40 % filter sterilised urea was added.


2.2. The Constant Depth Film Fermentor

In order to test the effect of antimicrobials on microbial biofilms, over a period of time, data must be comparable and reproducible from experiment to experiment. One approach to reproducibility is to develop constant depth reactors where surface growth is periodically removed to maintain a constant geometry. Such a device employing a mechanical scraper bar was first described by Atkinson and Daoud (1970). The idea was developed by Wimpenny (1981) for a fermentor designed to investigate the growth of dental plaque organisms. Early versions were used in dental plaque studies (Coombe et al., 1984) before Peters and Wimpenny (1988) improved the model further. The design of the fermentor was predicated on the need to provide answers which are not easily obtainable from natural microbial films (Kinniment and Wimpenny, 1990).

The constant depth film fermentor used throughout this study was a modified
version of the fermentor described by Peters and Wimpenny (1988) engineered by John Parry-Jones Engineering, Cardiff (Fig. 2.1).

![Figure 2.1. The Constant Depth Film Fermentor.](image)

A stainless steel turntable cut away at the centre holds fifteen polytetrafluoroethylene (PTFE) pans located flush around its rim (Fig. 2.2b). Each plug is inserted and held flush with the steel table by silicone rubber 'O' rings. Pans are each drilled with a central threaded hole into which a special tool is inserted for removal and replacement. Each of the pans
contains 5 or 6 cylindrical holes, 5 or 4.7 mm in diameter, which contain PTFE plugs. The biofilms were grown either on the PTFE plugs or on discs of various materials supported by these plugs. Two types of discs were used during the studies, bovine enamel discs (cut from bovine incisors which had been polished flat; Eastman Dental Institute, Biomaterials Dept.) and hydroxyapatite discs (Bio-Interfaces Inc., San Diego). The discs sit on the PTFE plugs of the same diameter and can be accurately recessed using a template to depths up to 500 μm. For the purpose of these studies depths of between 100 and 300 μm were used. The three substrata used were immersed firstly in sterile artificial saliva for 1 minute to form a conditioning film before the CDFF was assembled and autoclaved. Thus, the removal of each film pan produces 5 or 6 replicate biofilm samples, allowing different investigations to be performed or statistical variations to be determined.

The steel turntable was attached to a vertical central spindle running through the base plate via an autoclavable bearing assembly (Fig. 2.2a). The base plate is supported by three stainless steel legs to accommodate the detachable gearbox motor (RS). The gearbox is driven by a 15 V power supply (BBH Power Products) housed in the support in which the fermentor sits (Fig. 2).

The steel disc, rotating at 3 revolutions per minute (r.p.m.), passes beneath two angled PTFE scraper blades which are forced onto the surface of the steel disc by pressure exerted from loaded springs (Fig. 2.2a).

A QVF borosilicate glass tubing section, sealed to the top and bottom plates by PTFE seals, surrounds and completely encloses the turntable and scraper blades assembly. The top plate is fitted with a number of inlets for
medium and test agents as well as a sample port and air inlet. The air inlet had two Whatman Hepa-vent 0.2 μm pore filters (BDH/Merck) attached. Samples were taken using two sterilisable tools. First the extractor tool was inserted through the sample port and screwed into the appropriate film pan (this was simply positioned into place by reducing the voltage on the power supply to zero), which could then be removed through the sample port. The second tool was autoclaved with a replacement film pan already attached, this was held flush by a rim on the sample tool. The pan was inserted into the vacant hole and kept flush with the stainless steel turntable by the rim. The bottom plate is simply fitted with an effluent medium outlet.

Figure 2.2a. Schematic vertical section through the fermentor.
Figure 2.2b. Schematic horizontal section through the fermentor.

As with all fermentation work there is a large amount of tubing needed to supply media and other liquids to and from the fermentor. Silicone tubing (Merck/BDH) was used for the CDFF for several reasons, it is flexible, durable, non-toxic, autoclavable, and can be visually inspected for contamination. Separate silicone pump tubing (Watson-Marlow Ltd.) was required for use with the peristaltic pumps and, where required, quick-disconnect fittings (Nalgene) were used.
2.3 Organisms

The organism used in the single species work was *Streptococcus sanguis* NCTC 10904.

The six species comprising the six membered biofilm community were, *Actinomyces viscosus* NCTC 10951, *Neisseria subflava* ATCC 1078, *Streptococcus mutans* NCTC 10449, *Streptococcus oralis* NCTC 11427, *Streptococcus sanguis* NCTC 10904 and *Veillonella dispar* NCTC 11831.

The microcosm plaque communities were grown from pooled human saliva (2.4.3).

2.4. Inoculation of the fermentor

The fermentor was inoculated using different volumes of media, over various time periods depending on the number of organisms used for a specific run.
2.4.1 Inoculation of single species.

A 24 h culture of *S. sanguis* in nutrient broth (Oxoid Ltd, Basingstoke, UK) was inoculated into 2 litres of artificial saliva, this was recirculated through the CDFF by the pressure exerted by the variable speed peristaltic pump (Watson-Marlow) (Fig. 2.3a). Circulation of the inoculum took place over 24 h at 37°C using a flow rate of 1 ml/min.

2.4.2 Inoculation of multi-species.

The inoculation of the 6 membered biofilm community was carried out using *S. sanguis*, *S. mutans*, *S. oralis*, *A. naeslundii*, *V. dispar* and *N. subflava*.
Cultures of the above organisms were grown on blood agar plates and incubated anaerobically for 3-4 days except for *N. subflava* which was incubated aerobically overnight. A whole plate of each organism was aseptically placed into 10ml of suspending medium.

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone T</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.1 g</td>
</tr>
<tr>
<td>Cysteine hydrochloride</td>
<td>0.1 g</td>
</tr>
<tr>
<td>Bovine serum</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>Glycerol</td>
<td>10 ml</td>
</tr>
<tr>
<td>dH2O</td>
<td>88 ml</td>
</tr>
</tbody>
</table>

**Table 2.3. Composition of suspending medium**

(filter sterilised in 10ml amounts into sterile universals, stored at -20°C)

1 ml of this mixed culture was added to 9 mls of suspending medium in a sterile cryovial and these were stored at -70°C. For inoculation of the CDFF, a cryovial was thawed at room temperature and added to 5 mls of artificial saliva and vortexed for 10 seconds. Using a 10ml sterile pipette, the inoculum was aseptically added directly to the rotating pans via the sample port. This was left rotating for 1h and recirculated as previously described for the single species but with only 500 ml artificial saliva for 8 h.

2.4.3. *Inoculation of microcosm plaque.*

Saliva, obtained from 10 healthy individuals, was used as the inoculum to provide a multi-species biofilm containing species found in the oral cavity. Equal volumes from each person were pooled and 1 ml aliquots dispensed
into cryovials, and these were then frozen at -70°C for subsequent use. The cryovials were thawed and aseptically added to 500 ml sterile artificial saliva. This inoculum was then fed into the fermentor at a rate of 1 ml/min. over 8 h.

2.5. Continuous flow

The main batch of sterile artificial saliva was connected to the fermentor after inoculation, either at 8 or 24 h. At the same time the waste port was connected to a 10 or 20 litre effluent bottle, depending on the length of the run (Fig. 2.3b).
Figure 2.3b. Schematic diagram of fermenter with through-flow of medium.

Between the length of silicone tubing connecting the pump tubing (Watson-Marlow) from the peristaltic pump and the CDFF was a grow-back trap (Hampshire Glassware Co. Southampton, U.K.) to prevent contamination of the sterile medium. The artificial saliva was delivered at a rate of 0.72 litres per day, the resting flow rate of saliva in man (Lamb et al., 1991; Bell et al., 1980 and Guyton, 1992), via the peristaltic pump.
2.6. Culture methods

Pans were removed from the CDFF at various time intervals depending on the experiment, and the discs aseptically removed and placed into either 10 ml artificial saliva or 10 ml d/e neutralising broth (Difco Laboratories, Detroit, MI) if antimicrobial or anti-plaque agents were being used, this was to prevent any further action of the agent. The discs were then vortexed for 1 minute to disrupt the biofilm. Serial dilutions of *S. sanguis* were carried out in nutrient broth and 20 µl of each dilution plated in duplicate onto Tryptone Soya Agar (Oxoid). The plates were incubated anaerobically overnight at 37°C before the colonies were counted.

The multi-species biofilms and microcosm plaque were treated in the same way but dilutions were carried out in artificial saliva and plated onto various selective media. *Actinomyces* spp. were isolated on cadmium fluoride acriflavin tellurite (CFAT) agar plates, (Zylber and Jordan, 1982) *Veillonella* spp. on Veillonella agar (Difco) containing 7.5 µg vancomycin (Sigma) and *Streptococcus* spp. on mitis salivarius agar (Difco). The total anaerobic count was obtained on Wilkins-Chalgren agar (Oxoid). Plates were incubated anaerobically for 4 days at 37°C. Selective plates were also required for *Neisseria subflava*, Thayer-Martin Agar (Oxoid) was used for this purpose, plates were incubated aerobically at 37°C for 4 days. Total aerobic viable counts were performed on blood agar (Oxoid) plates and incubated in the same way.
2.7. Substratum preservation methods

2.7.1 Mineralising solution.

The object of the treatment was to promote re-mineralisation of the dentine prior to re-use. Causton and Johnson (1982) developed the treatment. Details of the preparation are given in g/L in Table 2.4. The final pH of the solution is 7.75.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium chloride</td>
<td>0.2 g</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>0.2 g</td>
</tr>
<tr>
<td>Magnesium chloride</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>0.16 g</td>
</tr>
<tr>
<td>Sodium hydrogen carbonate</td>
<td>0.02 g</td>
</tr>
<tr>
<td>Sodium dihydrogen orthophosphate 1-hydrate</td>
<td>0.05 g</td>
</tr>
<tr>
<td>Glucose</td>
<td>1 g</td>
</tr>
</tbody>
</table>

Table 2.4. Mineralising solution.

2.7.2. Cleaning discs which have been exposed to agents.

The substantivity of the antimicrobials being used, especially chlorhexidine is well documented and thus total removal of the agents from the discs was of great importance.

Exposure to Chlorhexidine

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetonitrile</td>
<td>40 %</td>
</tr>
<tr>
<td>Hydrochloric acid</td>
<td>0.4 %</td>
</tr>
</tbody>
</table>

5 ml / 5 min. exposure, dH$_2$O rinses in-between applications.
**Exposure to Triclosan**

Methanol, 5 ml / 1 min. exposure time.

**Exposure to Cetyl pyridinium chloride**

Sulphonic acid 0.05g / 20ml dH₂O

Acetonitrile 50ml

Tetrahydrofuran 30ml

pH adjusted to 3.8 with Orthophosphoric acid

5 min. exposure time.

### 2.8. Vital staining

Vital staining was carried out using a BacLight live/dead viability kit (Molecular Probes Inc. Eugene, USA). The kit contains two probes; calcein AM (Fig. 2.4.a), a fluorogenic substrate that is only cleaved in viable cells to form a green fluorescent membrane-impermeable product, and ethidium homodimer-1 (Fig. 2.4.b), a high-affinity red fluorescent DNA stain that is only able to pass through the compromised membranes of dead cells.

a.

![Chemical structure](attachment:image.png)
Figure 2.4. Structures of fluorescent probes.

The discs supporting the biofilms were vortexed for 1 minute and transferred into vials containing 1 ml of phosphate buffered saline (PBS; Oxoid). Equal volumes of reagent A (1.5μl) and reagent B (1.5μl) were mixed in a microfuge tube and vortexed, as per manufacturer’s specifications (stored at -20°C). 3μl of this solution was then added to the bacterial suspension and vortexed to mix. After 15 minutes incubation in the dark at room temperature, 5μl of the solution was added to a counting chamber. This was performed by trapping the stained bacterial suspension between the counting chamber slide and cover-slip until Newton’s Rings were visible. This allowed the use of an oil immersion lens (Zeiss, Ph3 x100) without displacement of the cover-slip. Microscopic visualisation showed green fluorescence indicating live bacteria whereas dead bacteria exhibited a red stain when viewed with light at a wavelength of 300 nm. The stain was shown to be effective with S. sanguis by testing on planktonic cultures. Staining took place as described above on an overnight culture resuspended in 1 ml of PBS before and after heat treatment (15 min. 100°C).
2.9. Cryosectioning

The freeze-sectioning method described below was based on work by Kinniment (1994). The aim of the method was to transfer the biofilm whilst still attached to the substratum, to the cryostat sectioning stub where it could be frozen and sectioned.

The fermenter pan was placed in 5ml of a 25% dextran solution, a cryoprotectant (Ashwood-Smith and Warby, 1971), in artificial saliva for 2 h, before being removed and placed in 5ml of 8% formaldehyde for 15 - 20 mins. One of the five PTFE plugs was forced upwards to expose the biofilm and substratum. The disc was removed and placed onto a thin layer of cryo-m-bed on the cryostat chuck. This was then placed in a -70°C freezer for 20 mins. until the cryo-m-bed and the biofilm were frozen. Further cryo-m-bed was then placed over the top of the biofilm so that the entire sample was embedded (Fig. 2.5).

![Diagrammatic cross-section of embedded sample for cryosectioning.](image)

This was then frozen in the same way for a further 20 mins. It was important
to freeze the biofilm so it retained its structure before the cryo-m-bed was used to cover the sample. Sections were cut on a cryostat at -19°C, 30μm thick sections for viable counts and 10 μm thick sections for live/dead staining. Using cold forceps, the sections were placed into 1 ml of PBS. These were vortexed for 1 min. and appropriate assays carried out as described previously.

2.10. Electron microscopy

2.10.1. Transmission electron microscopy.
Biofilms on selected substrata were fixed in 3 % gluteraldehyde in 0.1M sodium cacodylate buffer at 4°C overnight. The specimens were post-fixed in 1 % osmium tetroxide at 4°C for 2 h before being dehydrated in a graded series of alcohol (20 – 100 %, 15 mins. application time). The biofilms were embedded in fresh araldite CY212 and 90 - 100 nm sections cut. The sections were stained with lead citrate and uranyl acetate, and viewed with a Jeol 100 CX transmission electron microscope.

2.10.2. Scanning electron microscopy.
Biofilms were fixed in 3 % gluteraldehyde in 0.1M sodium cacodylate buffer at 4°C overnight. The specimens were post-fixed in 1 % osmium tetroxide at 4°C for 2 h before being dehydrated in a graded series of alcohol (20 – 100 %, 15 mins. application time). Alcohol was then exchanged with 100 % acetone for 3 x 10 min. rinses. The sample was then transferred into HDMS (Hexadimethylsilane) for 1-2 mins. and left to dry in a dessicator. Imaging was carried out using a Cambridge 90B stereoscan electron microscope.
2.11. Statistical Analysis

Unless otherwise stated, the statistical analysis carried out on the data was a Students two tail t-test, where p values less than 0.05 were regarded as being significant. In all cases the number of replicate samples taken from one run was four, and all experiments were carried out at least twice.
Chapter 3 - Effect of mouthwashes on the formation and growth of *Streptococcus sanguis* biofilms on various substrata.
3.1. Aims

The purpose of this study was to evaluate the effects of three antimicrobial-containing mouthwashes on biofilm formation and viability using a constant-depth film fermenter (CDFF) as a means of generating biofilms under conditions similar to those which would exist in vivo. The main objectives were to be able to produce a reproducible single species biofilm and to be able to rank the order of effectiveness of the antimicrobial agents whether they were being used either as antimicrobial or as an anti-plaque agent.

3.2. Materials and Methods

3.2.1. Growth of biofilms.

The single species biofilms were grown on either hydroxyapatite or bovine enamel. The fermentor was inoculated as described in 2.4.1 and the biofilms were grown for up to 4 days. The discs were immersed in sterile complete saliva for 1 min before the CDFF was assembled as described in Chapter 2.

3.2.2. Effect of mouthwashes on the viability of S. sanguis biofilms.

The three antimicrobial-containing mouthwashes and a placebo mouthwash were supplied by SmithKline Beecham Consumer Healthcare (Weybridge, U.K.). The four mouthwashes tested were as follows:

I. Colgate Actibrush Coolmint containing 0.03 % triclosan (T) and 0.025 % sodium fluoride.

II. 0.12 % chlorhexidine digluconate (CH) / 0.023 % sodium fluoride mouthwash found to be efficacious in the in vivo clinical study of
III. Macleans Mouthguard Coolmint containing 0.05 % cetyl pyridinium chloride (CPC) and 0.05 % sodium fluoride.

IV. The placebo mouthwash (P) was a typical mouthwash base containing colour and flavour but no antimicrobial or sodium fluoride.

After reaching a steady state (6 days), pans were removed aseptically from the fermenter and placed slowly, so as to avoid any disruption of the biofilm, into 5ml of the appropriate mouthwash, or deionised water in the case of the placebo, for either 1 or 5 mins. The pans were then carefully removed from the liquid and the discs removed and vortexed for 1 min in 10 ml neutralising broth (Difco Laboratories). Survivors were enumerated by viable counting on Tryptone Soya Agar (Oxoid Ltd.).

3.2.3. Effect of mouthwashes on biofilm formation.

The pans already containing the recessed bovine enamel or hydroxyapatite discs were placed in 5 ml complete saliva for 1 min before being placed in 5 ml of the appropriate mouthwash (3.2.2). 2 pans were used for each mouthwash and a water control, this allowed for a space between the differently-treated pans so as to prevent transfer of materials between them. The CDFF was then autoclaved and a culture of S. sanguis in complete saliva pumped through. Samples were removed at 4, 8 and 16 h intervals and immersed in neutralising broth. The number of viable bacteria on each disc was determined as described in 2.6.
3.3. Results

The electron micrograph (Fig. 3.1) shows a 24 h *Streptococcus sanguis* biofilm grown in the CDFF with nutrients supplied by artificial saliva (2.1.2). The characteristic cocci chains were still a dominant feature of the biofilm structure.

There was no statistically significant difference at 95 % confidence levels between the final cell density of *S. sanguis* biofilms grown on PTFE, hydroxyapatite or bovine enamel over the 192 h period. The growth rate was 0.8 h⁻¹ and the final cell density was approximately 2x10⁶ cfu per mm² on all three surfaces. The initial colonisation was rapid (1x10⁴ cfu per mm² at 24 h) but growth remained reasonably constant from 48 h onwards (Fig. 3.2).

Treatment of hydroxyapatite-grown (H) biofilms with the triclosan mouthwash for 1 min. resulted in appreciable kills of 74 % in the 6 d-old biofilms. Chlorhexidine (CH) achieved kills of 49 % in biofilms containing 10⁴ cfu/mm² over the same time period whereas the cetyl pyridinium chloride-containing mouthwash (CPC) did not achieve any significant decrease (p<0.05) in the viable count after 1 min. In the case of all three mouthwashes, the number of survivors decreased with increasing exposure time. CPC achieved significant killing (p<0.05) only after 5 min. Neither the placebo nor de-ionised water had a significant effect on the viable count of the biofilms after exposure for 60 s or 5 mins (Fig. 3.3).

The triclosan-containing (T) mouthwash was the most effective at killing 6 d-old biofilms grown on bovine enamel (BE), achieving reductions of 98 % and 99 % after exposure for 1 and 5 mins respectively. Chlorhexidine also showed appreciable kills (93 % and 94 % after 1 and 5 mins respectively)
while CPC exerted a significant (p<0.05) bactericidal effect (89 % kill) only after 5 mins. The placebo mouthwash had no effect on the viability of the organism (Fig. 3.4).

Treating the conditioned hydroxyapatite discs with chlorhexidine, CPC or triclosan resulted in no detectable viable bacteria on the discs after 8 h exposure to a *S. sanguis* suspension in artificial saliva. In contrast, the number of viable bacteria on discs treated with the placebo or water were $5.54 \times 10^3$ cfu/mm$^2$ and $1.54 \times 10^3$ cfu/mm$^2$ respectively. After 16 h there was an appreciable increase in the viable count of bacteria on the discs pre-treated with all four mouthwashes, although the T-containing mouthwash was the only one which achieved significant inhibition (p<0.05) of growth (73 % reduction) compared to the water placebo (Fig. 3.5).

The results obtained following treatment of conditioned BE with the mouthwashes were very different from those found when H discs were used. The accumulation of *S. sanguis* on the discs after 4 h was considerably reduced by chlorhexidine (91 %) and there were also statistically significant reductions (75 % and 56 %) in viable cell numbers when the discs were treated with mouthwashes containing CPC and T respectively. The placebo had no effect on the accumulation of viable cells compared with the water placebo. After 8 h, only discs treated with chlorhexidine showed a statistically significant reduction (p<0.05) in the number of viable organisms in the biofilms (Fig. 3.6).
Figure 3.1. Scanning electron micrograph of *Streptococcus sanguis* biofilm 24 h, bar represents 10 µm.
Figure 3.2. The growth of *Streptococcus sanguis* biofilms on various substrata. BE = Bovine enamel, H = Hydroxyapatite and PTFE = Polytetraflouroethylene. From Pratten *et al* (1998).
Figure 3.3. Effect of mouthwashes on the viability of *Streptococcus sanguis* biofilms grown on hydroxyapatite discs. C = Control, P = Placebo, CH = Chlorhexidine, CPC = Cetyl pyridium chloride and T = Triclosan. Bars represent the mean viable counts and error bars standard deviations (n = 8).

Figure 3.4. Effect of mouthwashes on the viability of *S. sanguis* biofilms grown on bovine enamel discs.
Figure 3.5. Formation of *Streptococcus sanguis* biofilms on hydroxyapatite discs treated with mouthwashes. C = Control, P = Placebo, CH = Chlorhexidine, CPC = Cetyl pyridium chloride and T = Triclosan. Bars represent the mean viable counts and error bars standard deviations (n = 8).

Figure 3.6. Formation of *S. sanguis* biofilms on bovine enamel discs treated with mouthwashes.
3.4. Discussion

This initial study has shown that using the CDFF to generate single species biofilms it was possible to (a) assess the antimicrobial activity of compounds against a plaque organism under conditions similar to those which would exist in vivo and (b) determine the ability of compounds to inhibit biofilm formation.

Work already carried out (Pratten et al., 1998) has demonstrated that the composition of the artificial saliva is of great importance in this study, through its chemical and physical properties, saliva exerts a profound influence on the adhesive interactions that occur in the mouth. Saliva may influence bacterial adhesion to teeth in several ways. Firstly through the formation of a pellicle, modifying the substratum for adhesion, through its physical and chemical properties as the suspension medium and through its interaction with the bacteria, modifying the bacterial surface or agglutinating potential colonisers (Ørstavik, 1978). Due to the obvious difficulty in using large volumes of human saliva, a synthetic substitute was needed to reproduce features of saliva which are considered important in cultural studies of plaque bacteria while remaining relatively inexpensive and simple to produce. The 'complete saliva' used in this study was developed from the work by Russell and Coulter (1975) and Shellis (1978).

Early in vitro models of dental plaque studied the accumulation of bacteria on extracted teeth but this has the disadvantage that large numbers of identical replicates are difficult to obtain. One of the advantages of the CDFF is that it can provide large numbers of identical biofilms so enabling sampling during the course of long runs and large numbers of biofilms at a given time.
point so permitting good statistical analysis. In order to take advantage of this, it was necessary to have a ready supply of large numbers of discs of a suitable substratum. It was decided therefore, to assess the use of hydroxyapatite and bovine enamel as possible substrata. Studies of the growth of \textit{S. sanguis} on these materials revealed that there was little difference between them in terms of the growth rate of the organism or the final yield. However, the nature of the substratum was found to have a profound effect on the ability of antimicrobial-containing mouthwashes to prevent biofilm formation or, strictly speaking, the survival of viable bacteria on the surface of the substratum. For example, triclosan was the most effective at preventing biofilm formation on hydroxyapatite discs whereas it was the least effective when bovine enamel discs were used. The differential accumulation of viable bacteria on the two substrata pre-treated with the various mouthwashes would be the result of a number of factors including (i) the ability of the antimicrobial agents to bind to the saliva-coated (and possibly uncoated) regions of the substrata during the initial 60 s period of immersion (ii) their ability to remain bound to the discs (i.e. their substantivity) (iii) their effectiveness, once bound to the discs, at inhibiting adherence of the bacteria (iv) the relative effectiveness of the antimicrobials at killing adherent bacteria. Retention of the agents on the discs involves binding to receptors on the conditioning film and, possibly, regions of the substratum not coated by this film. Such binding is governed by electrostatic, hydrophobic and lipophilic interactions as well as by hydrogen bonding and Van der Waals forces (Cummins and Creeth, 1992). Qualitative and/or quantitative differences in the conditioning films formed on the two substrata,
therefore, may account for the different effects of a particular agent on biofilm formation on the two substrata. Such differences, in terms of composition, packing distribution and configuration, have been reported for a range of substrata including glass, fused silica and Ge prisms (Baier and Glantz, 1978; Sonju and Glantz, 1975). The nature of the pellicle formed on a substratum has been shown to depend on its hydrophobicity (van der Mei et al., 1993). Few in vitro studies of the binding of anti-plaque agents to substrata have been reported. However, Freitas (1993) has shown that chlorhexidine binds to both hydrophobic and hydrophilic surfaces by hydrophobic and electrostatic interactions respectively via polar and non-polar regions of the molecule. Adsorption to the hydrophobic surfaces was greater than to the hydrophilic surfaces but rinsing with sodium acetate completely removed the chlorhexidine from both surfaces. Once retained on a substratum, chlorhexidine appears not to affect the adherence of bacteria or the viability of bound bacteria but has a prolonged bacteriostatic effect on adherent streptococci by prolonging the lag phase of the organisms (Reed et al., 1981). Despite greater oral retention (Bonesvoll and Gjermo, 1978) and equivalent antibacterial activity to chlorhexidine, CPC is not as effective at inhibiting plaque formation as the former (Gjermo et al., 1970). This may be attributable to rapid desorption from oral sites (Bonesvoll and Gjermo, 1978) and/or reduced antibacterial activity once it is adsorbed onto surfaces (Moran and Addy, 1984).

Whereas all three antimicrobial-containing mouthwashes were effective at reducing the number of viable bacteria accumulating on hydroxyapatite discs for 8 h, biofilm formation on bovine enamel over the same period of time was
reduced only by pre-treatment with chlorhexidine.

Although there are no similar in vitro studies with which these findings can be compared, Renton-Harper et al (1996) recently reported the results of an in vivo comparison of the effects of chlorhexidine, CPC and triclosan on plaque formation over a period of 4 days. As the subjects were rendered plaque-free at the commencement of the study, refrained from tooth brushing during the study and the test agents were administered as mouthrinses, it is meaningful to compare the results obtained with those found in the present laboratory study although the bioavailability of the antimicrobials would vary. Chlorhexidine was found to be the most effective at inhibiting plaque formation whereas CPC and triclosan were similar, but less effective, in this respect. These results are similar to those obtained in the present study using bovine enamel as the substratum. However, when hydroxyapatite was used as the substratum, triclosan proved to be the most effective. These results suggest that bovine enamel is preferable to hydroxyapatite for predicting the likely effectiveness of anti-plaque agents in vivo.

As might be expected, the nature of the substratum appeared to have less effect on the ability of the mouthwashes to kill bacteria in pre-existing biofilms. For biofilms grown on either substratum, the relative effectiveness at killing S. sanguis in the biofilms decreased in the order triclosan, chlorhexidine, cetylpyridinium chloride. The susceptibility of a biofilm to an antimicrobial agent is dependent, amongst other factors, on a number of attributes of the agent including its rate of penetration into the biofilm (which will depend on its charge, its size and its affinity for the biofilm matrix) and its
ability to exert an antibacterial effect in the microenvironment (pH, redox potential etc) within the biofilm. The molar concentrations of the three agents used were very similar (0.0013, 0.0014 and 0.001 for CH, CPC and T respectively) but little is known concerning their ability to penetrate biofilms or their ability to kill S. sanguis under the conditions existing within biofilms of the organism. The effectiveness of the three agents in terms of minimum inhibitory concentrations (MIC) shows that CPC is the most effective having an MIC of 0.00024 % compared with 0.001 % for triclosan and 0.0078 % for chlorhexidine (Lim, 1982). But much of this data is, of course, relevant only to planktonic bacteria. Work by Bonesvoll and Gjermo (1978) showed that quaternary ammonium compounds such as CPC are rapidly cleared from the oral cavity, the model could be mimicking this action and cause the CPC to act as in vivo. If the MIC data were used as a guide we would expect triclosan to be more effective as an antibacterial agent than chlorhexidine when used at the same concentrations. Both T and CH achieved greater kills in the biofilms grown on enamel, at least after short-term (1 min.) exposure, due possibly to the uptake of the agent by the biofilm and subsequent retention on it's surface. In a study of the effects of CH and CPC on biofilms of S. sanguis grown on PTFE (Wilson et al., 1996), similar kills after 5 mins were found although CPC appeared to be more effective than CH. Differences in the findings of the two studies may be attributable to a number of factors including the nature of the substratum, the presence of other ingredients in the mouthwashes used in the present study and the differing concentrations of the CH used.

The results of this study have demonstrated the importance of the
substratum when evaluating the effectiveness of agents suitable for preventing biofilm formation and for killing bacteria in pre-existing biofilms. This may be attributable to specific agent/substrata and bacteria/substrata interactions.

Summary:

• Nature of substratum influenced the susceptibility of biofilms to mouthwashes

• Able to rank the effectiveness of mouthwashes in terms of both anti-plaque and antimicrobial activity using the CDFF

• Results were comparable with those obtained in a clinical trial
Chapter 4 - The susceptibility to chlorhexidine of *Streptococcus sanguis* biofilms grown on bovine enamel.
4.1. Aims

Further to the investigations in Chapter 3, the aim of this part of the study was to determine the susceptibility of *Streptococcus sanguis* biofilms to pulsing with chlorhexidine, shown to be the most effective anti-plaque agent in clinical trials. This was carried out in an attempt to replicate conditions which would correspond to episodes of good oral hygiene *in vivo* (Bonesvoll *et al.*, 1974; Grossman *et al.*, 1989).

4.2. Materials and Methods

Biofilms of *Streptococcus sanguis* were grown as described in 2.4.1 using artificial saliva as the nutrient source.

Pulsing was carried out twice daily (9.00am and 5.00pm) for 1 minute with 10 ml of either 0.1 or 0.2 % (w/v) chlorhexidine digluconate (Sigma) delivered via a peristaltic pump. The pulsing was started once the biofilms had achieved a steady-state.

Live / dead staining was carried out as described in 2.8.

4.3. Results

Figure 4.1 shows a scanning electron micrograph of a *S. sanguis* biofilm. It can be seen that the depth of the biofilm ranged from approximately 210 - 300 μm. The effects of chlorhexidine pulses on the viability of *S. sanguis* biofilms are shown in Figures 4.2 and 4.3. The *S. sanguis* biofilms were pulsed with a 0.1 % solution of chlorhexidine at 120 h (Fig. 4.2). When sampled at 144 h an average reduction in total viable counts of 96.5 % was
observed. By 168 h there was an increase in the total viable count, although this was not significant, and after subsequent pulsing, growth of the biofilms continued to occur. However, the number of viable bacteria did not reach the levels seen prior to pulsing and there was still a 90.3 % reduction (compared to pre-pulsing levels) in the number of viable bacteria after 216 h.

Fig. 4.3 shows the total viable counts of S. sanguis biofilms after pulsing at 120 h with 10 ml of 0.2 % chlorhexidine. There was a similar reduction (99.1 %) in the number of viable bacteria to that achieved using 0.1 % chlorhexidine. This was followed by a significant rise (p<0.05) in numbers 24 h after the initial pulse. However, the viable count after 216 h was 87.6 % lower than that prior to pulsing.

Figs. 4.4 and 4.5 show the numbers of live and dead bacteria comprising the S. sanguis biofilms as revealed by vital staining for 0.1 and 0.2 % chlorhexidine respectively. The total number of bacteria counted before pulsing was $7 \times 10^7$ per mm$^2$ for Fig. 4.4 and $5.5 \times 10^7$ per mm$^2$ for Fig. 4.5. The results from pulsing with 0.1% chlorhexidine showed a 50 % reduction in the number of live bacteria counted after the initial pulse with chlorhexidine (120 h). The number of dead cells observed at 120 h was also reduced (31 %) compared to the pre-pulsing level. At 144 h the counts for both live and dead bacteria had remained similar and these only increased at 168 h. With direct visualisation, approximately a 1 log$_{10}$ reduction in live cells was observed as a direct response to the first pulse of 0.2 % chlorhexidine, a kill of 85.4 %. There was a corresponding increase of 52.2 % in the number of dead cells counted, which comprised 90.0 % of the total bacteria in the biofilm.
Figure 4.1. Scanning electron micrograph of *Streptococcus sanguis* biofilm
120 h, bar represents 300 μm.
A = Biofilm / air interface
B = Biofilm / substratum interface
C = Bovine enamel disc
Figure 4.2. Effect of pulsing with 0.1 % CH on the viability of *S. sanguis* biofilms. Dotted line represents the initial pulse (120 h) and pulsing was continued twice every 24 h. Error bars represent standard deviations (*n* = 4).

Figure 4.3. Effect of pulsing with 0.2 % CH on the viability of *S. sanguis* biofilms. Dotted line represents the initial pulse (120 h) and pulsing was continued twice every 24 h. Error bars represent standard deviations (*n* = 4).
Figure 4.4. Live / dead staining results following pulsing of *S. sanguis* biofilms with 0.1 % CH. Dotted line represents the initial pulse (117 h) and pulsing was continued twice every 24 h.
Figure 4.5. Live / dead staining results following pulsing of *S. sanguis* biofilms with 0.2 % CH. Dotted line represents the initial pulse (117 h) and pulsing was continued twice every 24 h.
4.4. Discussion

The scanning electron micrograph of the *Streptococcus sanguis* biofilm revealed a thick biofilm which appeared to vary in height from 210 - 300 μm. This variation, however is likely to have been an artefact resulting from the processing necessary prior to electron microscopy. The results from the previous chapter showed that chlorhexidine was the most effective of the agents tested at killing *S. sanguis* biofilms grown on bovine enamel using a 1 min. exposure time. However, these results did not indicate to what extent these biofilms were able to recover from such an exposure. In the oral cavity many other factors will affect the ability of the agent to be effective at killing plaque bacteria. For example, if bacteria are only killed and not removed from the surface of the enamel or salivary pellicle then re-colonisation of the surface may occur more quickly. Although re-colonisation in these experiments was not carried out, the results showed that the bacteria that survived the pulsing were able to re-generate and the biofilm reform.

The purpose of this part of the investigation was to determine the susceptibility of *S. sanguis* biofilms to pulsing with chlorhexidine over a period of 4 days.

The initial pulse of chlorhexidine produced reductions of approximately 1.5 and 2 log<sub>10</sub> in the total viable counts using 0.1 % and 0.2 % chlorhexidine respectively. Following the initial pulses, there was a recovery in the number of viable bacteria of biofilm despite the fact that they continued to be periodically exposed to chlorhexidine. However, the viable counts did not reach the levels that were seen prior to pulsing, the population level under these conditions represents an equilibrium achieved as a consequence of
two competing activities - bacterial growth during the twelve hour periods between antimicrobial pulsing and killing of bacteria during the short-term pulsing.

After 216 h there was no significant difference in the viability of the S. sanguis biofilms when pulsed with different concentrations of chlorhexidine. These results were corroborated with live-dead staining, where the viability of the biofilms was assessed in terms of the numbers of both live and dead bacteria. This surprising finding may be attributable to the dynamic nature of the experimental system used. Certainly, in a static system, it has been shown that the effect of chlorhexidine on S. sanguis biofilms is dose-related (Millward and Wilson, 1989). It is important to note that the 1 minute chlorhexidine pulses were followed by a continuous flow of artificial saliva (mimicking the in vivo situation) which would exert a flushing action so reducing the contact time of the chlorhexidine with the biofilm as well as the time available for biofilm penetration. Therefore, it may be that the similar kills attained with both chlorhexidine concentrations were because in each case only the superficial layers of the biofilm were affected. Interestingly, the absence of a concentration-dependent effect has also been observed in a clinical study with chlorhexidine, there being no significant differences in mean plaque scores following the use of 0.1 % and 0.2 % chlorhexidine (Addy et al., 1989).

The live / dead staining counts indicated similar trends to the viable count readings (in terms of live bacteria). However, higher numbers of live bacteria were counted using the live / dead staining technique compared to viable counting. This may be attributed to the ability of bacteria to take up the stain
i.e. Its cell membrane integrity (on which the staining relies to give a green
colour indicating viability), compared to the cells ability to replicate, on which
viable counting relies. Although vortexing takes place for 1 min. to disrupt the
biofilm, bacterial clumping may also affect the viable counts. A clump of
bacteria may be distinguished into individual cells using the direct
visualisation, however, a clump of cells would only appear as one colony
forming unit on an agar plate. Bacteria recalcitrant to growth may have an
adverse effect on viable counts and account for the variations observed
between methods. Other studies have indicated that previously viable
bacteria introduced to a model system can become unculturatable, although
this has not been indicated with single species biofilms (Leser et al., 1995).

The results from the live / dead staining would seem to indicate that although
a large number of bacteria comprising the biofilm were killed (not
metabolically active), these bacteria were still present in the biofilm and thus
constituted part of its mass. The flow rate of the antimicrobial into the CDFF
should have been sufficient to disrupt loosely adhered bacteria (although
difficult to corroborate) and therefore any bacteria detected in the live / dead
staining procedure would constitute part of the biofilm and not be 'free
floating' within the space between the biofilm and the top of the recess.

The results of this study have therefore demonstrated both the inability of
chlorhexidine to kill biofilms in vitro after pulsing twice daily over a period of 4
days and the lack of a concentration-dependant bactericidal effect over the
concentration range tested.

Summary:
• The use of the CDFF enabled pulsing of an agent into the model and thus mimic the use of a mouthwash in vivo
• Biofilms were able to recover from chlorhexidine pulsing even though pulsing continued twice daily
• Live / dead staining showed higher numbers of live bacteria compared to viable counts and indicated no loss of bacterial numbers after pulsing
Chapter 5 - Growth and susceptibility of a 6 membered biofilm community to chlorhexidine.
5.1. Aims

The purpose of this investigation was to generate multi-species biofilms similar to those that would exist in the oral cavity and to test their susceptibility to antibacterial agents. Consequently, we have used the CDFF to produce biofilms comprising six species of oral bacteria frequently encountered in supragingival dental plaque. The device was used to grow biofilms on bovine enamel (a substratum similar to human enamel) using an artificial saliva as the sole source of nutrients.

5.2. Materials and Methods

The majority of procedures carried out in the experiments described in this chapter are described in detail in Chapter 2; inoculation (2.4.2.) live / dead staining (2.8.1) and cryosectioning (2.9). The inoculation procedure was changed for the final experiment, where Streptococcus sanguis was inoculated over 24 h as described in 2.4.1., before the remaining 5 species were inoculated as described in 2.4.2.

At appropriate times, pans were removed and the susceptibility of the biofilms to 0.2 % (w/v) CH determined as follows. Each pan, containing five biofilms, was placed in a sterile container and 5.0 ml of antimicrobial-containing solution (or PBS as a control) was added to cover the biofilms. The solutions were pre-warmed to 37°C and added carefully down the side of the container so as not to disturb the biofilms. After incubation for either 1, 5 or 60 mins., the solution was removed and each PTFE plug and its associated biofilm was transferred to 10 ml of neutralising broth (Difco) to inactivate any antiseptic present and vortex-mixed for 60 s. Serial dilutions were then prepared and the number of
surviving organisms determined by viable counting as described in 2.6.

A 1 min exposure time to the CH was chosen as this was considered to be representative of the maximum length of time that an antimicrobial-containing mouthwash would be retained in the oral cavity during rinsing. Longer exposure times were also tested to give some indication of the likely effect of any antimicrobial retained in the oral cavity following the use of the mouthwash.

5.3. Results

Growth of the multi-species biofilms in the CDFF is shown in Fig. 5.1, which is based on data from a typical run. The total viable count of the biofilms reached a maximum after approximately 24 h and the viable cell density at this point was $7 \times 10^8$ cfu/mm$^2$.

Table 5.1. shows the total viable count and the proportions of each species in the biofilms after 216 h in three separate runs. The total viable counts range from $2.21 \times 10^7$ to $1.73 \times 10^8$. From the table it can be seen that streptococci constituted the predominant organisms in the biofilms with the three streptococcal species accounting for 49.6 % (mean of three runs) of the viable organisms in the biofilms. *N. subflava* was the next most prevalent organism, while *A. naeslundii* and *V. dispar* generally comprised smaller proportions of the total viable count.

The distribution of the various bacterial species throughout the biofilm as revealed by viable counting of 30μm sections through the biofilm is shown in Fig. 5.2, which presents the results of a representative biofilm. From this it can be seen that the proportion of the obligate aerobe *N. subflava* in each of the
sections decreased, in a fairly regular manner, with biofilm depth. In the section from the biofilm/air interface (i.e. a biofilm height of 270-300µm) this organism comprised 97.5 % of the total viable count whereas at the base of the film it accounted for only 0.002 % of the viable count. Only in the three uppermost sections (i.e. a biofilm height of 210-300µm) of the biofilm did it account for more than 50 % of the total viable count. In contrast, no general trend could be discerned for the obligate anaerobe V. dispar which comprised only a small proportion of the viable organisms in each section of the biofilm and was distributed fairly uniformly throughout the biofilm. There was no trend towards increasing proportions of the organism in the deeper layers of the biofilm. The proportion of V. dispar in this particular biofilm was lower than those generally found in other biofilms but the distribution of the organism within the other biofilms examined followed a similar pattern to that described. The distribution of A. naeslundii throughout the biofilm was similar to that found for V. dispar. Two of the three streptococcal species (S. sanguis and S. mutans) collectively comprised the dominant organisms in each section apart from in the uppermost 60 µm of the biofilm where N. subflava predominated. S. oralis generally comprised a lower proportion of the biofilms than the other streptococci.

Transmission electron micrographs of sections through the biofilms revealed high proportions of Gram-negative cocci (presumably Neisseria spp.) at the biofilm/air surface (Fig. 5.3.) while Gram-positive cocci were distributed throughout. ‘Ghost’ cells could be observed at the base of the biofilm (Fig. 5.4) together with a predominance of gram-positive cocci.

The Bac-Light vital staining kit was assessed for its ability to distinguish
between live and dead cells of all six species used to form the biofilms. 24h cultures of each organism displayed a predominance of green (i.e. live) cells while cultures boiled for 10 mins all contained red (i.e. dead) cells. The results of vital staining of the biofilm are shown in Fig. 5.5. from which it can be seen that the uppermost 240 μm of the biofilm consisted mainly of live bacteria while the two innermost 30 μm sections comprised mainly dead cells.

The effect of exposing the biofilms to 0.2 % CH for various periods of time on the viability of the various species comprising biofilms with different compositions is shown in Figs. 5.6a and b. Fig. 5.6a shows that exposure of these biofilms to the CH for 1 or 5 minutes had no statistically significant effect on the viability of any of the six species in the biofilms. However, a 60 min. exposure resulted in significant kills of all of the organisms. *S. sanguis* was the most susceptible, with approximately a 5 log₁₀ reduction whereas *V. dispar* was the least susceptible with a 2 log₁₀ reduction. Both *S. oralis* and *A. naeslundii* displayed susceptibilities similar to that of *S. sanguis*. Whereas *S. mutans* and *N. subflava* had susceptibilities intermediate between those of *S. sanguis* and *V. dispar*. In the case of the biofilms with lower proportions of *A. naeslundii*, *S. sanguis* and *S. oralis* (Fig. 5.6b), exposure to 0.2 % CH for 1 min again had no significant effect on the viability of any of the organisms. However, a 5 min exposure did achieve significant, though small, kills of *N. subflava*, *S. sanguis* and *S. oralis* - reflecting the greater susceptibilities of these organisms as found in the biofilms described above. However, substantial kills (> 2 log₁₀ reductions) of all species were achieved only after exposure for 60 mins. As for the other biofilms described above, *V. dispar* proved to be the least susceptible with a 2 log₁₀ reduction in viable count after
a 60 min exposure. The streptococci were particularly susceptible to CH although, once again, *S. mutans* was the least susceptible of the three streptococcal species.

Figure 5.7 shows the results of inoculating the fermentor with *S. sanguis* for 24 h prior to inoculation with the remaining 5 species. The growth of *S. sanguis* over 24 h was $1 \log_{10}$ lower than when all 6 species were inoculated together but the counts continued to rise until 96 h. The composition of the biofilm varied through the course of the run but was dominated by the streptococci and *N. subflava*. Both the number of viable bacteria and the proportions of these bacteria comprising the biofilm changed dramatically with duplicate runs. For example, the proportion of *N. subflava* varied from 14 to 63 %.
Figure 5.1. Growth of individual species comprising a multi-species biofilm formed on bovine enamel discs with artificial saliva as the sole nutrient source. Error bars represent standard deviations (n = 4).
<table>
<thead>
<tr>
<th>Species</th>
<th>Run.1 Cfu per mm²</th>
<th>s.d. Cfu per mm²</th>
<th>% of total cfu</th>
<th>Run.2 Cfu per mm²</th>
<th>s.d. Cfu per mm²</th>
<th>% of total cfu</th>
<th>Run.3 Cfu per mm²</th>
<th>s.d. Cfu per mm²</th>
<th>% of total cfu</th>
<th>mean %</th>
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</thead>
<tbody>
<tr>
<td><em>N. subflava</em></td>
<td>3.4x10⁷</td>
<td>1.2x10⁷</td>
<td>25.7</td>
<td>7.8x10⁷</td>
<td>5.9x10⁷</td>
<td>45.3</td>
<td>3.4x10⁷</td>
<td>1.2x10⁷</td>
<td>16.4</td>
<td>29.1</td>
</tr>
<tr>
<td><em>A. naeslundii</em></td>
<td>7.1x10⁶</td>
<td>1.1x10⁶</td>
<td>5.4</td>
<td>4.1x10⁶</td>
<td>1.5x10⁶</td>
<td>2.4</td>
<td>7.1x10⁷</td>
<td>1.1x10⁷</td>
<td>34.2</td>
<td>14</td>
</tr>
<tr>
<td><em>V. dispar</em></td>
<td>3.5x10⁶</td>
<td>1.5x10⁶</td>
<td>2.6</td>
<td>3.1x10⁷</td>
<td>9.2x10⁶</td>
<td>18</td>
<td>3.5x10⁶</td>
<td>1.5x10⁶</td>
<td>1.7</td>
<td>7.4</td>
</tr>
<tr>
<td><em>S. sanguis</em></td>
<td>4x10⁷</td>
<td>1x10⁷</td>
<td>30.3</td>
<td>2x10⁷</td>
<td>5.2x10⁶</td>
<td>11.6</td>
<td>4x10⁷</td>
<td>1x10⁷</td>
<td>19.3</td>
<td>20.4</td>
</tr>
<tr>
<td><em>S. mutans</em></td>
<td>4.8x10⁷</td>
<td>1.5x10⁷</td>
<td>36</td>
<td>1.8x10⁷</td>
<td>6.1x10⁶</td>
<td>10.4</td>
<td>4.8x10⁷</td>
<td>1.5x10⁷</td>
<td>23</td>
<td>23.1</td>
</tr>
<tr>
<td><em>S. oralis</em></td>
<td>1.1x10⁸</td>
<td>3.7x10⁴</td>
<td>0.1</td>
<td>2.2x10⁷</td>
<td>9.3x10⁶</td>
<td>12.6</td>
<td>1.1x10⁷</td>
<td>3.7x10⁶</td>
<td>5.5</td>
<td>6.1</td>
</tr>
</tbody>
</table>

Table 5.1. Mean total viable counts and percentage proportions of each species comprising 216 h biofilms formed during three separate runs. n = 4.
Figure 5.2: Viable counts of constituent bacteria in 30 μm sections of the 300 μm thick biofilms.
Figure 5.3. Transmission electron micrograph of a transverse section of biofilm (216 h) at the biofilm/air interface. Bar represents 2 μm.
Figure 5.4. Transmission electron micrograph of a transverse section of biofilm (216 h) at the biofilm/substratum interface. Bar represents 2 μm.
Figure 5.5. The relative proportions of live and dead bacteria in 30 μm sections of a 216 h old, 300 μm thick biofilm.
Figure 5.6.a and b. Response of individual species comprising multi-species biofilms to various periods of exposure to 0.2 % CH. Error bars represent standard deviations (n = 4).
Figure 5.7. Growth of 6 membered biofilm community inoculated with *S. sanguis* for 24 h prior to inoculation with the remaining 5 species. *N* = 4 Error bars represent standard deviations.
5.4. Discussion

Effective laboratory evaluation of antimicrobial agents for the control of supragingival plaque requires the use of a model which mimics the situation in the oral cavity. This could be achieved by growing multi-species biofilms comprised of those organisms most commonly encountered in supragingival plaque. The purpose of this part of the study was to determine whether the CDFF could reproducibly produce such biofilms and to determine their susceptibility to chlorhexidine.

Substantial biofilms (300 μm deep) were formed within 48 h of inoculation of the CDFF with the six oral organisms and the predominant organisms in these biofilms were found to be streptococci with lower proportions of *N. subflava* (although still substantial), *A. naeslundii* and *V. dispar*. Similar findings regarding the composition of supragingival plaque have been reported from a number of clinical studies (Theilade *et al.*, 1978; 1982; Bowden *et al.*, 1975). The biofilms, therefore, were similar in composition to supragingival dental plaques found *in vivo* bearing in mind the enormous variation in composition encountered from site to site within an individual and between the same sites in different individuals. Despite the fact that the inoculum in each case was derived from the same source, repeat runs resulted in biofilms with different compositions. Hence, the proportions of *N. subflava*, for example, varied from 16.4 % to 45.3 %. Such variations are most likely to be the result of unavoidable slight differences between runs in terms of the relative proportions of bacteria in the inoculum, composition of the artificial saliva, salivary flow rate etc. Such between-run variations are not surprising and have been reported in other attempts to model mixed communities of oral bacteria.
attempts to model mixed communities of oral bacteria. For example, Donoghue and Perrons (1988) grew multi-species biofilms consisting of *A. viscosus*, *S. oralis* and *V. dispar* on teeth for 66 h in an artificial mouth and reported that the proportions of these organisms in different runs ranged from 0.03-88.5 %, 7.9-97.2 % and 0-24.2 % respectively. Kinniment *et al* (1996) used the effluent from a chemostat culture of a nine-membered oral community to inoculate a CDFF. In different runs, the proportions of the various species at steady state ranged from 24-65.6 % for *N. subflava*, 4.6-21.64 % for *S. gordonii*, 17.91-52.28 % for *P. gingivalis* and 3.29-25.45 % for *F. nucleatum* (Fig. 5.8)

![Graph showing the composition of different species in a biofilm.](image)

**Figure 5.8.** Composition of nine-membered community biofilms - from Kinniment *et al* (1996). Bars represent means and error bars represent standard deviations.

Vital staining of biofilm sections revealed a high proportion of dead cells at the base of the biofilm and the total viable count was also much lower at the biofilm
such biofilms revealed that the basal layers contained high proportions of 'ghosts' implying a preponderance of dead, or compromised, cells. Such observations have been described in in vivo dental plaque, whereby a layer of ghost cells is present at the plaque / enamel interface (Schroeder, 1970). The distribution of the various species within the biofilms, however, revealed some surprises. It might have been predicted that the only anaerobic species in the community, V. dispar, would have been found mainly in the basal layers where anaerobic conditions would be likely to prevail. However, while this organism was found at the base of the biofilms, it was also abundant in the upper layers i.e. at the biofilm/air interface. As was expected, the only obligate aerobe, N. subflava, also predominated in these layers and comprised 97.5 % and 90.49 % of the viable count in the two uppermost layers. The organism comprised decreasing proportions of the total viable count in the deeper layers such that in the basal layer it comprised only 0.002 % of the total viable count. This is most likely to be related to the establishment of a decreasing oxygen gradient through these deep biofilms due to diffusion limitations coupled with oxygen utilisation in the upper layers (Wimpenny, 1982). Using a fluorescent antibody staining method, Ritz (1967) also found that Neisseria spp. predominated in the surface layers while streptococci were distributed throughout the plaques. However, in contrast to the findings in this study, they found that Veillonella spp. predominated in the lower two thirds of the plaques. It may be that the active consumption of oxygen by N. subflava provided suitable atmospheric conditions and redox potentials to enable survival of the V. dispar in the upper layers of the biofilms. The protection afforded by N. subflava to oral anaerobes in biofilms has been demonstrated previously by Bradshaw et al (1994).
Survival of *V. dispar* is also very much dependent on the metabolic activities of other species in the biofilm as it cannot utilise sugars as a carbon source and is dependent on lactate produced as a metabolic end-product of other bacteria such as streptococci (Mikx and van der Hoeven, 1975). The primary source of carbon in the artificial saliva is in the form of glycoproteins which must be degraded by the bacterial consortium and metabolised in order for the *V. dispar* to survive in the community (Bradshaw *et al.*, 1994; Bradshaw *et al.*, 1996). The numbers of streptococci present may therefore determine the distribution of *V. dispar* throughout the biofilm and this may also explain why the organism was undetectable until 48 h.

The well-known refractory nature of biofilms to antimicrobial agents was evident in this study where exposure of the biofilms to chlorhexidine concentrations as high as 0.2 % (the maximum concentration used in mouthwashes) for 1 minute (and in some cases for 5 minutes) had no significant effect on bacterial viability. Substantial reductions in bacterial viability resulted only from a 60 min exposure to the antimicrobial, illustrating that the effectiveness of an agent for use in the control of plaque-related diseases will depend very much on its substantivity i.e. its ability to be retained in the oral cavity following the application of a mouthwash, toothpaste etc. (Cummins and Creeth, 1992). It is of interest to note the relative susceptibilities to chlorhexidine of the constituent organisms of the biofilms. In general terms, the streptococci and *N. subflava* displayed the greatest susceptibilities to chlorhexidine, *V. dispar* was least susceptible while *A. naeslundii* displayed an intermediate susceptibility.

Emilson (1977) carried out a series of MIC tests involving chlorhexidine on
various oral bacteria. The findings showed there was a broad range of susceptibility to chlorhexidine among both Gram-positive and Gram-negative strains. Low MIC values were noted for S. mutans while strains of S. sanguis showed intermediate susceptibility with both low and high MIC values. The least susceptible strains were the Veillonella species. Wade and Addy (1989) have also observed that some strains of S. sanguis were relatively resistant to a mouthwash containing 0.2 % chlorhexidine.

There are few reports concerning the antimicrobial susceptibility of oral bacterial biofilms with which the results of this study can be compared. Nevertheless, Kinniment et al (1996) also found that V. dispar was the least susceptible to chlorhexidine pulsing when present in a CDFF-grown biofilm comprising nine oral bacterial species and that N. subflava was one of only two members of the community to be affected by the lowest concentration used.

When testing new antimicrobial agents it is important to know not only whether the agent is able to kill the biofilm, but also to what extent the proportions of the species comprising the biofilm have changed. Dental plaque is a complex community of interacting organisms and therefore inhibiting the growth of one or more species may have further implications. For example, if Veillonella species are not present then lactic acid (implicated in caries) may not be metabolised into propionic and acetic acids which are associated with fewer carious infections (Mikx and van der Hoeven, 1975). The ability, therefore, to produce biofilms that are consistent both in terms of viable counts and the proportions of species present is crucial. In order to compare the effectiveness of antimicrobial agents against
biofilms the composition of the biofilm obviously needs to be reproducible.

This investigation has shown that 6-membered biofilm communities grown in the CDFF had a similar susceptibility to *Streptococcus sanguis* biofilms grown under the same conditions (Chap. 4.3) and that these mixed-species biofilms had a composition similar (albeit variable) to supragingival plaque observed *in vivo* (Theilade *et al.*, 1982). However, the inability to produce biofilms that had a consistent population or proportions of species represents a major drawback. Using a 24 h biofilm of *S. sanguis* as a primary coloniser for further attachment did not improve the reproducibility of the community. Thus, due to the lack of reproducibility of the 6 membered community, an alternative approach needed to be investigated to provide a reproducible and stable community. Consequently saliva was used as an inoculum, the rational being that a more varied range of organisms would give rise to a community which, because of the complexity of interactions between the constituent species, would display greater reproducibility. The homeostatic mechanisms operating within such a community would be more likely to compensate for small variations in conditions likely to be operating in different experimental runs.

**Summary:**

- Unable to grow a reproducible 6 membered biofilm community
- Able to section the biofilm to determine proportions of species at various depths
- High proportion of dead and 'ghost' cells at the base of the biofilm
- High numbers of *Neisseria subflava* at the biofilm / air interface
• Chlorhexidine had no significant effect on the biofilms after 1 min. exposure
Chapter 6 - Growth of a microcosm biofilm and its susceptibility to chlorhexidine pulsing.
6.1. Aims

After failing to grow reproducible mixed-species biofilms in the CDFF, human saliva was used to inoculate the fermentor. The aims of these investigations were to; (i) generate reproducible biofilms consisting of a mixed oral bacterial population; (ii) test the susceptibility of these biofilms to pulsing with chlorhexidine; (iii) study the effect of pre-treating the substratum with the same agent prior to pulsing and (iv) investigate the structure of microcosm plaques in relation to their susceptibility to chlorhexidine.

6.2. Materials and Methods

6.2.1. Inoculation and sampling

The microcosm plaque biofilms were grown on bovine enamel discs which had been pre-treated with sterile artificial saliva for 1 min. to form a conditioning film. The inoculum used was 1 ml of pooled human saliva which was passed through the system for 8 h. Pans were removed for sampling at appropriate time intervals (usually 24 h) and discs removed for analysis as described in 2.4.3.

6.2.2. Chlorhexidine pulsing of biofilms

Pulsing was carried out twice daily (9.00am and 5.00pm) for 1 minute with 10 mls of 0.2 % (w/v) chlorhexidine digluconate (Sigma) delivered via a peristaltic pump. The pulsing was started once the biofilms had achieved a steady-state. However, when the discs were pre-treated (for 1 min.) with chlorhexidine prior to inoculation, pulsing took place 8 h after inoculation.

6.2.3. Chlorhexidine penetration through the biofilm
Chlorhexidine has a characteristic sharp absorption band with a $\lambda_{\text{max}}$ at 298nm. This was used to monitor its penetration through the biofilm following the cryosectioning of the latter. The microcosm plaques were exposed to CH for 1 min. and then sectioned as described in 2.9.

The absorbance of each of the sections (suspended in 0.1 ml dH$_2$O) at both 298 nm (absorbance of CH) and 288 nm (absorbance of cryo-m-bed) was then determined.

6.2.4. Susceptibility of varying thicknesses of microcosm plaque

The bovine discs on which the biofilms formed were recessed to depths of 100 µm or 300 µm. Microcosm plaques were grown for 96 h as described in 2.4.3. and susceptibility determinations were carried out as described in 3.2.2.

6.2.5. Scanning confocal laser microscopy

The bovine enamel discs containing the biofilms were placed in a well (constructed of RS plastic sheet) on a microscope slide as shown in Figure 6.1. For susceptibility testing, the well was filled with 1 ml of 0.2 % CH, or PBS in the case of the control, for either 1, 5 or 60 mins. This was then removed and 1 ml of Vectashield (Vector Labs. Inc. Burlingame, CA, USA), a mounting medium for fluorescence, containing 5 µl of live / dead stain (Molecular Probes) was added. Samples were examined at a wavelength of 488 nm using an inverted CLSM (Biorad Lasersharp MRC 500) with a long working-distance x 40 lens.
Figure 6.1 Section and plan schematic diagrams of sample holder for confocal laser scanning microscopy.
6.3. Results

6.3.1. Growth of microcosm plaques
The microcosm plaques produced total viable counts in the region of $5 \times 10^8$ cfu per mm$^2$. When these had reached a steady-state they consisted of 19 % *Actinomyces* spp., 25 % streptococci and 7 % *Veillonella* spp. (Table 6.1). The proportions of these species, expressed as a percentage of the total anaerobic count, are also given for the pooled saliva used as an inoculum (Fig. 6.2). The streptococci accounted for over 85 % of the enumerated species in the pooled saliva, which was considerably greater than that observed in the steady-state microcosm plaques.

Figures 6.3.a – e show the total counts from 5 pans for each of the species detected when sampled at 120 h. Neither the viable counts between the discs from the same pan or between 5 pans sampled from the same run were significantly different ($p<0.001$). All the viable counts for the species detected were between $2 \times 10^8$ and $9 \times 10^8$ cfu per mm$^2$ except for the *Veillonella* spp. which produced viable counts of approximately $5 \times 10^6$ cfu per mm$^2$. These differences between viable counts for the discs and pans were only studied in detail at 120 h. The reproducibility between runs, however, is shown in Figure 6.4. Each line represents the total anaerobic counts for 1 run. Over 168 h, sampling at 24 h intervals, there was no significant difference ($p<0.001$) at any of the time points for the 3 runs.

6.3.2. Susceptibility of microcosm plaques
When CH-pulsing was carried out on microcosm plaques, approximately 1 log$_{10}$ reductions were observed in the total viable counts (aerobic count...
p<0.05, anaerobic count p<0.001) after the first treatment with 0.2 % CH (Fig. 6.5). After this first pulse, the total aerobic count rapidly recovered and by 240 h the total viable count was not significantly different (p<0.05) from that prior to pulsing. The counts then remained at similar levels throughout the course of the experiment. With respect to the total anaerobic count, the number of viable bacteria did reach levels seen prior to pulsing after 336 h. The Actinomyces spp. and Veillonella spp. viable counts decreased significantly (p<0.001) after the initial pulse. Between 240 and 288 h the numbers did not significantly change but after this period there was an increase until at 336 h they had recovered to pre-pulsing levels. The proportions of these species, compared with the total anaerobic count, at 336 h had increased considerably, collectively making up over 90 % of the community. The streptococci seemed the most susceptible to CH in the microcosm community and, although the number of viable bacteria recovered to some extent following initial pulsing, this growth was not sustained. At the final sampling point the proportion of streptococci (as a percentage of the total anaerobic count) was 30 % less than that seen prior to pulsing.

In the case of the discs treated prior to inoculation and then subsequently pulsed (Fig. 6.6), after 8h the biofilms had total viable counts of $2.6 \times 10^5$ cfu per mm$^2$. This constituted approximately a $1 \log_{10}$ reduction in the number of colonising bacteria compared to untreated discs. After the initial pulse, a $3 \log_{10}$ reduction (99.8 % kill) was seen in the aerobic viable count, anaerobic viable count and streptococci (p<0.001). The Veillonella spp. and Actinomyces spp. were undetectable but numbers did recover later in the
run. However, the *Veillonella* spp. were again undetectable after 80 h. After sampling at 56 h, the total aerobic, total anaerobic and streptococci counts had all significantly (p<0.001) recovered. The total aerobic count continued to increase reaching levels similar to those prior to pulsing but still significantly lower than those found on untreated discs.

The susceptibility of biofilms of different thickness was investigated (Figs. 6.7a and 6.7b) using both CH and CPC for 1 and 5 mins. Approximately 1 log<sub>10</sub> reductions were seen in the 100 μm thick biofilms using either CPC or CH for 1 and 5 mins. The species comprising the microcosm plaques seemed equally susceptible to CPC, with the exception of the *Actinomyces* species which displayed greater susceptibility to CH than to CPC after a 5 min. exposure. Similar results were seen for the 300 μm thick biofilms. The viable counts of *Actinomyces* spp., *Streptococcus* spp. and *Veillonella* spp., as well as the total anaerobic count, were all reduced by approximately 1 log<sub>10</sub> unit when biofilms were exposed to either CH or CPC for 1 or 5 mins.

The penetration of chlorhexidine through the microcosm plaques was investigated by sectioning the biofilm and taking absorbance readings of each of the 10 sections. The mixed bacterial population and dextran (cryo preservative) showed low absorbance at 298 nm and 288 nm, the $\lambda_{\text{max}}$ of CH and cryo-m-bed respectively (Fig. 6.8a). The microcosm plaques were exposed to CH for 1 min. When these plaques were sectioned and their absorbance determined (Fig. 6.8b) it was shown that the highest absorbance at 298 nm was at the base of the biofilm (0-30 μm) and the absorbance readings then decreased steadily towards the biofilm / air interface.

Viable counts of sections of the microcosm plaques revealed counts from
each 30μm section of approximately $5 \times 10^7$ cfu per section (Fig. 6.9). However, from 60 – 180 μm the viable counts were approximately $1 \times 10^7$ cfu per section. The sections near the biofilm / air interface had high total aerobic counts, which would, of course, comprise both facultative anaerobes and obligate aerobes. Throughout the rest of the biofilm the *Actinomyces* spp. and *Streptococcus* spp. tended to be the numerically dominant species. After being exposed to 0.2 % CH for 1 min. the total viable counts of each section were reduced by approximately 1 log_{10} (Fig. 6.10). The relative proportion of species present, however, remained similar to those seen prior to chlorhexidine exposure. At the biofilm / air interface the highest numbers were seen for the total aerobic count, while at the base of the biofilm the *Streptococcus* spp. and *Actinomyces* spp. were dominant. Unlike in the untreated microcosm plaque there was a predominance of *Veillonella* spp. between 120 – 210 μm.

6.3.3. Structure

The structure of the microcosm plaques was investigated using two microscopic techniques, confocal scanning laser microscopy (CSLM) and transmission electron microscopy (TEM). However, these observations can also be compared to the cryosectioning results described in 6.3.2. The CSLM revealed that the control biofilms contained predominantly live bacteria, shown by green fluorescence with the live/dead stain, with some dead bacteria towards the base of the biofilm (Fig. 6.11a). In contrast, when the biofilms had been exposed to CH for 1 h most of the organisms in the biofilm appeared dead apart from a small area towards the base of the
biofilm in which live bacteria were apparently present (Fig. 6.11b).

Fig. 6.12 is a TEM of a vertical section through the biofilm. This shows that cocci predominated at the biofilm / air interface and the presence of 'ghost' cells at the biofilm / substratum interface. Some regions of the biofilm were dominated by a particular morphotype while others displayed greater heterogeneity.
<table>
<thead>
<tr>
<th>Organism</th>
<th>Approximal dental plaque</th>
<th>Microcosm plaques</th>
<th>Pooled human saliva</th>
</tr>
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<tr>
<td></td>
<td>mean</td>
<td>range</td>
<td>Mean of 4 runs</td>
</tr>
<tr>
<td>Actinomyces spp.</td>
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<td>4-47</td>
<td>19</td>
</tr>
<tr>
<td>Streptococci</td>
<td>24</td>
<td>4-61</td>
<td>25</td>
</tr>
<tr>
<td>Veillonella spp.</td>
<td>11</td>
<td>&lt;1-30</td>
<td>7</td>
</tr>
</tbody>
</table>

Table 6.1 Comparison of the bacterial composition of approximal dental plaque (data from Newman and Nisengard, 1988), microcosm plaques and the pooled human saliva used as the inoculum. Figures represent the proportion of each type of organism expressed as a percentage of the total anaerobic viable count.

Figure 6.2. Proportions (expressed as % of total anaerobic count) of species comprising pooled saliva from 5 separate inocula.
Figure 6.3a. The total aerobic viable counts (120 h) from 5 pans of the same run. 4 samples (discs) were taken from each pan. Error bars represent standard deviations.

Figure 6.3b. The total anaerobic viable counts (120 h) from 5 pans.
Figure 6.3c. The *Actinomyces* spp. viable counts (120 h) from 5 pans.

Figure 6.3d. The *Streptococcus* spp. viable counts (120 h) from 5 pans.
Figure 6.3e. The *Veillonella* spp. viable counts (120 h) from 5 pans.

Figure 6.4. Graph showing the anaerobic counts from three separate runs over 192 h. Bars represent standard deviations.
Figure 6.5. Effect of pulsing 0.2 % CH on the viability of microcosm plaques. Dotted line at 168 h represents the initial pulse and pulsing continued twice daily from 168 – 264 h. n = 4. Error bars represent standard deviations.
Figure 6.6. Growth of a microcosm plaque community on bovine enamel discs treated with 0.2 % CH and then pulsed with CH after 8 h. Dotted line represents initial pulse, pulsing continued twice daily to 80 h. n = 4. Error bars represent standard deviations.
Figure 6.7a Viable counts of 100 μm thick microcosm plaques before and after exposure to CPC and CH for 1 and 5 mins. \( n = 4 \). Error bars represent standard deviations.
Figure 6.7b: Viable counts of 300 μm thick microcosm plaques before and after exposure to CPC and CH for 1 and 5 mins. n = 4. Error bars represent standard deviations.
Figure 6.8a. Absorbance readings of constituents used in cryosectioning microcosm plaque biofilms over a range of wavelengths.
Figure 6.8b. Absorbance readings taken from sections through a microcosm plaque community.
Figure 6.9. Viable counts from 30 μm thick sections through a 120 h microcosm plaque. Bars represent means. Values of 4 replicates.
Figure 6.10. Viable counts from 30 μm thick sections through a 120 h microcosm plaque which has been exposed to 0.2 % CH for 1 min. Bars represent means. Values of 4 replicates.
**Figure 6.11a.** CLSM image of a microcosm plaque on bovine enamel viewed after live/dead staining. Step 10.44 μm, 27 images, thickness 281 μm (estimated not calibrated).

**Figure 6.11b.** CLSM image of a microcosm plaque on bovine enamel exposed to 0.2 % CH for 1 h, viewed after live/dead staining. Step 10.44 μm, 26 images, thickness 260 μm (estimated not calibrated).
Figure 6.12. Transmission electron micrograph of a vertical section through a microcosm plaque (120 h) grown in the CDFF. Bars represent 20 µm.
6.4. Discussion

The aims of this study were to generate reproducible biofilms comprised of a mixed oral bacterial population and to determine the effects of chlorhexidine pulsing on the viability of these microcosm plaques.

Due to their increased complexity, the use of microcosm dental plaques mimics the in vivo situation more closely than when single species biofilms are used (Chap. 4). Furthermore, the proportions of species detected in the microcosm community were similar to those observed in supra-gingival plaque (Newman and Nisengard, 1988). Although over 80% of the saliva inoculum consisted of streptococci, these organisms comprised only 25% of the microcosm plaques, suggesting that colonisation of the enamel and subsequent growth of the biofilm community was similar to that which occurs in the oral cavity.

The reproducibility of the microcosm plaques in terms of both growth and relative proportions of species present was very important. It had been shown in the previous chapter that the reproducibility of the 6-member multispecies biofilm was poor. However, the microcosm plaques showed good reproducibility between runs and the proportions of species detected comprising the biofilm remained similar, although a more detailed analysis (e.g. to the species level) may well have revealed differences. Because of the large number of species present, however, such an undertaking was not possible because of time constraints.

The initial pulse of chlorhexidine produced reductions of approximately 1.5 \( \log_{10} \) in the total viable counts of the microcosm plaques. Following the initial pulses, there was a recovery in the number of viable bacteria in the biofilms.
despite the fact that they continued to be periodically exposed to chlorhexidine. Streptococci appeared to be the most susceptible to chlorhexidine of those organisms comprising the microcosm plaques grown on untreated discs. The results were comparable with those obtained with the single species biofilms in that the number of viable streptococci also recovered during subsequent pulsing, but did not reach the levels seen prior to pulsing. It appears therefore that, although they were present in a more complex microbial community, the susceptibility of these organisms was not altered. Following pulsing with chlorhexidine, the total aerobic count of the microcosm plaques soon recovered to pre-pulsing levels. In contrast, the anaerobic count took much longer to reach the same pre-pulsing levels. This may be attributable, in part, to the aerobic atmosphere within the CDFF; growth of anaerobes may have been limited until an appreciable community had developed to allow the establishment of an anaerobic environment within the biofilm.

Work of a similar nature has been carried out on a nine-membered community grown in a conventional chemostat used to inoculate the CDFF over an 8 h period. The biofilms were challenged with eight 10 min. pulses of two concentrations of chlorhexidine (0.0125 and 0.125 % w/v). The lower concentration had a limited effect on the composition of the biofilms while a differential and substantial inhibition was obtained with the higher concentration. Actinomyces naeslundii was lost from the biofilm, and the viable counts of streptococci, Fusobacterium nucleatum and Porphyromonas gingivalis were reduced by over three orders of magnitude by 0.125 % chlorhexidine, whereas Veillonella dispar was only transiently affected
(Kinniment et al., 1996). However, the relative proportions of the species present in the nine-member community (8.1 % streptococci, <0.1 % Actinomyces naeslundii and <0.01 % Veillonella dispar) were very different from those found in the microcosm plaques used in the present study. Because of the far greater species-diversity within the microcosm plaques, detailed analysis of the relative proportions of individual species was not possible in the present study. This greater complexity may account for the ability of the microcosm plaques to withstand (in terms of the total number of viable organisms) repeated chlorhexidine pulsing although it is likely that the relative proportions of the individual species would have been altered. Certainly, the proportions of streptococci and Actinomyces spp. in the biofilms were different after chlorhexidine pulsing. Prior to pulsing these genera constituted approximately 20 % and 25 % of the biofilm respectively, however, after pulsing the proportion of streptococci increased to approximately 53 % and the proportion of Actinomyces spp. increased to 48 %. Chlorhexidine, therefore, although unable to kill all the bacteria in the biofilm affected the composition and hence species that are less susceptible to the agent are able to become dominant in the biofilm after pulsing. When the enamel substrata supporting the microcosm plaques were pretreated with chlorhexidine the numbers of viable bacteria after 8 h were 1 log₁₀ lower than when untreated discs were used, with Veillonella spp. being undetectable. When these biofilms were subsequently pulsed with chlorhexidine, greater log₁₀ reductions were seen than with biofilms formed on untreated enamel, with the Actinomyces spp. falling below the detection limit. In previous studies (Pratten et al., 1997) of the colonisation of
chlorhexidine-treated enamel by *S. sanguis*, after 8 h the viable counts were 2 log₁₀ lower than those achieved with the microcosm plaques in this study. This difference may be a consequence of the availability of a greater variety of adhesion sites (due to the greater range of primary colonisers) for secondary colonisation in the multi-species biofilms thereby enabling the more rapid formation of a biofilm community.

It would be of great interest to compare the results of this study with those of clinical trials involving chlorhexidine. However, such comparisons are difficult as the mouthwashes used often contain other agents, such as fluoride, which may increase or reduce their effectiveness, especially when the test compound is used in lower concentrations (Thrower *et al.*, 1997; Addy *et al.*, 1989). Nevertheless, the results from plaque regrowth trials, where the patients' teeth are cleaned, treated and subsequently rinsed twice daily, are comparable with those obtained in the pre-treatment and pulsing experiments in this study. This method of delivery has consistently been shown *in vivo* to be the most effective way of administering chlorhexidine (Jenkins *et al.*, 1994).

Biofilm thickness may affect the transfer of essential nutrients into biofilms by increasing the diffusional length of penetration to the base of the film (Christensen and Characklis, 1990). Thinner biofilms may, therefore, have a faster growth rate due to the possible increase in nutrients from increased penetration. A number of investigations have concluded that slowly growing cells are less susceptible to the action of inimical agents (Gilbert and Brown, 1980). Hence if bacteria are growing faster in a 100 μm thick biofilm compared to a 300 μm thick biofilm they may be more susceptible to the
action of antimicrobial agents. In this study the depth of the biofilms seemed to have little effect on their susceptibility to CPC or chlorhexidine, however, the *Actinomyces* species appeared to be more susceptible to the action of chlorhexidine in 100 μm biofilms than in the thicker biofilms. The absorbance reading of chlorhexidine showed it was able to penetrate through to the base of the 300 μm biofilm. This is of great importance as it implies that the biofilm matrix had little effect on the passage of antimicrobial through it, at least with the concentration of chlorhexidine used and this particular biofilm thickness. One of the reasons often suggested for the reduced susceptibility of bacteria in biofilms to antimicrobial agents is that the copious extracellular matrix protects the bacteria by hindering penetration of the antimicrobial agent by acting as a molecular seive, by ionic interaction between the negatively charged matrix polymer and positively charged antimicrobial agents or by chemical reaction between the polymer and the agent (Wilson, 1996). However, it would appear that even when there is a strong ionic interaction between a negatively charged matrix and a positively charged antimicrobial agent, diffusion of the agent is not hindered to a great extent and once all of the binding sites have been filled the matrix would not present any further barrier for diffusion (Nichols *et al.*, 1988). For example, in a study of the diffusion of the positively charged antibiotic tobramycin through negatively charged alginic acid (which is very similar to the polysaccharide produced by *P. aeruginosa*), Nichols *et al* (1988) found that the alginate decreased the rate of diffusion of tobramycin by less than one-third. It is unlikely that this factor alone could account for the 1000-fold decrease in susceptibility of biofilm-grown *P. aeruginosa*
compared to planktonic cells of the same organism (Nichols et al., 1989). Interestingly, the results of this study, where chlorhexidine-treated biofilms were sectioned and viable counts of the constituent bacteria carried out, showed an equal susceptibility of the bacteria to the agent throughout the biofilm.

These findings could not be correlated with CLSM as observations for 1 min. exposure to chlorhexidine showed no difference compared with the untreated biofilms. In order to gain a significantly different image, the biofilms had to be exposed to chlorhexidine for 1 h. With these initial studies it was important to determine whether using the live / dead stain it was possible to visualise both green and red cells in contrasting conditions.

The ability to visualise biofilms is important in defining their architecture and the interactions occurring between the cells and between the cells and the substratum to which they are attached. A number of different microscopic techniques have been widely used for the direct visualisation of initial attachment and subsequent biofilm formation (Marshall, 1989, Keevil et al., 1987 and Geesey and White, 1990) and for the determination of phenotypic changes which occur following adhesion (Costerton et al., 1995).

Studies using bright-field and phase contrast microscopy coupled with image analysis have examined colony development, effects of nutrient concentration on attachment and the initial stages in attachment (Marshall, 1989). Phase contrast microscopy has been used to demonstrate reversible and irreversible attachment of marine bacteria to glass surfaces (Busscher et al., 1992). However, most bright-field and phase contrast microscopy is heavily reliant on the use of transparent surfaces, severely limiting their
application to the study of biofilms on opaque materials, such as enamel, except in situations where fluorescent stains such as INT-formazan and Molecular probes live / dead stain can be used (Yu and McFeters, 1994). The major advantage of electron microscopy, used in this study, is its ability to resolve objects which cannot be seen using light microscopy due to an increased level of resolution: approximately 0.5 nm compared to the 0.2 μm resolution of differential light absorption microscopes. Unlike conventional bright field and phase microscopy, Scanning Electron Microscopy (SEM) removes the requirement for a transparent surface. In transmission electron microscopy (TEM), the electrons are scattered as they pass through the specimen, then focused by magnetic lenses to form an image on a fluorescent screen. TEM has been used to produce information on biofilm thickness and on the interactions occurring at a cellular level between members of a biofilm. It enables detailed analysis of the spatial arrangements and cellular structure of cells present within the biofilm. This is true of this study where observations have been made showing dense populations of mixed-species at certain levels within the biofilm together with sparsely populated areas. Other areas appear to be colonised by single species within the matrix of the mixed-species community. Observations from in vivo plaque studies have also shown similar findings (Fig. 6.13).
The microcosm plaques contained a predominantly coccoid flora with most cells exhibiting cell wall characteristics compatible with those of Gram-positive micro-organisms. Cells with the appearance of Gram-negative bacteria were usually not observed in direct contact with the enamel surface. *Streptococcus* spp. are the obvious candidates for coci bacteria at the substratum interface, however, *Actinomyces* spp. have also been reported to assume coccoid shapes *in vivo* (Listgarten, 1976) and due to the complexity of the microcosm plaque this may also be occurring in this model.
In contrast to conventional microscopy techniques, confocal laser scanning microscopy (CLSM) utilises krypton/argon lasers, enabling penetration into in vivo biofilms. The lasers excite natural fluorescence within the biofilm or stains can be added to the sample resulting in fluorescence which can be detected by photomultiplier tubes and a digital image is obtained. Alteration of the focal (z plane) depth and the subsequent collection of the x-y plane images (parallel to the surface) enables the collection of a series of optical sections which can then be computer-processed using image analysis software to create a three-dimensional image (Gorman et al., 1993). CLSM is an effective tool for the study of a wide range of biofilm features, including physiological profiles and structural heterogeneity. It has enabled the in situ study of intact, fully hydrated biofilms; the measurement of pH and dissolved oxygen profiles (Caldwell, 1992a) using microelectrodes; an analysis of liquid flow in biofilm systems (Caldwell, 1992b) and chlorine penetration (Yu and McFeters, 1994). As such, CLSM represents a technique of major importance in the study of medical, industrial and environmental biofilms (Gorman et al., 1993). Electron microscopy requires specimen preparation involving dehydration, which may cause disruptive shrinkage, and slicing, which dictates that three-dimensional images can be obtained only by lengthy reconstructions from hundreds of serial sections. Confocal scanning laser microscopy (CLSM) forms a bridge between light and electron microscopy, affording penetrative views of specimens.

In this study of microcosm plaque the depth of the biofilm caused the greatest problems. CLSM is best suited to biofilms of a few cells in thickness and thus, biofilms of around 300 μm were too thick for accurate
measurements to be made. As the laser penetrates through the biofilm there is a certain amount of distortion, if there is too much distortion then measurements of the depth cannot be made but only estimated. However, for the purpose of this study, comparison of the results with other techniques, especially cryosectioning, has shown that using the CLSM for investigating the structure of microcosm plaque, in terms of viability, may be a useful tool.

Susceptibility testing of oral bacteria has previously relied on MIC testing. The results of one study showed that at concentrations as low as 0.025 %, chlorhexidine was able to inhibit the growth of all the bacterial species present in 89 % of plaque samples tested (Wilson et al., 1990). However, these results have demonstrated the complexity of biofilms and the inability of chlorhexidine to kill biofilms in vitro. The use of microcosm plaques obviously mimics the in vivo situation more closely than when single species are used, the interactions between bacteria of the same and different species being of great importance in the formation and maturation of dental plaque. Hence, by using reproducible microcosm plaques that have a similar composition to supra-gingival plaque, it should enable better predictions of antimicrobial and/or anti-plaque effectiveness of test compounds in vitro. One of the advantages of this model is that it enables pulsing of these agents into the system and pre-treatment of the substratum with the same or different agents. Plaque re-growth clinical trials with chlorhexidine have shown this to be the most effective way of preventing plaque formation and this was also found to be the case in this study.
Summary:

- Microcosm plaques appear more reproducible than the 6 membered biofilm community.
- Live / dead studies carried out on cryosections of microcosm plaques correlated with CLSM.
- The thickness of microcosm plaques appeared to have little effect on their susceptibility to chlorhexidine, at least over the range 100 μm to 300 μm although their composition had altered.
- The viability of microcosm plaques recovered after chlorhexidine pulsing.
- Pre-treatment and subsequent pulsing with chlorhexidine resulted in the greatest reductions in the viability of the biofilms.
Chapter 7 - Growth and susceptibility of microcosm plaques grown in the presence of sucrose.
7.1. Aims

The aim of this part of the study was to produce microcosm dental plaques grown in the presence of sucrose and to test the susceptibility of these biofilms to chlorhexidine pulsing. This was carried out in order to take account of an important dietary constituent in Western societies, sucrose. Supplementing the artificial saliva with sucrose may influence the structure and the composition of the biofilms and hence their susceptibility to antibacterial agents.

7.2. Materials and Methods

7.2.1. Growth of sucrose-supplemented biofilms

The microcosm plaques were grown on bovine enamel discs in the CDFF with nutrients supplied by artificial saliva delivered at a rate of 0.72 L per day supplemented with sucrose. 33.3 ml of a 10 % (w/v) aqueous solution of sucrose was pumped over the biofilms for 30 mins. via a second peristaltic pump. This was carried out thrice daily at 9 am, 1 pm and 5 pm, thereby equating to the total mean daily intake of sucrose by an adult in the U.K. (Burt, 1993).

7.2.2. Determination of pH

The pH of the biofilms was determined by using a pH meter (pH-boy, Camlab, U.K.). This had a flat electrode probe approximately 6 mm in diameter onto which an inverted disc containing the biofilm was placed. The probe was cleaned and re-calibrated before application of each sample. The accuracy of the instrumentation was ± 0.2 pH units.
7.3. Results

The microcosm plaques supplemented with sucrose produced total viable anaerobic counts in the region of $5 \times 10^8$ cfu per mm$^2$ (Fig. 7.1). After 120 h they consisted of approximately 12% Actinomyces spp., 85% streptococci and 0.2% Veillonella spp. (Table 7.1). The Veillonella spp. were undetectable until 24 h, however at 96 h their numbers had reached $5 \times 10^5$ cfu per mm$^2$ and continued to increase until 192 h when the counts were $6 \times 10^7$ cfu per mm$^2$.

When these microcosm plaques were pulsed with 0.2% chlorhexidine at 120 h (Fig. 7.2) there was a reduction in the total aerobic and anaerobic counts of approximately 1.3 log$_{10}$. The viable counts of the Streptococcus spp. and Veillonella spp. were reduced by less than 1 log$_{10}$. However, as the initial viable counts of the Veillonella spp. were lower, this amounted to a much smaller number killed than in the case of the streptococci. The greatest reductions in counts were seen for the Actinomyces spp. - from $4 \times 10^7$ cfu per mm$^2$ to $8 \times 10^5$ cfu per mm$^2$. When the CDFF was sampled at 192 h all the viable counts had significantly increased (except for the total aerobic count) and by 216 h all the counts had reached at least their pre-chlorhexidine pulsing levels. In fact, the viable counts of the Actinomyces spp. and Veillonella spp. post-pulsing had increased compared to those found prior to pulsing.

Figure 7.3 shows the pH results from the run shown in Fig. 7.1. These results are compared with those obtained using microcosm plaques grown in the absence of sucrose, and S. sanguis biofilms grown in the absence of
sucrose. The pH of biofilms which had been grown in the absence of sucrose remained relatively constant at around pH 6.8 (range - pH 6.1 to 7.2). However, the pH of the microcosm plaques supplemented with sucrose dropped steadily from 6.8 at 24 h to 4.2 at 192 h, the pH then increased slightly to 4.8 when sampled at 264 h.

When the sucrose-supplemented biofilms were sectioned (Fig. 7.4) the counts from each section showed considerable variation. At the biofilm / air interface the total aerobic count (i.e. including both facultative anaerobic species and aerobic species) comprised 23 % of the entire biofilm. In sections taken from depths of from 180 µm to 300 µm the viable counts were lower but the counts from the 150 µm to 180 µm sections showed that the numbers of each genus had significantly increased (p<0.05) in this region. Towards the base of the biofilm there were again generally low numbers of viable bacteria with significantly higher (p<0.05) counts in the 60 µm to 90 µm section.

When these biofilms were exposed to 0.2 % chlorhexidine for 1 min. and subsequently sectioned there was approximately a 1.5 log_{10} reduction in the total viable counts of the entire biofilm. The total aerobic count at the biofilm / air interface was greatly reduced by approximately 4 log_{10}. The total anaerobic count for each of the sections remained similar. However, the viable counts for the Veillonella spp. were reduced by between approximately 1.5 log_{10} and 2.5 log_{10} towards the base of the biofilm (0 – 120 µm). The Actinomyces spp. in the centre of the biofilm (150 – 210 µm) were also significantly reduced (p<0.05) by the action of chlorhexidine.
Figure 7.1. Growth of various groups of bacteria comprising a microcosm plaque community pulsed thrice daily with sucrose. Error bars represent standard deviations, n = 4.

<table>
<thead>
<tr>
<th>Genus</th>
<th>120 h (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Veillonella</td>
<td>0.18</td>
</tr>
<tr>
<td>Actinomyces</td>
<td>11.67</td>
</tr>
<tr>
<td>Streptococcus</td>
<td>84.7</td>
</tr>
</tbody>
</table>

Table 7.1. Percentage of genera (relative to the total anaerobic count) comprising sucrose-supplemented microcosm dental plaque (from Figure 7.1).
Figure 7.2. Viable counts of sucrose-pulsed microcosm plaques additionally pulsed twice daily with 0.2 % CH at 120 h. Dotted line at 120 h represents the initial pulse and pulsing continued twice daily from 120 –216 h. Error bars represent standard deviations, n = 4.
Figure 7.3. Comparisons between the pH of three different types of biofilms. pH measurements taken with pH-boy, pH +/- 0.1. Figures represent means, n = 4.
Figure 7.4. Viable counts of 30 μm thick sections through a 120 h microcosm plaque grown in the presence of sucrose. Bars represent means.
Figure 7.5. Viable counts of 30 μm thick sections through a 120 h microcosm plaque grown in the presence of sucrose following exposure to 0.2 % CH for 1 min. Bars represent means.
7.4. Discussion

The purpose of this part of the study was to determine the effect of sucrose pulsing on the composition of microcosm plaques and their susceptibility to chlorhexidine. Despite the complexity of the human diet, the only class of compounds found to greatly influence the ecology of the resident microflora is that of fermentable carbohydrates (Marsh and Martin, 1992). Such carbohydrates can be broken down to acids by the microflora of the mouth. High pH values are known to be associated with periodontal disease and low pH values (resulting from a dietary intake high in sugars) are associated with acid demineralisation, the major destructive process in enamel caries (Russell and Coulter, 1975). The role of specific micro-organisms in dental caries is governed by a variety of environmental factors which determine the ecology of the microbial dental plaque (Fitzgerald, 1975) and thus the interactions between the various components of the system may contribute to the overall disease process.

Many in vivo and in situ studies have reported few significant differences in plaque accumulation after sucrose rinsing (Stratt et al., 1975). However, the frequent consumption of dietary carbohydrates is associated with a shift in the proportions of the microflora of dental plaque in vivo (Sissons et al., 1992). Mühlemann and de Boever (1970) observed that when sugar was applied to plaque in vivo strict anaerobes were significantly absent from the plaque, due possibly to the low pH and high Eh values resulting from the acidogenic fermentation by streptococci. S. mutans and lactobacilli numbers have also been reported to increase following sucrose rinsing (Stratt et al., 1975). The difference in bacterial composition between sucrose-grown and
sucrose-free biofilms may be attributed to the presence of acidogenic species, which are able to produce acid from sucrose, and aciduric species that are able to tolerate the resulting low pH. *S. mutans* is both acidogenic and aciduric and thus when sucrose is in the diet is able to produce environmental conditions in which other species (e.g. *S. sanguis*) are not able to thrive.

In these experiments the addition of sucrose into the system also had an effect on the bacterial composition of the biofilm. Microcosm plaques supplemented with sucrose had a much greater proportion of streptococci (85%) than non-supplemented microcosm plaques (25%). The pulsing of sugars, in the absence of pH control, into a modified chemostat system has also been shown to result in the selection of cariogenic species such as *S. mutans* (Bradshaw *et al.*, 1996b). Indeed a large number of studies have focussed on the predominance of *S. mutans* when challenged by sugars both *in vivo* and *in vitro* (Igarashi *et al.*, 1990). Such speciation was not carried out in this study because of technical and time constraints and therefore direct comparisons are difficult.

The sucrose-supplemented microcosm plaques also produced a lower proportion of *Veillonella* species (<1%) compared to non-supplemented biofilms (7%). Similar findings have been reported both *in vitro* (Wilson *et al.*, 1997) and *in vivo* (Dawes *et al.*, 1982). *Veillonella* spp. are unable to metabolise normal dietary carbohydrates, they use lactate produced by other micro-organisms and convert it into a range of weaker, and possibly less cariogenic, organic acids. Therefore, the establishment of a food chain (i.e. *Veillonella* spp. using metabolites from streptococci) should account for
higher numbers of *Veillonella* spp. because of the higher numbers of streptococci present. However, this was not seen in the present study and *in vivo* studies have also failed to demonstrate such a relationship (Igarashi *et al.*, 1990). This is probably because of the influence of other environmental factors and the presence of a range of other organisms in the biofilm.

The pH of the microcosm plaques reached levels as low as pH 4.2 when pulsed with sucrose compared to approximately 6.2 to 6.8 in non-supplemented biofilms. Studies by Sissons *et al* (1992) on microcosm plaques have also shown that the addition of 10 % sucrose can cause a reduction of 2 pH units in such 'plaques'. However, acidic pH fluctuations in plaque induced by dietary carbohydrate have a typical profile, the Stephan curve (Stephan, 1944; Kleinberg and Jenkins, 1964). The results from such studies have indicated that low plaque pH *in vivo* are able to return to 'normal' pH levels within 2 hours. Studies using computer modelling, however, have indicated that plaque thickness can have a profound effect on the Stephan curve response (Dawes and Dibdin, 1986). In approximal sites, plaque is potentially 1-2 mm thick and these are known to be sites of caries susceptibility (Igarashi *et al.*, 1989). The inner regions of such plaques are inaccessible to saliva exchanges, and the plaque can remain at a low pH for long periods (Schachtele and Jensen, 1982). Such findings may explain the consistently low pH observed in this study. However, factors such as sampling times, which were within 2 hours of the addition of sucrose, also have to be taken into account. It may be possible that the pH was fluctuating in response to sucrose pulsing but that this was not detected. Due to the constraints of the system, and the number of samples that can be removed
from the CDFF, it was not feasible to monitor pH continuously.

No ideal procedure for the long-term measurement of plaque pH *in situ* has yet been devised. Calibration, drift and the response of the measurement system to the plaque environment are issues that need to be addressed *in vitro* (Russell and Coulter, 1975; Hudson *et al.*, 1986) and in intra-oral studies (Schachtele and Jensen, 1982 and Igarashi *et al.*, 1989,1990) of plaque pH. The method employed in this study to measure biofilm pH was restricted by the amount of biofilm in contact with the probe. However, the probe could be re-calibrated between measurements and thus avoid drift.

The viable counts from the 30 µm sections through the sucrose-supplemented microcosm plaques showed a different pattern to those from non-supplemented plaques. Similar numbers of viable bacteria were observed in each of the 10 sections comprising the microcosm plaque grown in the absence of sucrose. The total anaerobic count varied from $8 \times 10^6$ cfu per section (60 – 90 µm) to $7.6 \times 10^7$ cfu per section (210 – 240 µm). However, the viable counts from sections through the microcosm plaques grown in the presence of sucrose showed greater variation between sections. The total anaerobic and aerobic counts varied from $4.8 \times 10^5$ to $4 \times 10^7$ cfu per section and $7 \times 10^5$ to $2 \times 10^8$ cfu per section respectively. The differences between microcosm plaques supplemented with sucrose and those not supplemented suggest that an overall change in the structure of the biofilm has taken place. Studies using computer modelling, have shown that the availability of substrates may play a major role in the structure of biofilms (Wimpenny and Colasanti, 1997). As described previously in Section 1.2.1, the computer model implies that biofilms may have structures
that reflect the availability of nutrients. The model suggests that high concentrations of substrates result in the formation of dense films. It is difficult to compare these computer-modelling studies with the cryosectioning results observed in this study. The variability between viable counts from the 10 sections comprising the sucrose-supplemented biofilms may indicate channels or pores through the biofilm. It is also documented that biofilms are able to produce larger amounts of exopolysaccharide material under nutritionally-rich conditions in the oral cavity (Marsh et al., 1989). Therefore, the 'gaps' in the biofilm structure observed by sectioning may be due to the accumulation of exopolysaccharide in these regions of the microcosm plaque and thus the density of bacteria comprising the biofilm may have remained similar to non-sucrose supplemented biofilms.

The effects of pulsing with chlorhexidine on sucrose-supplemented biofilms indicated that the biofilm was able to recover to the numbers seen prior to pulsing. However, the composition, and possibly the structure, of the biofilm appeared to change. Compared to the non-supplemented microcosm plaques, the Actinomyces spp. and Streptococcus spp. comprising the sucrose-supplemented biofilms were more susceptible to the action of 0.2 % chlorhexidine. The Veillonella spp., however, were less susceptible to the action of the antimicrobial agent in sucrose-supplemented biofilms. In these biofilms there were fewer Veillonella spp. than in the non-supplemented biofilms and although a similar log_{10} reduction was achieved fewer bacteria were killed. Indeed, although overall log_{10} reductions, compared to non-supplemented biofilms, were similar, the viable counts for each of the species detected were very different to non-supplemented biofilms making
direct comparisons difficult. As CH-pulsing continued the number of bacteria comprising the sucrose-supplemented biofilms increased, and at the final sampling point the viable counts were higher, in respect to each of the genera detected, than those seen prior to chlorhexidine pulsing. This may be due to the greater volume of fluid flowing through the system (an additional 100 ml per day) with sucrose pulsing, compared to the sucrose-free system, thus producing a greater washout effect and reducing the substantivity of chlorhexidine in the biofilm. Also coupled with this, the increase in nutrient availability may cause both an increase in the growth of the biofilm and the growth rates of the species present, the latter possibly explaining the differences in the susceptibility of the individual species (Brown et al., 1988).

Directly after the first pulse, the cryosectioning results indicated a change in the structure of the biofilm, the large variations in the viable counts for each section seen prior to pulsing with chlorhexidine were no longer evident. As pulsing continued the bacterial population of the entire biofilm also changed, now comprising a higher proportion of Veillonella spp. This may have implications for the cariogenicity of the microcosm plaque. There are conflicting reports on the role Veillonella spp. may play in caries. Early reports (van der Hoeven et al., 1978) suggested that Veillonella spp. reduce experimental caries by metabolising the lactate produced by S. mutans. However, Hamilton and Ng (1983) have found that Veillonella spp. can stimulate the growth and glycolytic activity of streptococci by the continual removal of lactate.

The results of this part of the study have demonstrated the versatility of the CDFF, in that it permits supplementation with dietary nutrients to mimic
some of the external factors influencing plaque *in vivo*. A sucrose rinse lasting a few minutes, followed by clearance by artificial saliva, simulates the processes that arise *in vivo* during the intake of sucrose drinks (Lagerlof *et al.*, 1984; Lindfors and Lagerlof, 1988) and this rinse is commonly used in modelling cariogenic challenges (Dawes, 1989; Igarashi *et al.*, 1989, 1990).

It was also possible to monitor the pH of the biofilm, an important aspect of caries and its control, and to test the susceptibility of these biofilms to an antimicrobial agent.

**Summary:**

- Attempted to mimic the *in vivo* situation by supplementing microcosm plaques with sucrose
- Lower pH values were observed in sucrose-supplemented microcosm plaques
- The composition of the sucrose-supplemented plaques was different to non-supplemented microcosm plaques
- Chlorhexidine was shown to be ineffective at reducing the total viable counts in the biofilms over long periods of time but did alter their composition
Dental plaque was probably the first biofilm to have been studied in terms of either its microbial composition or its sensitivity to antimicrobial agents. The term biofilm was not described in the scientific literature until 1981 (McCoy et al., 1981) but literature reviews on the composition and formation of dental plaque date back to the 19th Century, where plaque was described in a dental context as a felt-like mass of micro-organisms on carious enamel (Black, 1898). However, in the late 17th Century, Anton van Leeuwenhoek pioneered the approach of studying biofilms by direct microscopic observation when he reported on the diversity and high numbers of 'animalcules' present in scrapings taken from around human teeth. He also conducted early studies on biocides when he established the resistance of these 'sticky animalcules' to salt and vinegar (Brock et al., 1984). The realisation that bacteria may prefer to grow as a biofilm and that these bacteria are far less susceptible to the action of antimicrobial agents has caused many studies to concentrate on this aspect of microbiology (Marsh, 1995).

Plaque control plays a central role in the therapeutic arsenal directed against caries, gingivitis and periodontal disease. There is considerable interest in developing chemical agents to supplement mechanical means (i.e. toothbrushing) of controlling these diseases. The principal mechanisms of action of these agents are either reduction of plaque formation by direct or sustained antimicrobial activity, inhibition of bacterial adsorption to the tooth surface or interference with the metabolic processes of dental plaque bacteria.

The antimicrobial properties of an agent do not necessarily correlate with its
anti-plaque activity. However, an antimicrobial agent could be of considerable clinical value even if it had only marginal effects on the plaque mass if it suppressed the selection of potentially cariogenic or periodontopathogenic bacteria under conditions where they would otherwise flourish. Furthermore, an agent, which interfered with the rate of accumulation or metabolism of supra-gingival plaque, may inhibit the production of inflammatory compounds and therefore reduce the risk of gingivitis developing.

Dental plaque contains a diverse mixture of micro-organisms that co-exist in a relatively stable system due to the dynamic balance of synergistic and antagonistic interactions among the component species. Hence, an antimicrobial agent can inhibit an organism by both direct and indirect means. An organism that was dependant for growth on the provision of essential nutrients or co-factors by 'directly-inhibited' species, would be 'indirectly-inhibited'.

Although MIC data may be available for a particular compound showing that compound has antimicrobial activity, it is difficult to extrapolate from such studies and predict the effectiveness and behaviour of the compound in vivo. The MIC test is concerned with inhibition of growth of the organism, no attempt is made to distinguish between inhibition and death. In systemic infection a bacteriostatic effect would be sufficient to allow the host immune system to clear the invader. Host defences are, however, less effective in the oral cavity and hence a bactericidal effect is desirable (Wilson, 1993).

The aims of this study, therefore, were to develop and test a laboratory model suitable for evaluating the effectiveness of antimicrobial agents
against oral bacterial biofilms. A constant depth film fermentor was used to grow the biofilms. In order to mimic the situation *in vivo*, the biofilms were grown in an aerobic environment on a substratum similar to human enamel, with nutrients supplied by artificial saliva.

Studies were carried out using a mono-species biofilm grown on a variety of substrata; polytetrafluoroethylene, hydroxyapatite (composed of the mineral content of enamel) and bovine enamel. While the nature of the substratum had little effect on the growth of biofilms of *S. sanguis* they did influence the susceptibility of the biofilms to the various mouthwashes used (chlorhexidine, cetyl-pyridinium chloride and triclosan). With these initial studies it was possible to rank the effectiveness of each of the mouthwashes in terms of both their anti-plaque and antimicrobial activity, the results of which were comparable with those obtained in a clinical trial (Jenkins *et al.*, 1993). Further studies on the single species biofilms indicated the importance of the mode of application of the agent. In the previous study it was only shown whether the biofilm could be killed or growth on the substratum inhibited. However, *in vivo*, the agent would be delivered twice daily in the form of a rinse. Thus, in order to evaluate the effectiveness of an agent, its ability to kill the biofilm over time is of great importance. The results of this study indicated that the first pulse (10 ml / min.) reduced the viable counts of the biofilm by approximately 2 log_{10}. These results were corroborated with live / dead staining which indicated that the dead bacteria were still present and not removed from the biofilm. After this initial pulse the viable counts recovered to pre-pulsing levels.

Further studies were carried out on the effect of chlorhexidine on multi-
species biofilms and microcosm plaques. Chlorhexidine was used in the majority of the investigations as it has been shown to be the most effective agent in anti-plaque clinical studies and thus the majority of references involving oral clinical trials and screening trials (Addy and Wade, 1995) use chlorhexidine as either a test or control agent (Moran et al., 1992). This is an important aspect of the work as few in vitro studies have been carried out in this field. Therefore, comparisons between the model plaque and plaque in vivo are vital.

The reproducibility of the multi-species biofilms was poor, with regard to the total viable counts and the proportions of species present in each run. The microcosm plaques appeared to be far more reproducible and the proportions of the predominant species were comparable with those found in supra-gingival plaque. However, this 'reproducibility' is likely to have been an artefact of the lack of discrimination of the analytical methods used. Hence, bacteria were identified only to the genus level and not the species level. Therefore, although the proportions of the various genera determined were found to be similar in different runs, the proportions of individual species may have varied widely. Microcosm plaques were also grown supplemented with sucrose to take into account dietary intake of this fermentable carbohydrate.

The effects of a number of treatment regimes (involving chlorhexidine) against these biofilms were investigated primarily involving chlorhexidine pulsing twice daily to mimic the use of an agent in vivo. When challenged with chlorhexidine pulses there was an initial $2\,\log_{10}$ reduction in the number of viable organisms, however, although pulsing continued, the biofilms recovered in terms of both the viability and relative proportions of the
constituent species. The greatest kills were achieved when the pellicle-coated bovine enamel discs were pre-treated with chlorhexidine for 1 min. prior to inoculation. In these studies the microcosm plaques were pulsed with chlorhexidine after 8 h and therefore not allowing the biofilm to reach a steady state. Approximately a $1 \log_{10}$ reduction in the total viable counts was achieved by pre-treating the discs and a further $2 \log_{10}$ reduction after the initial pulse with chlorhexidine. After this time the numbers of bacteria recovered but only reached the numbers of bacteria counted at 8 h, prior to pulsing. This method of pre-treatment and subsequent pulsing (or rinsing) has shown to be the most effective way of administering chlorhexidine in vivo (Jenkins et al., 1994).

Cryosectioning of the microcosm plaques, both with and without sucrose supplementation, was carried out to determine the viability and proportions of species present at various depths throughout the biofilm. This showed that both the proportions and structure of the biofilm differed depending on the nutrient source. However, both microcosm plaques showed similar susceptibility to chlorhexidine pulsing.

There are several suggested explanations for the reduced antimicrobial susceptibility of biofilms, as described in the introduction, including reduced growth rate of bacteria comprising biofilms; the production of EPS by the bacteria forming a barrier to antimicrobial agents and that bacteria in biofilms are genetically different from their planktonic counterparts. The results of this study have shown that when biofilms were supplemented with sucrose the increased growth rate from the rich nutrient source did not affect the susceptibility of the biofilm to chlorhexidine. The results from cryosectioning
also showed that the antimicrobial was able to penetrate through the biofilm and accumulated at the base of the structure.

The structure of the biofilm prior and subsequent to chlorhexidine treatment was also investigated using confocal laser scanning microscopy. This was carried out using the same live / dead staining techniques used for the cryosectioning work. The results obtained from the CLSM corroborated the findings from the cryosectioning studies, indicating that there was a predominance of dead cells at the base of the biofilm not treated with an antimicrobial agent. The most susceptible bacteria comprising the biofilm appeared to be at the biofilm / air interface where the largest numbers of dead bacteria were observed. The CLSM proved to be a convenient method of observing the susceptibility of the biofilm, however, two main problems were encountered. Firstly a long application time of the agent was required to observe differences in the susceptibility and secondly, the depth of the biofilm could not be determined accurately. An interesting finding from the confocal work was that the dead cells at the biofilm / air interface were not removed from the biofilm. Indeed further studies would need to be carried out to investigate whether bacteria are being removed from the biofilm surface during pulsing. If still present and attached after an antimicrobial application, although dead, these bacteria may still provide binding sites for new colonisers.

Interest in the use of antimicrobial agents for the treatment of plaque-related diseases such as caries and periodontitis has generated a need for laboratory models for the evaluation of agents effective against oral bacterial biofilms and the CDFF has proved to be useful in this respect. This study has shown that a
biofilm-based model is able to (a) assess the antimicrobial activity of compounds against dental plaque bacteria under conditions similar to those which would exist in vivo and (b) to determine the ability of compounds to inhibit biofilm formation.

The Constant Depth Film Fermentor proved to be a convenient and versatile means of generating large numbers (up to 75 replicates) of bacterial biofilms. The model is particularly suited to studies of biofilms of oral bacteria in that it provides an environment similar to that found in the oral cavity. The biofilms were grown on a solid substratum with nutrients being provided in a thin film of liquid, continually replenished, trickling over the surface of the biofilm. The removal of the surfaces of the biofilms by the scraper blade simulates the continuous removal of the outermost layers of supragingival plaque due to chewing and tongue movements. Most studies, however, have employed single-species biofilms of oral bacteria rather than the complex communities characteristic of supragingival plaques.

The results of this study have demonstrated that the CDFF can be used to grow single or multi-species biofilms under conditions similar to those prevailing in the oral cavity. In terms of the microcosm plaques the bacterial composition, and the spatial distribution of the various species within these biofilms were similar to that found in supragingival dental plaques. Such biofilms were found to be less susceptible to chlorhexidine, a commonly-used oral antiseptic, than the bacterial components of the biofilm when these were in a planktonic form.

In this investigation the CDFF has allowed us to evaluate the ability of a range of compounds to kill bacteria in biofilms and to prevent biofilm
formation. The screening of potential anti-plaque and antimicrobial agents for use in preventing and/or treating plaque-related diseases using this approach is of considerable value. It may be able to reduce the need for large numbers of expensive clinical trials by selecting compounds, and the concentrations at which to use them, prior to expensive clinical trials.
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205


