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MOLECULAR IDENTIFICATION OF CARIOGENIC MICRO-ORGANISMS AND A POSSIBLE EFFECT OF FLUORIDATED MILK ON THEIR PROPORTIONS IN DENTAL PLAQUE.

by

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THESIS

Presented for the Degree of Doctor of Philosophy

in the

Faculty of Medicine, University of Glasgow

Infection Research Group,

Glasgow Dental Hospital and School,

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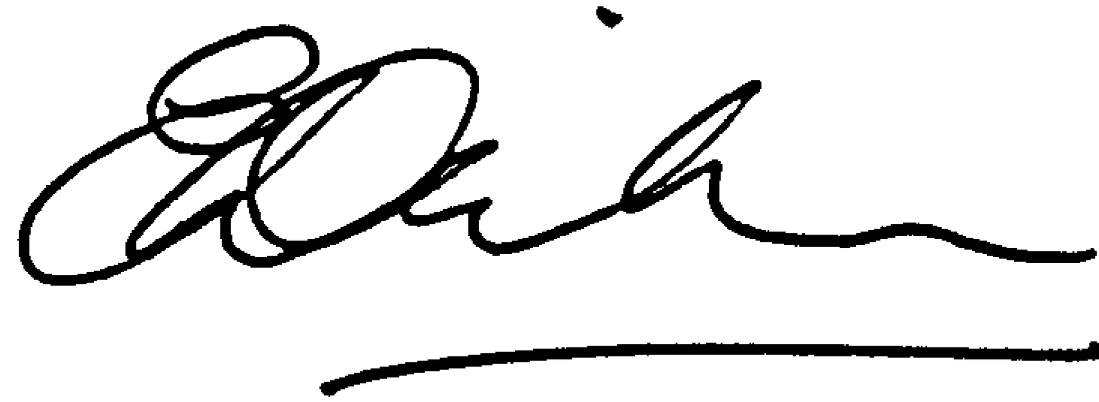
DECLARATION

This thesis is the original work of the author.



Elizabeth Marion Dickson

SUMMARY

The effect of fluoridated milk on plaque bacteria was investigated in fourteen complete denture wearers. These subjects were randomly split into two groups, seven in the treatment only group and seven in the treatment + dentifrice rinse group. There were five treatment regimes, each of six-week duration. The subject either consumed milk once or three times a day; milk with fluoride once or three times a day; or no beverage consumption as a control. The purpose of the additional dentifrice rinse was to mimic the effects of brushing twice daily with a fluoridated toothpaste. Six enamel slabs were embedded at strategic locations on the upper denture to investigate the influence of salivary flow rate on aciduric bacteria within dental plaque. Plaque samples were obtained from each enamel slab at Weeks 1 and 6 of the experimental treatments, and subjected to microbiological analysis. Enumeration of the bacteria was carried out to obtain the total counts of plaque bacteria, the counts of *S. mutans* on selective MSB agar and counts of *Lactobacillus* spp. on selective Rogosa agar. Statistical analysis revealed a main effect of treatment within the treatment only group for the lactobacillus counts. No beverage consumption and milk with fluoride consumed three times a day produced the lowest counts. Proportions of lactobacilli within the overall plaque bacteria were compared at the two buccal sites and one lingual site. The proportions at the buccal sites with a good salivary flow rate were lower than those that were experienced at the lingual site where there is a poor salivary flow.

To investigate strain variation among clinical *S. mutans* isolates identified from unrelated individuals, ribotyping was employed. Chromosomal DNA from the

clinical isolates was digested with restriction enzyme *Hin*dIII and the resulting fragments were transferred to a membrane by Southern blotting. The DNA fragments were hybridised to a digoxigenin-labelled probe (pKK3535) and detected with an antibody detection system. Profiles were obtained for each isolate and genotypes were compared within individuals and across a group of seven individuals. Analysis revealed that carriage of *S. mutans* genotypes was stable within an individual but that strain variability was diverse when analysed across all individuals. Longitudinal investigation revealed the possibility that *S. mutans* genotypes could be lost and then reappear at the same tooth site.

Identification of *Lactobacillus* spp. is routinely carried out to genus level but further speciating of these organisms in clinical samples is infrequent, due to the general inaccuracy of biochemical identification methods. A PCR-RFLP method was developed to speciate clinical *Lactobacillus* isolates identified from dental plaque. The technique was developed by digesting *Lactobacillus* reference strains with *Mnl*I and *Cfo*I. Restriction enzyme *Mnl*I generated profiles which allowed good discrimination of Lactobacillus spp., and further digestion with *Cfo*I of *L. casei*-group isolates enabled discrimination between these organisms. PCR-RFLP of clinical isolates revealed five groups of isolates which did not correspond to any of the reference strains. In order to identify these isolates, a representative from each group was processed for DNA sequencing. Overall analysis revealed that the most frequently isolated species was *L. casei/paracasei* with *L. gasseri* the second most common organism isolated from dental plaque.

A species-specific PCR assay for *L. fermentum* was developed. Oligonucleotide primers were selected from regions of the 16S rRNA gene with signatures unique to *L. fermentum*. Following confirmation of the sensitivity of the assay and optimisation of the primers, the species-specific PCR assay was then applied to clinical samples in an attempt to identify *L. fermentum*. *L. fermentum* was not found in any pus samples from dento-alveolar abscesses examined, and was infrequently identified from supragingival plaque samples. This assay was also applied to isolates that had similar profiles to *L. fermentum* in the PCR-RFLP study but which could not be classified as any other oral *Lactobacillus* species. The assay confirmed the unknown clinical isolates were not *L. fermentum*.

Methodological problems regarding the execution of the fluoridated milk study are highlighted and further approaches to monitoring and analysing potential cariogenic micro-organisms with molecular techniques are discusssed.

ABBREVIATIONS

AP-PCR	arbitrarily primed-polymerase chain reaction
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
bp	base pairs
dH_2O	distilled water
DNA	deoxyribonucleic acid
EPS	extracellular polysaccharide
F	fluoride
FAA	fastidious anaerobe agar
FAB	fastidious anaerobe broth
FTF	fructosyltransferase
GTF	glucosyltransferase
IgA	immunoglobulin A
IPS	intracellular polysaccharide
kb	kilobases
kbp	kilo base pairs
LC	lactobacillus counts
MSB	mitis salivarius-bacitracin
NCTC	National Collection of Type Cultures
NLC	non-lactobacillus counts
PCR	polymerase chain reaction
ppm	parts per million
PTS	phosphotransferase system
RAPD	randomly amplified polymorphic DNA

REA	restriction enzyme analysis
RFLP	restriction fragment length polymorphism
rRNA	ribosomal ribonucleic acid
SFT	sugar fermentation test
spp.	species
subsp.	subspecies
TC	total counts
T+D	treatment + dentifrice
TOnly	treatment only
UV	ultraviolet

CHAPTER 1: GENERAL INTRODUCTION

1.1 MICROBIOLOGY OF THE ORAL CAVITY

1.1.1 Introduction

Bacteria are a ubiquitous, nutritionally and environmentally diverse group of microscopic, unicellular prokaryotic organisms. Each bacterial cell retains its autonomy, that is, its ability to metabolise, grow and reproduce independently of other cells.

The first direct evidence of the role of bacteria in causing disease came from the study of anthrax by the German physician Robert Koch (1843-1910). His criteria for proving the casual relationship between a micro-organism and a specific disease are known as the Koch's postulates:

1. The organism must be isolated from every patient with the disease and its distribution in the body corresponds to that of the lesion observed.
2. The organism must be isolated and cultured outside the body (*in vitro*) in pure culture.
3. The pure organism must cause the disease in healthy, susceptible animals.
4. The organism must be recovered from the inoculated animal.

Clearly, these are ideal criteria and are not always attainable in practice but they provide a framework for establishing an aetiological role of organisms in infectious diseases.

Classification of bacteria makes it possible to identify the aetiologic agents of infectious disease. Taxonomy is the means of classifying microbes. The species is the basic unit of classification and a bacterial species is a group of organisms that share a set of characteristics. Phenotypic analysis includes microscopic examination of individual bacteria or their colonies and the study of their metabolic traits. A thorough genetic analysis is essential for accurate classification because the sequence and arrangement of nucleotide base pairs on the bacterial chromosome determine all other cellular characteristics. Additional information can be obtained from analysis of bacterial metabolic requirements and capabilities and from serologic studies that reveal similarities in the surface structures of bacteria and that bind the same immunoglobulin molecules.

1.1.2 Microbial Ecology

The specific area which supports a bacterial flora is the habitat and the micro-organisms growing in a particular habitat constitute a microbial community. The role of an organism within its community is termed niche, and this role is dictated by the biological properties of each microbial population (Marsh and Martin, 1999). Populations of bacteria that occupy similar niches in the community compete with each other and the one most suited to that niche will overgrow the other populations (Bowden and Edwardsson, 1994).

Many of the species inhabiting earth today, and that are still being discovered, reside in harsh environments that resemble those which existed at the beginning of our planet.

Almost every possible environmental pocket is occupied by micro-organisms that have specifically adapted to live in those conditions. These diverse habitats also exist to a certain extent on and in the human body, ranging from the dry, salty conditions of the skin to the acidic stomach (Bagg *et al.*, 1999). The majority of these bacteria reside as commensals, where they coexist with their host in a state of equilibrium. However, many of the commensals that are part of the normal microflora may, when the opportunity presents, become opportunistic pathogens. These opportunities usually occur when the host becomes immunocompromised and therefore susceptible to diseases caused by opportunistic pathogens.

1.1.3 Oral Microbial Ecology

The oral ecosystem comprises several distinct habitats each supporting the growth of a characteristic microbial community adapted to the local environment (Theilade and Theilade, 1985). Such habitats are supra- and sub-gingival plaque in the gingival region of the teeth, occlusal fissure plaque and coronal smooth surface plaque, denture plaque on removable dentures, the papillary surface of the dorsum of the tongue, and the smooth oral mucous membranes. Each location has a characteristic microflora (Theilade and Theilade 1985). Bacteria are able to adapt their physiology to changing environments, although the nature and extent of the ability to adapt varies among different strains and species. For example, *Streptococcus mutans* is able to adapt to acid environments that suppress or eliminate other oral streptococci (Bowden and Hamilton, 1987; McDermid *et al.*, 1986), and many other oral streptococci are able to adapt to enable them to grow in relatively high concentrations of fluoride (Hamilton, 1990).

Changes in the balance between the host and the oral microbial flora may lead to mucosal infections and increase the prevalence of both dental caries and periodontal disease. Oral bacteria have also been implicated in many systemic diseases such as bacterial endocarditis (Goldenberger *et al.*, 1997; Guaduchon *et al.*, 2001), bacterial arthritis (Kohashi *et al.*, 1976), aspiration pneumonia (Shay, 2002), coronary heart disease (Meyer and Fives-Taylor, 1998; Dorn *et al.*, 1999) and strokes (Genco *et al.*, 2002). These associations have proved that it is important to discover what organisms are present in the oral cavity for the diagnosis and treatment of systemic as well as oral diseases.

1.1.4 Acquisition of the Oral Flora

It has previously been estimated that there are about 500 species of bacteria inhabiting the oral cavity (Moore and Moore, 1994; Socransky and Haffajee, 1994; Wilson *et al.*, 1997; Paster *et al.*, 2001). The oral cavity of a new-born baby is usually sterile and from first feeding, micro-organisms are transferred from the surrounding environment to the infant through maternal saliva, from food, milk and water, and from the saliva of individuals in close proximity to the child (Marsh and Martin, 1999). Evidence for maternal acquisition comes from studies on serotype tracing, bacteriocin patterns and more recently by restriction endonuclease mapping (Berkowitz and Jordan, 1975; Kulkarni *et al.*, 1989).

The first micro-organisms to colonise are termed pioneer species and these continue to grow and colonise until environmental resistance is encountered. In the oral cavity, the shedding of epithelial cells (desquamation) and the shear forces from chewing and saliva

flow act as physical limiting factors. Nutrient requirements, redox potential, pH and the antibacterial properties of saliva can act as chemical barriers (Marsh and Martin, 1999). Streptococci, in particular *Streptococcus salivarius, S. oralis* and *S. mitis* bind to epithelial cells and are usually the first to colonise. These early colonisers rapidly modify their environment by producing extracellular products which improve conditions for the growth of other species. These microbial interactions and improvements eventually result in the formation of a highly stable climax community, containing a high diversity of species.

In a new-born infant, there are usually only epithelial surfaces available for colonisation. The pioneer populations consist of mainly aerobic and facultatively anaerobic species. The pioneer community increases during the first few months of life, and several Gram-negative anaerobic species appear. By the age of one, when teeth have erupted, *Streptococcus, Neisseria, Veillonella and Staphylococcus* species are predominantly isolated (Bowden and Edwardsson, 1994; Marsh and Martin, 1999). *Lactobacillus, Actinomyces, Prevotella* and *Fusobacterium* species are isolated less frequently (Theilade *et al.*, 1982). The acquisition of the oral flora continues with age and following tooth eruption the isolation frequency of spirochaetes and black-pigmenting anaerobes increases. Studies on the latter group found that they could be recovered from 18-40% of children aged five years but this increased to 90% carriage in teenagers aged 13-16 years. In adults, the microflora remains relatively stable and this stability (microbial homeostasis; *see* Section 1.2.4) is due to a dynamic balance among the members of the resident flora, resulting from numerous inter-bacterial and host bacterial interactions (Marsh and Martin, 1999).

1.2 FORMATION OF DENTAL PLAQUE

1.2.1 Introduction

Dental plaque is a mixed microbial biofilm growing on teeth and is the prime aetiological agent of the two main oral diseases, dental caries and periodontal disease. In common with other biofilms, the microbial composition of dental plaque is capable of change in response to changes in the environment, notably the diet. These responses are modulated by homeostatic mechanisms inherent in the plaque. The major sites of plaque accumulation are in the fissures of molar teeth, in the area bounded by the margin of the gum and the tooth and between adjacent teeth. In addition, plaque can cause gingival inflammation which may result in loss of epithelial attachment to the tooth leading to the formation of sub-gingival pockets. These pockets may also harbour subgingival dental plaque, which is significantly different from supra-gingival plaque in a number of important respects. In particular, a much lower redox potential which selects for a variety of anaerobic bacterial species is observed in subgingival plaque when compared to supragingival plaque.

1.2.2 Dental Plaque and Microbial Biofilms

The vast majority of micro-organisms that exist in nature are associated with a surface (Marsh and Martin, 1999) and these communities are termed as 'biofilms'. The microbes within a biofilm are organised into a three-dimensional structure and are enclosed in a matrix of extracellular material derived both from themselves and the

external environment. Biofilms possess unique properties which can have great clinical significance and many attribute resistance or resilience to be related to their distinctive architecture (Helmerhorst *et al.*, 1999; Reid, 1999). They offer protective properties to the micro-organisms that enable them to evade host defences and predators, avoid dessication and provide shelter from antimicrobial agents. It has been established that micro-organisms in a biofilm can be up to a thousand times more resistant to antimicrobial agents than the same cells grown in a liquid culture. This resistance is thought to be related to modified nutrient environments, direct interactions between extrapolymer matrices and the development of biofilm/attachment-specific phenotypes (Gilbert *et al.*, 1997).

Antonie van Leeuwenhoek (1632-1723) pioneered the study of biofilms when he first reported on the diversity and high numbers of 'animalcules' present in scrapings taken from around human teeth (Prescott *et al.*, 1993). He also reported on his findings that repeated and prolonged rinsing with wine-vinegar did not kill plaque bacteria *in situ* on his teeth. However, if the organisms were first removed from his molars and mixed with vinegar in his laboratory, they then appeared to be 'killed' (Marsh and Martin, 1999). These studies on dental plaque are probably the earliest records of research into biofilms in terms of either its microbial composition or its sensitivity to antimicrobial agents.

1.2.3 Composition and Establishment

Formation of microbial plaque appears to be an extremely complex and dynamic process, encompassing a number of interrelated factors (Saxton, 1973), but it has been

possible to piece together a sequence of events which lead to its establishment. Plaque formation has been shown to be preceded by the development of an organic film, the acquired pellicle, on the surface of clean enamel. In a number of studies (McDougall, 1963; Björn & Carlsson, 1964; Meckel, 1971) this film has been shown to be a requirement for plaque formation. The pellicle forms within the first few hours of a tooth surface being cleaned and is composed of salivary proteins, glycoproteins (Sönju and Rölla, 1972) and gingival crevicular fluid (Marsh and Bradshaw, 1995). Numerous studies have indicated the role of the pellicle in providing specific receptors for bacterial attachment (Duan *et al.*, 1994; Gibbons *et al.*, 1991; Hsu *et al.*, 1994; Scannapieco, 1994; Scannapieco *et al.*, 1989; Scannapieco *et al.*, 1995).

The pellicle is then colonised by so-called "pioneer species" which include *Neisseria* spp. and streptococci, predominantly members of the *S. mitis*-group. *Actinomyces* spp. are also commonly isolated after 2 hours, but obligately anaerobic species are detected only rarely at this stage and usually occur in low numbers. These pioneer organisms then multiply, forming first a monolayer and, subsequently, palisades of cells perpendicular to the tooth surface (Guggenheim *et al.*, 2001). Figure 1.1 shows an overview of the development of plaque over 48 hours on a clean enamel surface.

During and after this outgrowth period secondary colonisation by a variety of Gram positive and Gram negative species occurs, leading to a large increase in species diversity. Foremost among the events contributing to this secondary colonisation is the process known as co-aggregation whereby colonising microbes attach to cells which are already part of the developing biofilm. This mechanism is based on specific interactions

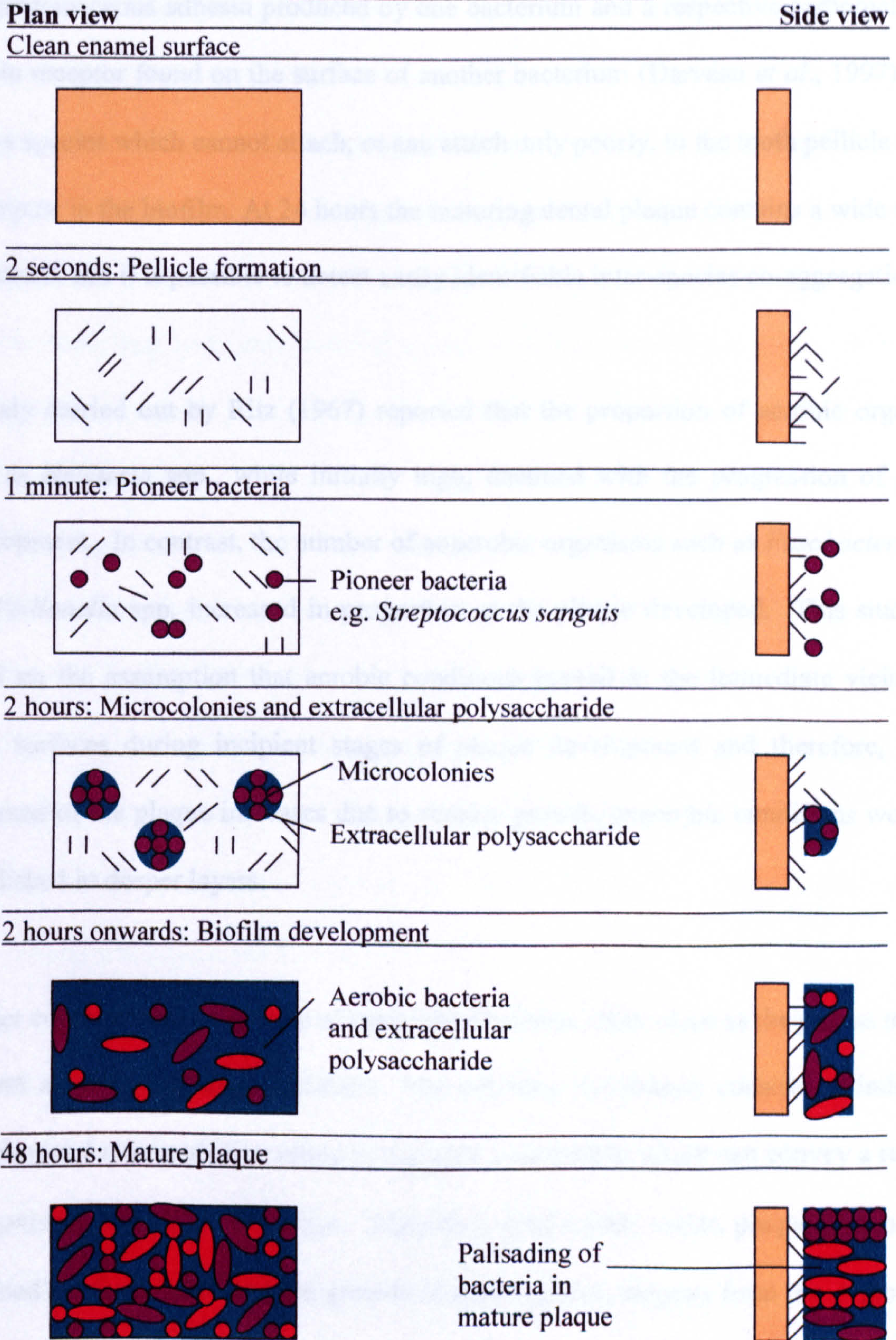


Figure 1.1 Summary of the development of dental plaque on a clean enamel surface (Bagg *et al.*, 1999).

of a proteinaceous adhesin produced by one bacterium and a respective carbohydrate or protein receptor found on the surface of another bacterium (Darveau *et al.*, 1997). This allows species which cannot attach, or can attach only poorly, to the tooth pellicle to participate in the biofilm. At 24 hours the maturing dental plaque contains a wide variety of bacteria and it is possible to detect easily identifiable inter-species co-aggregations.

A study carried out by Ritz (1967) reported that the proportion of aerobic organisms such as *Neisseria* spp., while initially high, declined with the progression of plaque development. In contrast, the number of anaerobic organisms such as *Fusobacteria* spp. and *Veillonella* spp. increased in proportion as the plaque developed. This study was based on the assumption that aerobic conditions prevail in the immediate vicinity on tooth surfaces during incipient stages of plaque development and therefore, as the thickness of the plaque increases due to aerobic growth, anaerobic conditions would be established in deeper layers.

Further colonisation and growth of established bacteria takes place as the plaque matures to form a stable, climax community. The resulting community consists of individual microbes and microcolonies acting in complex associations which can convey a range of antagonistic/synergistic properties. Microbial metabolism within plaque will produce localised gradients affecting the growth of other species, ranging from the depletion of essential nutrients with the simultaneous accumulation of toxic inhibitory by-products, to the consumption of oxygen enabling the growth of obligate anaerobes.

1.2.4 Microbial Homeostasis in Dental Plaque

The composition of dental plaque at any site is characterised by an extraordinary degree of stability or balance among the component species despite its microbial diversity. This is maintained in spite of the host defences and exposure to a variety of environmental stresses, including diet, antimicrobial agents in mouthwashes and saliva flow. The ability of the community to maintain this stability in its variable environment is termed microbial homeostasis (Marsh, 1989). This results from a balance of the dynamic microbial interactions, including synergism and antagonism. Self-regulatory mechanisms, also known as homeostatic reactions, restore the original balance if the environment is disturbed. Host defences acting synergistically with the resident microflora maintain microbial homeostasis in plaque. However, deficiencies in the immune response and other non-immune factors can cause a breakdown in homeostasis. This can lead to colonisation by exogenous species and the invasion of host tissues by opportunistic pathogens which cause oral disease.

1.2.5 Bacterial Attachment

Bacterial attachment is a complex process comprising four main stages (Bagg *et al.*, 1999). Micro-organisms are transported passively to the tooth surface by the flow of saliva (Marsh and Martin, 1999), although some use chemotactic activity (Bagg *et al.*, 1999). There are two types of forces involved in the initial adhesion. At distances of 10-100 nm, weak forces such as van der Waal's and electrostatic forces come into effect. These are highly dynamic and are influenced by the ion content of surrounding saliva.

As the bacterium gets within 2 nm of the surface, strong forces such as hydrogen bonding between hydroxyl groups in the pellicle and phosphate groups in the bacterial cell wall will exert an effect.

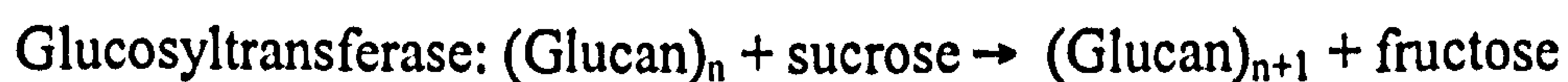
Following initial adhesion, a more permanent attachment can occur by covalent, ionic or electrostatic bonding. Oral bacterial attachment, and therefore plaque formation, is affected by a number of host and microbial factors. Once bound, the bacterium can replicate and remain attached. These pioneer organisms then produce microcolonies which in time become confluent. An extracellular matrix also develops simultaneously. This comprises microbial polysaccharides and additional layers of salivary glycoprotein (or crevicular fluid, depending on the site). Doubling times of plaque bacteria can vary considerably, both between different bacterial species and between members of the same species, depending on the intraoral environmental conditions.

1.2.6 Extracellular Polysaccharide (EPS)

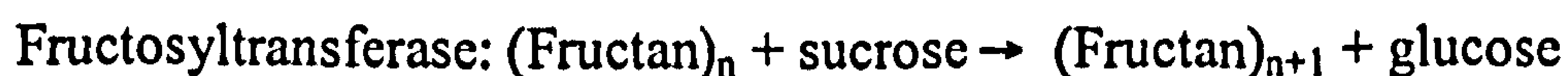
Many oral bacteria synthesise extracellular polysaccharides from sucrose in the diet, which have been shown to lead to heavy plaque accumulation and also increased cariogenicity (Carlsson and Egelberg, 1954; Carlsson and Sundström, 1968; Hojo *et al.*, 1976). There are two main types of glucans (glucose polymers) synthesised by oral bacteria. Dextrans are water-soluble glucans containing α-1:6 linkages with α-1:3 branches and are utilised as a food reserve. The α-1:6 linkages contain an extra bond between residues which allows the glucose moeities extra freedom of rotation, therefore the sugar rings are further apart and less likely to interact to restrict rotation. Mutans are

glucans containing α-1:3 linkages with α-1:6 branches, and aid bacterial attachment. The structural conformation of α-1:3 linked glucans permits interaction between adjacent residues, and consequently the chains are rigid. These rigid chains pack together efficiently and thus tend to form fibrous aggregates that are insoluble in water (Rölla *et al.*, 1985). These sticky insoluble polymers accumulate in plaque and have been frequently postulated to form a barrier to restrict the free movement of molecules between plaque fluid and saliva that create anaerobic conditions and increase acid production (McNee *et al.*, 1982; Van Houte *et al* 1989). Another type of EPS are water-insoluble fructans (polymers of fructose) called levans, which are found to a lesser extent and have β-2:6 linkages with β-2:1 branches. These also serve as a carbohydrate reserve, and are more rapidly metabolised since they are more susceptible to degradation by oral bacteria than dextran. *Streptococcus salivarius* primarily produces levans (Hamada and Slade, 1980).

The enzymes involved in synthesis of EPS are either extracellular or bound to the bacterial surface. They catalyse the following reactions:



Glucosyltransferase: $(\text{Glucan})_n + \text{sucrose} \rightarrow (\text{Glucan})_{n+1} + \text{fructose}$



Fructosyltransferase: $(\text{Fructan})_n + \text{sucrose} \rightarrow (\text{Fructan})_{n+1} + \text{glucose}$

There are two types of glucosyltransferase: dextransucrase and mutansucrase, which catalyse the synthesis of dextrans and mutans, respectively. Fructosyltransferase is also known as levansucrase. Bacterial virulence and increased adherence can be substantially attributed to active glucosyltransferase (Kuramitsu, 1974; Tanzer *et al.*,

1974). It has been reported that it is not the synthesis of glucan that is essential but the production and release of glucosyltransferase as it mediates the binding between the glucan chain and solid surfaces in the environment (Kuramitsu, 1974).

1.2.7 Lipoteichoic Acid (LTA)

Lipoteichoic acid is a polymer of bacterial origin that is present in the cell membrane and also released in relatively high amounts from bacteria (Rölla *et al.*, 1985). The molecule contains high numbers of phosphate groups and possesses a lipid moeity. LTA can form complexes with soluble and insoluble polysaccharides and such polymers have been found to be prevalent in sucrose-induced plaque *in vivo* (Melvær, *et al.*, 1974; Doule *et al.*, 1975, Ciardi *et al.*, 1977; Rölla, *et al.*, 1980). LTA is able to bind directly to teeth or enamel and when it is exposed on the surface of the bacterium, and could thus provide a bacterial binding mechanism. LTA has also been shown to interact with blood group reactive substances in the pellicle. As antibodies are found in the pellicle, a number of antigens may also indirectly act as 'adhesins' (Jenkinson and Lamont, 1997).

1.3 BACTERIAL TRANSPORT SYSTEMS

1.3.1 Introduction

Bacteria in the oral cavity reside in a 'feast or famine' environment and their persistence is dependent on their ability to obtain nutrients and grow (Carlsson and Hamilton, 1994). Although saliva is the main source of nutrients for the oral bacteria, the concentration of

readily available nutrients in salivary secretions is very low. Exogenous nutrients are supplied intermittently via the diet, most significantly dietary carbohydrates and casein (Marsh and Martin, 1999). The consumption of food results in a transitory burst of nutrients to the oral microflora. The fluctuating supply of nutrients and the resulting environmental changes have required the organisms to bring many adaptive mechanisms into play to exploit the available nutritional resources (Carlsson and Hamilton, 1994).

1.3.2 Functions of Transport Systems

The first step in nutrient utilisation is uptake of required nutrients by the microbial cell. Uptake mechanisms must be specific so that only necessary substances are acquired. Since micro-organisms often live in nutrient-poor habitats, they must be able to transport nutrients from dilute solutions into the cell against a concentration gradient. The most important transport mechanisms are facilitated diffusion, active transport, ion-linked transport and group translocation (phosphotransferase system) (Prescott *et al.*, 1993).

Many of the proteins in cell membranes function to carry out selective transport. A uniport transport system carries a single solute from one side of the membrane. Co-transport of two substances in the same direction is symport and the co-transport of two different substances in opposite directions is antiport. These proteins usually span the whole membrane, making contact with the outside environment. They often require the expenditure of energy to help the compounds move across the membrane (Stryer, 1975).

1.3.3 Passive Transport

Some substances, such as glycerol, can cross the plasma membrane by passive diffusion. This is the process in which molecules pass from an area of high concentration to one of low concentration. For molecules in passive transport, laws of simple diffusion direct their movement. There is no transport protein, it is non-specific, and energy is not required. A reasonably large concentration gradient is required for adequate nutrient uptake and the rate of uptake decreases as more nutrient is acquired unless it is used immediately. Thus, passive diffusion is an inefficient process and is not employed extensively by bacteria.

Carrier proteins greatly increase the rate of diffusion and some called permeases are embedded in the plasma membrane. This process is called facilitated diffusion and the rate increases with the concentration gradient much more rapidly and at lower concentrations of the diffusing molecule than that of passive diffusion. Although a carrier protein is required, the process is still only diffusion therefore no extra energy input is required (Prescott *et al.*, 1993).

1.3.4 Active Transport

Facilitated diffusion carriers only move molecules when the solute concentration is higher on the outside; they cannot move against a concentration gradient. When micro-organisms reside in habitats of very dilute nutrient sources, such as the oral cavity, they must be able to transport and concentrate these molecules. Active transport utilises a

protein carrier therefore energy input is required to transport solute molecules against a concentration gradient. Binding protein transport systems employ special substrate binding proteins which bind the molecule to be transported and then interact with the cell membrane transport proteins to move the solute molecule inside the cell (Prescott *et al.*, 1993). The energy source is ATP and a specific ATPase is required (Stryer, 1975).

1.3.5 Ion-Linked Transport

This is also known as secondary active transport, which depends on an ionic concentration gradient across the membrane. Ionic gradients/membrane potential provide energy for active transport of compounds, such as the co-transport of sugar/amino acid with an ion along the ionic concentration gradient. An example of this is the H^+/lactose symporter system where H^+ translocation by the electron transport chain creates an electrochemical gradient that drives the active uptake of lactose.

1.3.6 Phosphotransferase System (PTS)

In some bacteria, the active transport of sugars is coupled to their phosphorylation, for example glucose enters the cell as glucose-6-phosphate. PTS is the most significant high affinity transfer system for mono-and di-saccharides in acidogenic oral bacteria, especially *Streptococcus, Actinomyces* and *Lactobacillus* species. Figure 1.2 is a simple diagrammatic representation of the steps involved in the PTS. The phosphotransferase reaction is a group transfer pathway (Rohwer *et al.*, 2000) and begins inside the cell when the phosphoryl group of phosphophenol pyruvate (PEP) is transferred to a specific

Figure 1.2 The phosphotransferase system

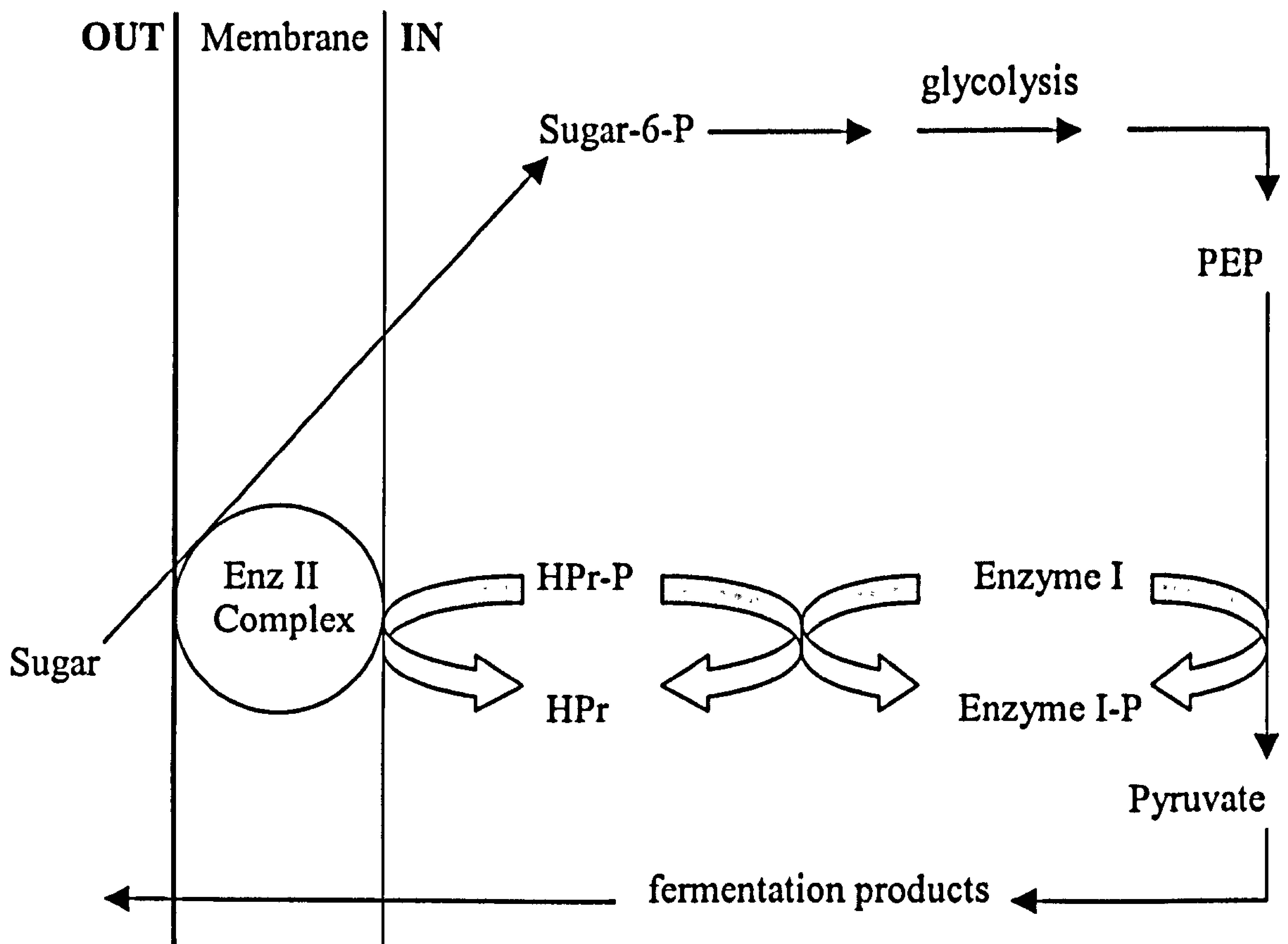


Figure 1.2 Simple diagrammatic representation of the steps involved in the phosphophenol-pyruvate (PEP)-mediated sugar phosphotransferase system (PTS). Enzyme I and HPr are cytoplasmic proteins; Enzyme II is a sugar-specific protein that is associated with the membrane and may exist on its own or complexed with sugar-specific cytoplasmic-associated proteins such as IIA, IIB and IIC (Marsh and Martin, 1999).

histidine residue of a small protein called HPr in a reaction catalysed by enzyme I. The phosphorylated form of HPr then transfers its phosphoryl group to a sugar in a reaction that is catalysed by membrane-bound enzyme II.

$$\text{Sugar}_{\text{(outside)}} + \text{PEP} \rightarrow \text{Sugar-P}_{\text{(inside)}} + \text{Pyruvate}$$

HPr, enzyme I and part of enzyme II (II-B) participate in the transport of many sugars. However, the other part of the enzyme II complex (II-A), is much more specific (Stryer, 1975). There are many different II-A proteins each responsible for the uptake and phosphorylation of different sugars. PTS is constitutive for some sugars, for example, glucose, mannose and sucrose, but it must be induced for others, such as lactose and sugar alcohols (mannitol and sorbitol). PTS activity in oral streptococci is optimal under conditions of carbohydrate limitation, neutral pH and slow bacterial growth rates. However, PTS activity is repressed under conditions of excess sugar, low pH and high bacterial growth rates (Vadeboncoeur and Pelletier, 1997).

1.3.7 Multiple Sugar Metabolism (Msm)

Many strains of *S. mutans* possess a second means of transporting sugars into the cell, known as the multiple sugar metabolism (Msm) transport system. This system is similar to the binding-protein-dependent system that is normally found in Gram-negative bacteria, and is capable of transporting various common sugars. The exact role of this system in plaque ecology is unknown but it has been hypothesised that it might play a role in the transport of breakdown products of EPS degradation during intervals between

meals when the supply of refined mono- and di-saccharides is negligible (Marsh and Martin, 1999).

1.3.8 Glucose Permease

When phosphotransferase system activity is repressed as a result of exposure to high sugar concentrations, sugar transport is increased by an ATP-dependent glucose permease. The sugar is transported into the cell where it is phosphorylated on the inner surface of the membrane. Cells have the tendency to form glycogen when under conditions of carbohydrate excess in order to reduce the toxic intracellular levels of glycolytic intermediates. This system allows organisms to cope better in situations of 'feast and famine' in the mouth in terms of dietary carbohydrates (Marsh and Martin, 1999).

1.4 BACTERIAL METABOLISM AND ACID PRODUCTION

1.4.1 Introduction

The persistence of members of the resident oral microflora depends on their ability to obtain nutrients and grow in the mouth. These nutrients are derived primarily from the metabolism of endogenous substrates that are present in saliva and gingival crevicular fluid (Marsh and Martin, 1999). Oral bacteria employ the various systems mentioned previously to transport these nutrients across their membranes where they must then be utilised. There are two main groups of bacteria, those that utilise nitrogenous materials

and produce basic products which lead to an increase in pH, and those that convert carbohydrates into organic acids which lower the pH.

1.4.2 Carbohydrate Metabolism

Particular attention has been paid to carbohydrate metabolism because of the relationship between dietary sugars, low pH and dental caries (see section 1.5). Sugar is also the main energy source for the microflora in dental plaque (Carlsson and Hamilton, 1994; Bowden and Hamilton, 1998). Starches contain a mixture of amylose and amylopectin which can be broken down into their constitutive sugars by amylases derived from saliva or the bacteria themselves. Some streptococci, e.g. *S. gordonii* and *S. mitis* can bind amylase, which is thought to provide additional metabolic capabilities (Marsh and Martin, 1999). Lactose is obtained in the diet mainly from milk while sucrose is the main carbohydrate of relevance in terms of caries. In plaque, energy is required for the biosynthetic reactions that convert sugars to pyruvate (glycolysis) (Prescott *et al.*, 1993), and the fate of pyruvate is dependent on the availability of oxygen.

In the aerobic conditions that are present on the outer surface of plaque, pyruvate is completely oxidised to carbon dioxide (CO_2) and water (H_2O) via the Krebs cycle and the electron transport chain. In the anaerobic conditions that are found in the interior of plaque, pyruvate is converted into lactate when the sugar supply is plentiful. Other organic acids, such as acetic, butyric, formic and propionic are also formed via an alternative pathway under conditions of low sugar supply, and these all serve to lower the pH in dense plaque (Geddes, 1975).

1.4.3 Aerobic Respiration

The pyruvate produced in glycolysis undergoes further breakdown through a process called aerobic respiration which requires more oxygen and yields much more energy than glycolysis. It is divided into two processes: the Krebs Cycle (also known as the Tricarboxcylic Acid [TCA] cycle or the citric acid cycle) and the electron transport chain (or respiratory chain). The pyruvate molecules produced during glycolysis contain energy-rich bonds. In order to use that energy, the cell must convert it into the form of ATP. To achieve this, pyruvate molecules are processed through the Krebs cycle. The pyruvate dehydrogenase complex first oxidises pyruvate to form CO_2 and acetyl coenzyme A (acetyl-CoA), an energy-rich molecule composed of coenzyme A and acetic acid joined by a high energy bond (Stryer, 1975). Acetyl-CoA arises from the catabolism of many carbohydrates, lipids and amino acids and is the main substrate for the Krebs cycle (Prescott *et al.*, 1993). Because glycolysis produces two pyruvate molecules from one glucose, each glucose is processed through the cycle twice. For each molecule of glucose, six NADH, two $FADH_2$ and two ATP molecules are produced.

The NADH and $FADH_2$ are then oxidised to NAD^+ and FAD, respectively, in the electron transport chain (Stryer, 1975). Electrons are passed along the chain, with oxygen being the final electron acceptor, being ultimately reduced to water. If the oxygen levels are depleted, electrons cease to flow through the electron transport system and the proton concentration gradient will not be sufficient to power the synthesis of ATP. When this happens, the bacteria are not able to survive long without oxygen.

1.4.4 Fermentation

Production of ATP through the Krebs cycle and electron transport chain requires the presence of oxygen. In the absence of oxygen, NADH is not usually oxidised by the electron transport chain because no external electron acceptor is available. However, it must still be oxidised back to NAD^+ in order to allow glycolysis to continue. Since glycolysis occurs without oxygen, anaerobic organisms are able to convert glucose into pyruvate by slowing or stopping pyruvate dehydrogenase activity and using pyruvate or one of its derivatives as an electron and hydrogen acceptor in the re-oxidation of NADH. This may lead to the production of more ATP. This energy-yielding process is called fermentation, where organic molecules serve as both electron donors and acceptors (Prescott *et al.*, 1993). The energy conserved, called the proton motive force, can be used in the generation of ATP, as well as for flagellar motion and energy for various cellular transport processes (Carlsson and Hamilton, 1994).

Oral streptococci convert pyruvate to lactate (catalysed by lactate dehydrogenase) when sugars are in excess, while mutans streptococci and *S. sanguis* produce formate, acetate and ethanol when metabolising sugars under carbohydrate limitation (Carlsson and Griffith, 1974; Hamada and Slade, 1980). Figure 1.3 is a diagrammatic representation of the formation of acid end products by mutans streptococci under various growth conditions.

Figure 1.3 Formation of end products of metabolism by mutans streptococci

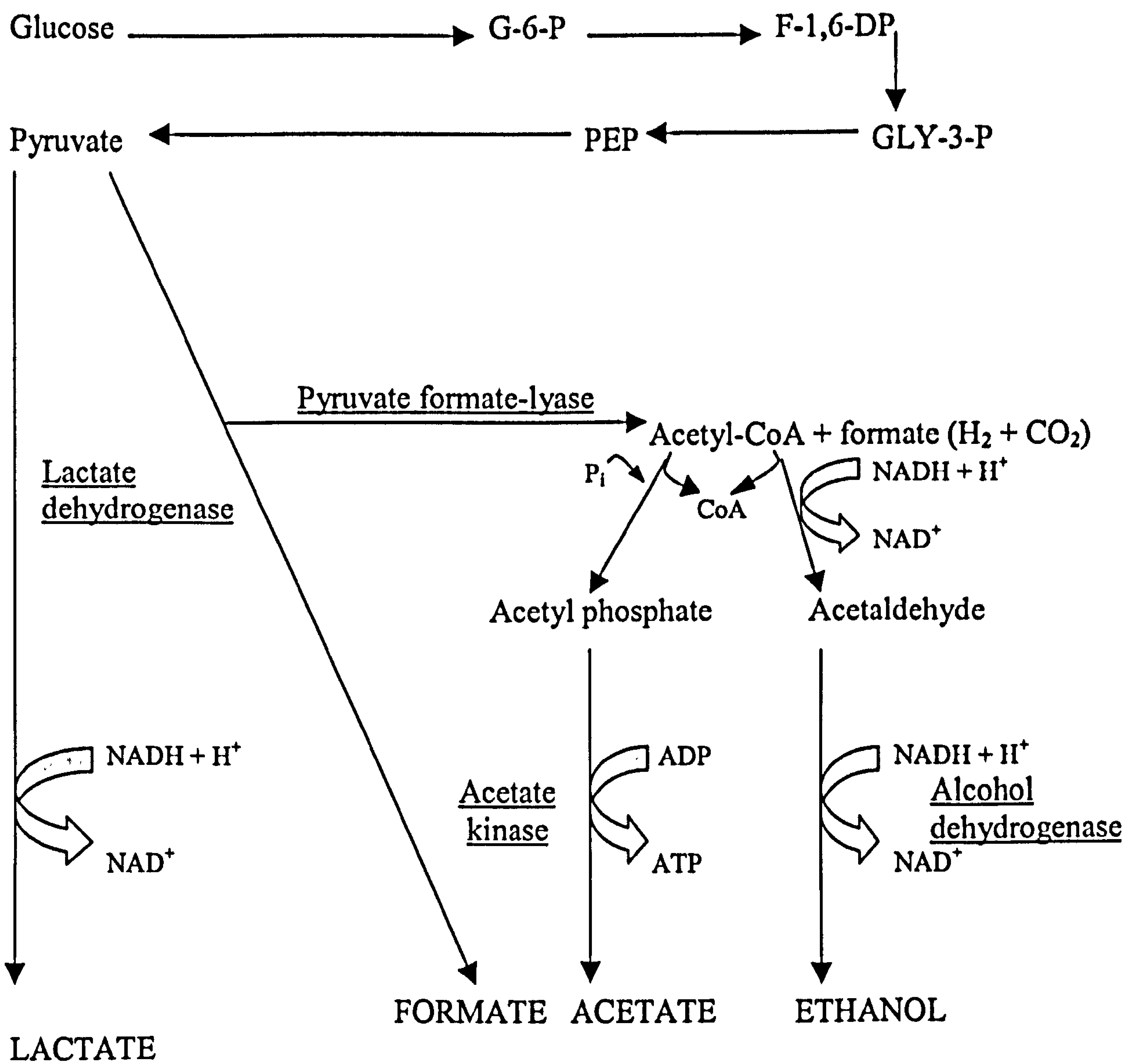


Figure 1.3 Formation of end products by mutans streptococci and alternative pathways when under various growth conditions. Lactate is the end product when sugar is in excess and formate, acetate and ethanol are the end products when sugar supply is low. The enzymes that catalyse these reactions are underlined. G-6-P, glucose-6-phosphate; F-1,6-DP, fructose-1,6-diphosphate; GLY-3-P, glyceraldehyde-3-phosphate; PEP, phosphoenolpyruvate. (Adapted from Marsh and Martin, 1999).

1.4.5 Acid Production/Tolerance

Geddes (1975) reported that after exposure to sugar under dietary conditions, a high concentration of lactic acid rapidly built up in human dental plaque as the pH decreased. Some members of the oral microflora can further degrade the acid end-products formed during sugar metabolism by other bacteria (Carlsson and Hamilton, 1994). For example, lactic acid that is released by *Lactobacillus* spp. and *Streptococcus* spp., is utilised by *Veillonella* spp. and *Peptostreptococcus* spp. to produce acetic and propionic acid (Geddes, 1972).

Streptococci are important organisms in terms of acid production as they display special features of metabolism, as mentioned previously, such as the uptake of sugars by the high affinity PTS system, and the possession of a second, low affinity sugar uptake system (Msm). One of the characteristics of lactobacilli and mutans streptococci is their ability to produce acids, whilst another is their acid tolerance which enables them to survive and reproduce at a low pH in dental plaque (Borgström, *et al.*, 1997). Strains of *S. mutans* produce acid at the fastest rate (McGhee and Michalek, 1985) and *Lactobacillus* spp. generate the lowest environmental pH (Strålfors, 1950). These organisms can tolerate acid conditions that other oral bacteria find inhibitory or lethal. Survival depends on the ability of the cell to maintain internal pH homeostasis and this will be addressed in the respective sections later in the introduction.

1.4.6 pH Changes in Plaque

The initial drop in plaque pH is due to the rate at which plaque microbes are able to metabolise carbohydrates. The lowest pH achieved depends greatly on the microbial composition of the dental plaque, the nature of the fermentable carbohydrate source and the rate of diffusion of substrates and metabolites into and out of the plaque (Geddes, 1975; Marsh and Martin, 1999).

Accumulation of ammonia or organic acids influences the acid-base ratio and pH of plaque. Open plaque is the term given to thin, loose plaque that has little EPS associated with it and a good flow of saliva. Any pH changes within this plaque are resisted primarily by the mechanical washing action of saliva removing bacterial waste. Ammonia is also produced at an acidic pH, which then neutralises accumulated acid and *vice versa*. Pyruvic and 2-oxoglutaric acids take up ammonia to form amino acids which remove the basic and acidic metabolites.

Carbohydrate which is metabolised more slowly to a plaque community, such as starch, with fewer aciduric, acidogenic microbes results in less acid production and a higher terminal pH. The density of the plaque and access by saliva governs the rate of diffusion of material into and out of plaque. Thus, less dense plaque fully exposed to saliva flow will more rapidly exchange metabolites with the surroundings. This will enable the substrates to diffuse into the plaque rapidly and at the same time allow microbial by-products to diffuse out. Conversely, the presence of significant numbers of aciduric, acidogenic bacteria in plaque developing in a sheltered site with a low diffusion rate coupled with readily fermentable carbohydrate such as sucrose or glucose would produce the lowest pH. Under these conditions a pH in the region of 4.5, or even lower,

might be attained. This poor saliva access and the presence of EPS will result in the retention of organic acids in the matrix to create a pH change that can cause dental caries.

1.5 DENTAL CARIES

1.5.1 Introduction

Dental caries is the single most common chronic disease of childhood (Becker *et al.*, 2002) and results in a considerable direct burden of pain and suffering as well as poorer general health. The carious lesion results from the production of acids, particularly lactic acid, as plaque micro-organisms metabolise dietary carbohydrates on the tooth surface which causes demineralisation of enamel and later of dentine (Samaranayake, 1996; Pratten, *et al.*, 2000). The initial process of enamel demineralisation is usually followed by remineralisation, and cavitation occurs when the former process overtakes the latter. Cavities begin as small demineralised areas below the surface of the enamel and once the enamel has been affected, the caries can progress through the dentine and into the pulp. Lesion formation involves the dissolution of the enamel and the transport of calcium and phosphate ions away into the surrounding environment (Marsh and Martin, 1999).

The major factors involved in the aetiology of caries are host factors (including the tooth and saliva), plaque micro-organisms, diet and the time necessary for caries development (Bagg, *et al.*, 1999). Figure 1.4 shows the interaction of each of these four factors that results in the occurrence of dental caries.

Figure 1.4 Interaction of factors involved in dental caries

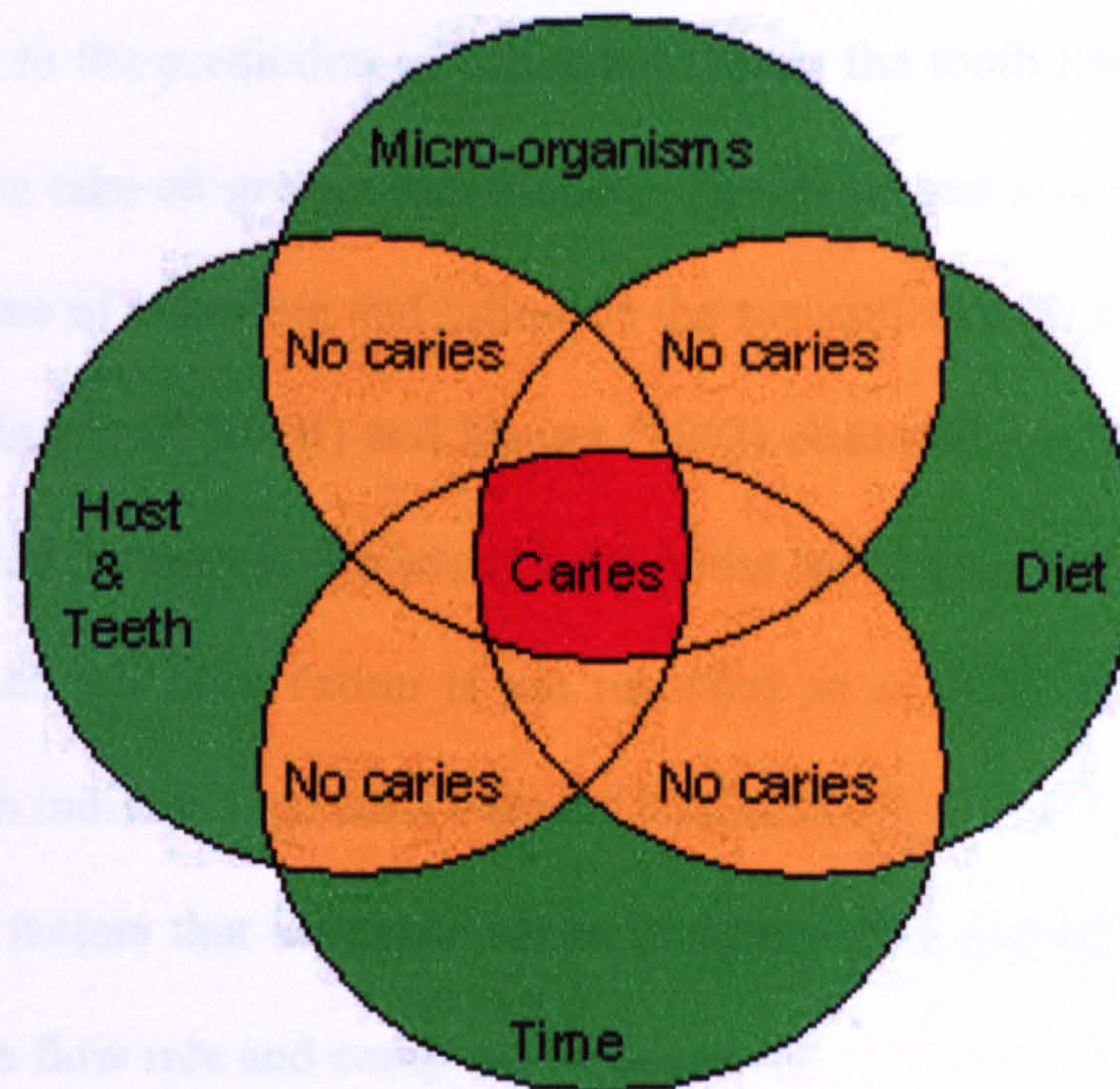


Figure 1.4 Aetiological factors in dental caries. The four circles represent the parameters involved in the carious process. All four factors must be acting at the same time (causing the circles to overlap) for caries to occur (Fejerskov and Thylstrup, 1994).

1.5.2 Host Factors

One approach to the prediction of caries is to study the tooth itself. Various aspects of tooth resistance take on greater importance. The shape and size of the whole tooth may affect the degree of crowding and influence the susceptibility to caries (Axelsson, 2000). Grahnen and Ingervall (1963) and Hunter (1967) observed a relationship between tooth width and caries resistance, the larger teeth being more frequently restored than smaller ones. However this observation is not regarded as a useful tool in the prediction of dental caries in individual patients because it is such a multifactorial disease. Therefore, the main host factors that influence caries initiation are regarded as the structure of the enamel and the flow rate and composition of saliva.

1.5.2.1 Enamel

Some areas of the same tooth have been found to be much more susceptible to carious attack than others. Susceptibility to demineralisation by acid is probably related to many factors including the mineral and fluoride content. Demineralised enamel is more porous and quantitation of changes in tissue porosity can be used as an indicator of mineral loss from the enamel (Thylstrup and Fejerskov, 1994). A defective or rough enamel surface, as well as the chemistry of the enamel, may also be determinants of tooth resistance to caries (Axelsson, 2000).

1.5.2.2 Saliva

Mixed or whole saliva consists of secretions from the major (parotid, submandibular, and sublingual) and minor salivary glands, with a variable input from the gingival crevicular fluid (Bagg *et al.,* 1999). Saliva plays an important role in maintaining an appropriate balance within the ecosystem associated with tooth surfaces (Bowden and Edwardsson, 1994). The mechanical washing action of saliva is effective in the removal of food debris and oral micro-organisms. Salivary components (agglutinins) can aggregate certain bacteria which facilitate their removal from the mouth by swallowing (Scannapieco, 1994). Salivary buffer in the form of bicarbonate and phosphate has long been identified as significant in the control of plaque pH and, through this, demineralisation of enamel. Maintenance of neutral pH levels removes the advantage given to aciduric cariogenic bacteria by lower pH environments (Bowden and Edwardsson, 1994). It is also highly saturated with inorganic compounds of calcium, phosphate and fluoride which increase the resistance of hydroxyapatite against challenges from cariogenic bacteria and enhance the saturation of plaque fluid with respect to the tooth surface structures (Tenovuo and Lagerlöf, 1994). Remineralisation of early caries lesions may also be attributed to the calcium and phosphate ions and it is remarkably enhanced by the presence of fluoride ions, for which saliva is a delivery vehicle.

Secretory IgA (sIgA) is the predominant immunoglobulin found in all mucosal secretions, including saliva (McNabb and Tomasi, 1981). In general sIgA is thought to participate in the local disposal of soluble antigens by providing an immunologic "first

line of defence" in the oral cavity (Scannapieco, 1994). Local sIgA antibody can also play a role in viral neutralisation, attenuation of viral growth and replication within oral tissues, and effect the neutralisation and disposal of toxins and food antigens (McNabb and Tomasi, 1981). Lysozyme is also an important antimicrobial constituent of saliva that lyses the cell walls of bacteria by hydrolysing the β1,4 glucosidic linkages between N-acetlymuramic acid and N-acetylglucosamine of the peptidoglycan. Although members of the normal oral flora have been found to be relatively insensitive to lysis by lysozyme (Gibbons *et al.*, 1966), it may bind to and aggregate Gram positive bacteria such as *S. mutans* (Pollock *et al.*, 1976) and other streptococci (Laible and Germaine, 1982). Binding is thought to involve cationic portions of the molecule interacting with anionic bacterial surface components (Laible and Germaine, 1982), as well as specific recognition of bacterial cell wall sugars by the enzyme active site (Pollock *et al.*, 1976). Lysozyme is also known to inhibit glucose fermentation by oral streptococci (Wang and Germaine, 1993).

When the supply of saliva is compromised, the important role that saliva plays in maintaining a healthy balance becomes apparent (Bowden and Edwardsson, 1994). Salivary shear forces and lubrication are reduced, increasing the time by which food is retained in the mouth. This retention of food and a reduction in the buffering effect of saliva causes lower pH environments in plaque. These environments favour aciduric bacteria, such as *S. mutans*, and place the tooth at greater risk of demineralisation. Reduction in saliva also means a reduction in the non-specific and specific (immune) factors.

1.5.3 Diet

Diet can be considered to influence the composition of the microflora and its metabolic activities in three ways. These are the chemical composition of the diet, its physical consistency and intake frequency (Bowden and Edwardsson, 1994). There is a direct relationship between dental caries and carbohydrate intake, the most cariogenic of which is sucrose. Sucrose is highly soluble and diffuses easily into dental plaque, acting as a bacterial substrate for the production of extracellular polysaccharides and acids (Bagg *et al.*, 1999). Carbohydrates such as glucose and lactose are also cariogenic, but less so than sucrose. In general, liquid foods such as fruit juices are swallowed quickly and are less available to bacteria compared to sticky foods. Also, foods used frequently will be present in the mouth and available for bacterial metabolism for longer periods of time (Bowden and Edwardsson, 1994). The frequency of sugar intake rather than the total amount of sugar consumed also appears to be of importance (Koch *et al.*, 1994). Diets that are optimal for health in terms of caries are those that provide minimum amounts of readily fermentable carbohydrates but that are still compatible with the health of the host. This will keep the plaque pH relatively high, thereby removing any benefit that a low pH confers on aciduric bacteria.

1.5.4 Microbiology of Dental Plaque

Micro-organisms that are isolated from dental plaque play an important role in the initiation and development of dental caries. They are surrounded by an organic matrix that acts as a food reserve and cement, binding organisms both to each other and to

various surfaces. The microbial composition of plaque can vary widely between individuals and there are also further variations in plaque composition within a single individual whether at the same site on different teeth, at different sites on the same tooth or at different times on the same tooth site. Babaahmady *et al.* (1997) observed variations in bacterial populations in gingival plaque that appear to relate to the location of the plaque sample.

A high proportion of plaque bacteria metabolise dietary carbohydrates to produce a variety of organic acids, causing a drop in environmental pH (Geddes, 1972, 1975; Featherstone and Rodgers, 1981). Lactate can be metabolised by certain plaque bacteria (e.g., *Veillonella, Neisseria*) to produce propionate, acetate, CO_2 and H_2 which can return environmental pH and acid composition of plaque to around 7.0, close to resting plaque pH. Plaque also contains bacteria which can produce alkaline compounds and this may explain why the resting pH of plaque can be higher than that of saliva.

As mentioned earlier, the production of EPS plays an integral role in plaque formation but it is apparent that they also play a role in the caries process and enhance the pathogenicity of plaque. The insoluble mutan form of glucan polymers is responsible for large amounts of 'sticky' plaque, which are thought to convey cariogenicity to *S. mutans*. *In vivo* tests and *in vitro* measurements of the impact of these insoluble polymers on diffusion and demineralisation suggest that large amounts of glucans in plaque facilitate both these processes (Macpherson *et al.*, 1990; Van Houte *et al.*, 1989).

Streptococcus spp. including *S. mutans*, as well as *Actinomyces* spp. and *Lactobacillus* spp., form intracellular polymers during periods of nutrient excess. Intracellular polysaccharides (IPS) can be degraded when supplies of nutrients are low (Bowden and Edwardsson, 1994). The majority of research has been carried out on *S. mutans* models, either from developing mutant strains lacking the ability to produce IPS, which were less cariogenic than their IPS-producing counterparts, or from the comparison of caries-active and caries-inactive plaque, which showed an association between IPS-production and caries-active plaque. It has become apparent, through extensive research, that dental caries does not occur *in vivo* if micro-organisms forming dental plaque are absent, therefore dental caries is regarded as a plaque-associated disease.

1.5.5 Specific & Non-Specific Plaque Hypotheses

As discussed earlier, Koch's postulates are applied for any microbe considered responsible for a given condition. Despite extensive sampling of plaque in health and disease, no single microbe has been found which completely satisfies Koch's postulates for plaque mediated diseases (Marsh and Martin, 1999). Thus another version has had to be devised to explain the role of individual bacteria from plaque in caries:

1. A microbe should be present in sufficient numbers to initiate disease.
2. The microbe should generate high levels of specific antibodies.
3. The microbe should cause disease in an appropriate animal model.
4. Elimination of the microbe should result in clinical improvement.

There are two hypotheses surrounding the primary bacteria involved in the initiation of caries and upon which a lot of debate has centred. The specific plaque hypothesis is the belief that the disease is caused by one or more specific bacteria. *S. mutans* is often thought to initiate almost all carious lesions, but some believe that *S. mutans* is important although not essential. The non-specific plaque hypothesis suggests that the disease is caused by a heterogenous mixture of non-specific bacteria and is not dependent on the presence of *S. mutans.*

Loesche (1976) who was continuing research that originated from Fitzgerald & Keyes (1960) first introduced the specific-plaque hypothesis. Research into caries initiation in hamsters and gnotobiotic rats (Van Houte, 1980) has shown that only certain species of *Streptococcus* and *Lactobacillus* lead to the initiation of caries. Prior to this, Klock and Krasse (1977) reported that caries risk was especially increased when high numbers of *Lactobacillus* spp. and *S. mutans* occurred together. Emilson and Krasse (1985) reviewed the biochemical properties required for caries initiation and the evidence supporting the specific plaque hypothesis and concluded that although several micro-organisms possess some of the biochemical properties, *S. mutans* is the only one which carries them all. Support for the non-specific hypothesis is limited, but both Mikkelson and Poulsen (1976) and Hardie *et al.* (1977) suggested that there was no correlation between *S. mutans* and *Lactobacillus* spp. with regard to the initiation of caries.

The microbial composition of supragingival plaque collected from the same site in the same mouth varies substantially with respect to time. Considering this wide variability, it is unreasonable to assume that the initiation and progression of all carious lesions are

associated with identical or even similar plaques from either a qualitative or a quantitative viewpoint. However, for the purpose of this thesis, there is good evidence to believe that *S. mutans* and *Lactobacillus* spp. are more important than others in the development and initiation of dental caries. Each of these micro-organisms will be discussed individually later in this chapter.

1.5.6 Prevention and Control

Interventions that disrupt the pathobiology of caries are needed to prevent and treat this aggressive infectious disease. This can be achieved in a number of ways, including a dietary change, improving general oral health by mechanical cleansing techniques, the use of topical applications such as fluoride supplements, antimicrobial agents, fissure sealants; and also vaccination.

1.5.6.1 Diet

It has been firmly established that dietary carbohydrates are caries-conductive and that they exert their cariogenic effect locally on the tooth surface (Johansson and Birkhed, 1994). All common dietary carbohydrates such as sucrose, glucose, fructose and lactose are used in the metabolism of many plaque bacteria which results in the production of organic acids (Geddes, 1972, 1975). However, it has been shown that sucrose possesses a greater caries-inducing potential when it is utilised by bacteria for the synthesis of insoluble glucans (Gustafsson, 1954; Newbrun, 1967, 1982; Minah and Loesche, 1977; Hamada and Slade, 1980). Therefore the use of artificial sweeteners is based on the

premise that they cannot be absorbed and metabolised by plaque bacteria to produce acid. Sugar alcohols (e.g. sorbitol) and lycasin are produced from corn-starch syrup and have a calorific value (nutritive sweetners); xylitol and saccharin are non-nutritive sweeteners. The reversal of caries by xylitol is due to the stimulation of salivary flow and the attendant rise in pH and calcium concentration when a sweet substance is introduced into the mouth (Leach and Green, 1981). Xylitol also interferes with acid production in *S. sobrinus* (Bagg *et al.*, 1999) which is regarded as one of the most cariogenic bacteria. The most important goal of preventing caries must be to reduce the consumption of carbohydrate products to a minimum, although this may be a difficult if not impossible task. The use of low-cariogenic sweeteners is therefore an attractive alternative as well as attempting to reduce the frequency of sugar intake.

1.5.6.2 Improving Oral Health

Dental health educators as well as their patients have regarded carbohydrate-containing residues at the tooth surface as the most important caries-promoting factor. As a result of this, toothbrushing after meals of sugary substances has been regarded as an important method of avoiding dental decay. Brushing of the teeth has for a long time been one of the basic components in programmes aiming at the prevention of dental caries (Koch *et al.*, 1994). As plaque removal is the goal of toothbrushing, caries reduction should be expected. However, the results of studies are still inconclusive. Conventional toothbrushing with a fluoridated toothpaste, even though it depends very much on the motivation and skill of the patient, is related to an overall reduction in the incidence of caries (Glass, 1986). Other aids for plaque removal, such as interdental

brushes, wood sticks and dental floss, may achieve some reduction in interdental caries, but there is little evidence for this as it seems unlikely that mechanical cleansing alone will reduce or prevent caries in fissures or pits significantly.

1.5.6.3 Fluoride

A more in depth analysis of the role of fluoride in caries prevention and control will be provided later in this chapter.

1.5.6.4 Antimicrobial Agents

The use of antimicrobial agents to control plaque has been advocated for a number of years and some of these products are anti-plaque rather than just anti-caries. Mouthrinses have proved to be an excellent vehicle for the delivery of anti-plaque agents. Chlorhexidine as a 0.12% to 0.2% mouthwash is the most successful agent in controlling dental plaque formation and has activity against many Gram-positive and Gram-negative oral bacteria (Emilson, 1994). At high concentrations, chlorhexidine is bactericidal and acts as a detergent by damaging the cell membrane. It binds to oral surfaces (especially teeth) and is released slowly into saliva over many hours at bacteriostatic concentrations that exert a number of effects:

1. Reduces plaque acid production by abolishing the activity of the PTS sugar transport system.
2. Inhibits amino acid uptake and catabolism in *S. sanguis.*

3. Affects various membrane functions, including the ATP-synthase and the maintenance of ion gradients in streptococci.
4. It is also possible that its presence on enamel surfaces and in saliva interferes with the adherence of plaque-forming bacteria, thus causing a reduction in the rate of plaque accumulation.

Mutans streptococci tend to be more sensitive to chlorhexidine than other streptococci commonly involved in plaque development, such as *S. sanguis*. This is convenient in that the antiseptic not only reduces the number and the end-products of the major group of cariogenic bacteria, but also tends to favour the growth of streptococci that are associated with health. Chlorhexidine can reduce plaque, caries and gingivitis in humans, although it is not used for prolonged periods of treatment because of side-effects such as tooth staining, mucosal irritation and its unpleasant taste.

Caution still needs to be taken regarding the regular use of antimicrobial agents from toothpastes and mouthrinses, since this could lead to the disruption of the ecology of the microflora by either:

1. Perturbing the balance among the resident microflora, which might lead to the overgrowth of potentially more pathogenic species, or
2. Development of resistance.

Manufacturers now have guidelines to adhere to for performing long-term clinical trials to confirm that these eventualities do not occur (http://home.intekom.com/pharm/group/corsodyl.html).

1.5.6.5 Fissure Sealants

Fissure sealants were developed in the early 1970's for occlusal caries prevention. They are regarded as a safe and effective measure in dental health care for the prevention of caries related to pits and fissures (Meurman and Thylstrup, 1994). Fissure sealants eliminate stagnation areas in the pits and fissures and block potential routes of infection by oral bacteria deep within the tooth (Bagg *et al.*, 1999). Sealants are effective as long as they remain firmly adherent to the tooth. Subsequently, the evaluation of their effectiveness involves the determination of occlusal caries reduction (Ripa, 1985). Studies by Meurman (1977), Mertz-Fairhurst *et al.* (1984) and Simonsen (1991) all record excellent caries reduction with retention rate of 28% in permanent first molars fifteen years after a single application.

Early carious lesions in fissures that are sealed tend not to progress, possibly because the source of microbial nutrition has been blocked. However, more extensive lesions will probably extend into the pulp as the bacteria will obtain sufficient nutrients from the carious dentine (Bagg *et al.*, 1999). Sealants can be applied in less time than amalgam restorations and they can be placed by dental auxillaries (Meurmann and Thylstrup, 1994). A sealant application has been estimated to cost almost half that for a one-surface amalgam, although Mitchell and Murray (1989) question the lack of valid information relating to the cost-effectiveness of this treatment.

1.5.6.6 Immunisation

Since vaccination has become one of the most valuable and successful means of preventing infectious disease, it seems reasonable to consider the development of a vaccine against dental caries. Theoretically, dental caries is no different from other diseases caused by micro-organisms, in being dependent on the microbial attack on one hand and the resistance of the host on the other (Killian and Bratthall, 1994). However, microbial attack and host resistance are much more difficult to define in the case of dental caries. While the microbial aetiology of dental caries is not totally specific, there is substantial evidence to implicate mutans streptococci as the major group of causative bacteria. This has led to the concept of using mutans streptococci (whole cell vaccines), or molecules derived from these bacteria (sub-unit vaccines), as an immunogen (active immunisation) (Marsh and Martin, 1999). Immunisation with either cell-wall associated antigens or glucosyltransferases from *S. mutans* is effective in reducing experimental dental caries in rats and monkeys. It is not entirely clear how the vaccine exerts its effect, although the following mechanisms have been suggested:

1. Inhibition of bacterial colonisation of enamel by secretory IgA.
2. Interference with bacterial metabolism.
3. Enhancement of phagocytic activity in the gingival crevice area due to the opsonisation of *S. mutans* with IgA or IgG antibodies.

A number of cell-wall associated vaccines have been tested and all have produced good protection against caries in monkeys, but no trials have yet been performed on humans.

Some of the antigens that were tested elicited antibodies that cross-reacted with heart-tissue and although this problem has been eliminated through the development of more defined and purified antigens (Bowen, 1996), other side-effects could emerge. This raises the question of balancing the risk of using a potentially hazardous vaccine in the prevention of a non life-threatening disease. Regulatory agents have made it apparent that tolerance levels would be extremely low to adverse effects from a vaccine when dental caries can be treated or prevented by alternative means (Bowen, 1996). Until immunisation has been tested *in vivo* it can only be regarded as a potential method of preventing dental caries.

1.6 *STREPTOCOCCUS MUTANS*

1.6.1 Introduction

Streptococci have been isolated from all sites in the mouth and comprise a large proportion of the resident oral microflora (Marsh and Martin, 1999). They constitute almost 50% of the total cultivable flora from saliva and the tongue, but only 30% of the total flora from the gingival crevice and supragingival plaque. The term 'viridans streptococci' has often been used alongside that of 'oral streptococci' (Gibbons, 1972) and refers to the type of haemolysis produced on blood agar. It produces a partial clearing of the erythrocytes around the colony, often accompanied by a green colouration of the agar, termed α- or partial-haemolysis (Whiley and Beighton, 1998). This use of the term 'viridans' arises from the employment of haemolysis to discriminate

between strains of streptococci, whether β-haemolytic (complete), α-haemolytic, or γ-haemolytic (non-haemolytic).

Eventually the term *Streptococcus viridans* was used to describe all the oral streptococci, despite reports of heterogeneity within strains being tested biochemically and serotypically (Sherman *et al.*, 1937). However, this is not strictly accurate as many of the currently recognised species include strains that are β-haemolytic or non-haemolytic, as well as α-haemolytic and some of these organisms can also inhabit other sites, such as the upper respiratory tract, gastrointestinal tract, and the female genital tract (Kennedy and Smith, 2000). Traditionally, oral streptococci have been differentiated by simple biochemical tests, but recent studies comparing DNA homology, whole cell protein profiles and the detection of glucosidase activity have clarified the taxonomic relationship between many species (Marsh and Martin, 1999).

1.6.2 Classification of Oral Streptococci

Early studies of the oral streptococci (Andrews and Horder, 1903; Gordon, 1905; Holman, 1916) characterised strains using a combination of carbohydrate fermentation tests, morphology (chain length) and growth on gelatin. *Streptococcus salivarius* and *S. mitis* were both described as being characteristic of the mouth and Coykendall (1974) named a third β-haemolytic group as *S. anginosus*, which was considered to be a pathogenic form of *S. salivarius*. In 1943, Sherman *et al.* observed a tendency in the literature for strains of *S. salivarius* to be characterised on the basis of fewer tests and that the group *S. mitis* should encompass the other α-haemolytic streptococci.

Since the study by Sherman *et al.* (1943), oral streptococci have been classified into one or more species groups. The most recently designated oral streptococcal groups were reported by Kawamura *et al.* (1995), and are based on 16S rRNA gene sequence comparisons (Table 1.1). The phylogenetic relationships for 34 species of streptococci, including the oral species, determined by rRNA gene sequencing are shown in Figure 1.5.

1.6.2.1 'salivarius'-group streptococci

This group of streptococci are facultative anaerobes and are either non-haemolytic on blood agar or produce alpha haemolysis (Whiley and Beighton, 1998). *Streptococcus salivarius* prefers to colonise mucosal, keratinised surfaces, especially the tongue. Strains produce an extracellular fructan (levan) and this gives rise to characteristically large mucoid colonies when grown on sucrose-containing agar (Gibbons, 1972). It also produces insoluble glucans (dextrans) from sucrose, giving a harder colonial texture with pitting of the agar, but it is not considered a significant opportunistic pathogen. *S. vestibularis* is isolated most commonly from the vestibular mucosa of the human mouth and is usually α-haemolytic on blood agar (Whiley and Hardie, 1988; Whiley and Beighton, 1998). These strains do not synthesise extracellular polysaccharides from sucrose, but Marsh and Martin (1999) report that they produce a urease (which can generate ammonia and hence raise the local pH) and hydrogen peroxide (which can contribute to the salivary peroxidase system, and inhibit competing bacteria). *S. thermophilus* strains are either α- or non-haemolytic on blood agar and also do not

Table 1.1 Current classification groups of oral streptococci (Marsh and Martin, 1999).

Group	Species
mutans-group	*S. mutans*, serotypes *c, e, f*
	S. sobrinus, serotypes *d, g*
	S. cricetus, serotype *a*
	S. rattus, serotype *b*
	S. ferus
	S. macacae
	S. downei serotype *d*
salivarius-group	*S. salivarius*
	S. vestibularis
anginosus-group	*S. constellatus*
	S. intermedius
	S. anginosus
mitis-group	*S. sanguis*
	S. gordonii
	S. parasanguis
	S. oralis
	S. mitis
	S. pneumoniae

Figure 1.5 *Streptococcus* phylogenic relationships

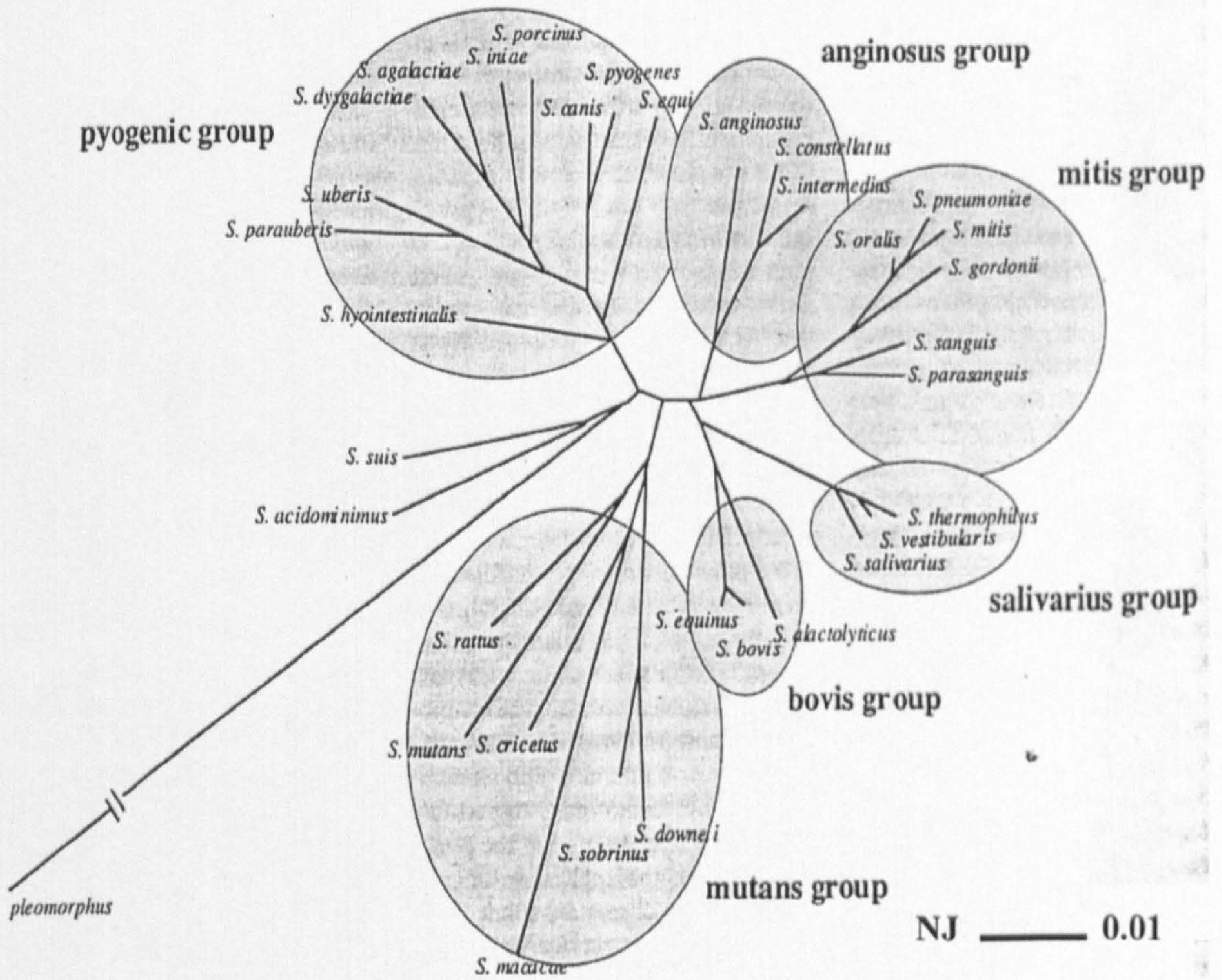


Figure 1.5 Phylogenetic relationships among 34 *Streptococcus* species by 16S rRNA gene sequence analysis. Distances were calculated by the neighbour-joining (NJ) method (Kawamura *et al.*, 1995).

produce extracellular polysaccharides on sucrose-containing media. The source of this organism is milk but the natural habitat is not known (Whiley and Beighton, 1998).

1.6.2.2 'milleri'-group streptococci

This group includes the organisms *S. anginosus, S. constellatus* and *S. intermedius* which form a biochemically and serologically heterogeneous collection of strains (Jacobs, 1997). The majority of strains in this group are either α- or non-haemolytic on blood agar but some strains can produce β-haemolysis (Whiley and Beighton, 1998). These oral streptococci are readily isolated from dental plaque and mucosal surfaces, but members are also an important cause of purulent disease in humans. *S. milleri* strains are commonly found in abscesses of internal organs and their predisposition for abscess formation was first noticed by Parker and Ball (1976), who compared the distribution of streptococcal species recovered from purulent lesions. *S. milleri* has been isolated from 56-81% of bacterial brain abscesses in either pure or mixed culture (Gossling, 1988) and implicated in abscess formation in liver, joints, myocardium and spleen (Barnham, 1989).

The taxonomy of this group of organisms has been subject to much debate and the initial grouping of *S. milleri* was separated into the distinct species *S. anginosus, S. constellatus* and *S. intermedius* (Jacobs, 1997). However in the 1980s, DNA-DNA re-association studies yielded yet more controversy until Coykendall *et al* (1987) published the unification of all *S. milleri* strains into a single species , *S. anginosus* which is the oldest approved name for these bacteria. Eventually, Whiley and Beighton (1991) published

an amended description of the three species and consequently re-instated the nomenclature *S. anginosus, S. constellatus* and *S. intermedius*. Confirmation of the current taxonomy has been shown by rRNA gene restriction patterns and 16S rRNA sequences (Bentley *et al.*, 1991).

1.6.2.3 'mitis'-group streptococci

Recent molecular research into this group of bacteria has clarified many earlier classification anomalies. *S. sanguis* and *S. gordonii* are now individual species and although they both produce extracellular soluble and insoluble glucans from sucrose, *S. sanguis* produces a protease that can cleave surface IgA (sIgA) (Kilian, *et al.*, 1989; Whiley and Beighton, 1998), while *S. gordonii* can bind α-amylase, enabling these strains to break down starch (Marsh and Martin, 1999). *S. oralis* is the officially recognised name for *S. mitior* and these strains produce neuraminidase (sialidase) and an IgA protease (Whiley and Beighton, 1998) but cannot bind α-amylase. *S. mitis* strains do not produce any extracellular polysaccharides from sucrose or produce IgA protease. *S. parasanguis* can be isolated from clinical specimens such as the throat, blood and urine (Whiley and Beighton, 1998). Strains hydrolyse arginine and can bind salivary α-amylase, but cannot produce extracellular polysaccharides from sucrose. *S. crista* has been proposed for strains that resemble *S. sanguis* but Handley, *et al.* (1991) characterised them by tufts of fibrils observed on their cell surface. Some of these strains can produce extracellular polysaccharide (glucan) from sucrose (Whiley and Beighton, 1998). *S. pneumoniae* is isolated from the nasopharynx, and can acquire and transfer antibiotic resistance genes among the other members of the *S. mitis*-group.

Strains produce strong α-haemolysis on blood agar and colonies can be mucoid due to the production of a polysaccharide capsule (Hardie and Whiley, 1994; Whiley and Beighton, 1998).

1.6.2.4 'mutans'-group streptococci

Most of the research performed in recent years into the role of micro-organisms in caries has concentrated on mutans streptococci, especially *S. mutans*. They are characteristically non-haemolytic on blood agar. Their name derives from the fact that cells can lose their coccal morphology and often appear as short rods or as cocco-bacilli. Among strains formerly assigned to the single species *S. mutans* on the basis of phenotypic characters defined by Facklam (1974), eight serotypes were recognised (*a-h*) based on the serological specificity of carbohydrate antigens located in the cell wall (Bratthall, 1969, 1971; Perch *et al.*, 1974; Beighton *et al.*, 1981). Subsequent work has shown that significant differences existed between clusters of these serotypes to warrant their sub-division into seven distinct species (Table 1.1); these are collectively described as mutans streptococci (Coykendall, 1977, 1983; Beighton *et al.*, 1981; Whiley *et al.*, 1988).

1.6.3 Mutans Streptococci

S. cricetus (serotype *a*) was isolated from a decayed hamster tooth and was described by Fitzgerald and Keyes (1960). It can be found in the human mouth but is not as common as *S. mutans* in man (Bratthall, 1972; Perch *et al.*, 1974). *S. rattus* (serotype *b*) was also

identified by Fitzgerald and Keyes (1960) and refers to the source of the original isolate, the rat. Like *S. mutans*, *S. rattus* can store, and then catabolise, intracellular polysaccharide (Freedman and Coykendall, 1975). The species is not as common as *S. mutans* in humans (Bratthall, 1972; Perch *et al.*, 1974), although Bratthall (1972) has suggested it to be more common in certain populations.

S. downeii (serotype *h*) was proposed as a distinct species by Whiley *et al.* (1988) on the basis of data collected from biochemical characteristics together with data from chemotaxonomic and immunological studies and DNA-DNA hybridisation experiments.

S. ferus was isolated by Coykendall (1974) from the mouths of wild sucrose-eating rats living in sugar cane fields. Freedman *et al.* (1982) demonstrated that strains were non-cariogenic in animal models and genetic relationship studies carried out by Gilmour *et al.* (1987) suggest that it is phylogenetically closer to species of the sanguis complex. Another species, *S. macacae*, was isolated from dental plaque of monkeys by Beighton and colleagues (1984) and this has been shown to carry the serotype *c* antigen. Neither of these two species is found in human dental plaque.

The second most commonly isolated species from human dental plaque is *S. sobrinus* (serotypes *d* and *g*), which has also been associated with human dental caries (Bratthall, 1972; de Soet *et al.*, 1987; Köhler, 1995). Research carried out by de Soet (1989) found that of all the mutans streptococci they tested, *S. sobrinus* produced acids more rapidly from glucose than *S. mutans*, especially at low pH levels. It has been reported that *S. sobrinus* produces less intracellular polysaccharide than *S. mutans*, therefore all

available glucose can be used for acid production (Hamada and Slade, 1980). Results presented by de Soet (1991) suggest that *S. mutans* and *S. sobrinus* might play different roles in the aetiology of dental caries, which was supported by findings from Huis in't Veld (1982) and Lindquist and Emilson (1989) who reported that *S. mutans* and *S. sobrinus* have different habitats. Previous studies into dental caries are misleading as they do not attempt to differentiate between *S. sobrinus* and *S. mutans*, while some commonly used selective media for the isolation of mutans streptococci from dental plaque contain bacitracin which can be inhibitory to the growth of both *S. sobrinus* (Emilson and Bratthall, 1976; Little *et al.*, 1977; Van Palenstein Helderman *et al.*, 1983; Tanzer *et al.*, 1984; Jordan, 1986; Schaeken *et al.*, 1986; Wade *et al.*, 1986) and *S. cricetus* (Shklair and Keene, 1974).

The most commonly isolated species of mutans streptococci from dental plaque is *S. mutans*, which is the appellation given to isolates previously described as serotypes *c, e* and *f* (Marsh and Martin, 1999). Epidemiological studies have implicated it as the primary pathogen in the aetiology of enamel caries in children and young adults, root surface caries in the elderly and nursing (or bottle) caries in infants. The rest of this section will focus on this species.

1.6.4 Early Microbiological Studies

Clarke (1924) first isolated *S. mutans* from carious teeth, and shortly afterwards Abercrombie and Scott (1928) recorded an association with a case of infective endocarditis. It was a number of years later before interest was renewed in this species.

In 1960, Keyes demonstrated that tooth decay could be transmitted between strains of hamsters in a manner similar to other infectious diseases. Subsequent studies by Fitzgerald and Keyes (1960) led to the isolation of *S. mutans* from rodent carious lesions which were able to initiate rampant decay when introduced into the mouths of caries 'inactive' hamsters. These studies led to the development of animal models for the assessment of the cariogenic potential of pure cultures of human oral bacteria. These include the two main models using pathogen-free hamsters (indigenous flora does not include any bacteria that can initiate caries) and germ free rats (completely free of bacteria).

1.6.5 Cariogenic Potential of *S. mutans*

The optimum approach for identifying a virulence factor is to isolate a mutant defective in the specific property under scrutiny. Initial investigations isolated relevant strains either as spontaneous mutants or following treatment with chemical mutagens (Loesche, 1986; Lang *et al.*, 1987). Although useful information was obtained from these studies, it was not possible to define precisely the nature of the mutation and therefore multiple defects in the strains were still a consideration. Advances in molecular biology have made it possible to construct defined mutants that are defective only in a particular gene. These mutants can then be tested in animal model systems, in an attempt to identify direct relationships between a particular trait and cariogenicity.

S. mutans possess an impressive combination of commonly accepted cariogenic traits and it is considered to be the prime aetiological agent of human dental caries. These

include sophisticated sugar transport systems, a high rate of acid production, a high acid tolerance, sucrose-mediated extracellular polysaccharide synthesis, and intracelluar glycogen synthesis from a variety of dietary carbohydrates. They also exhibit, in a variety of experimental animals, a high ability to induce dental caries on multiple dental surfaces in the presence of sucrose (Tanzer, 1989; Van Houte *et al.*, 1991).

1.6.5.1 Sugar Transport Systems

As mentioned in Section 1.3, bacteria must first be able to facilitate the transfer of dietary carbohydrates across their membrane before they can metabolise them and produce acid. The most significant system is the PTS (*Section 1.3.6*), which is a high affinity sugar transport system for mono- and di-saccharides in acidogenic bacteria. It is constitutive for glucose, mannose and sucrose, but must be induced for lactose, mannitol and sorbitol. When grown in the presence of any of these sugars, there is the induction of distinct PTS systems, although they are rapidly repressed by glucose (Vadeboncoeur and Pelletier, 1997). Regulation of this system is important during periods of low pH and high sugar concentrations and it has been established that Enzyme I, HPr and membrane and cytoplasmic sugar-specific components of the PTS play key and global regulatory roles (Carlsson and Hamilton, 1994; Vadeboncouer and Pelletier, 1997; Burne, 1998). Mutations in Enzyme II of the PTS caused a decrease in sugar transport, but this did not have an effect on cariogenicity a finding which may not be altogether surprising since *S. mutans* possesses multiple strategies for transporting sugars (see section 1.3) (Marsh and Martin, 1999).

1.6.5.2 Acid Production

An efficient glycolytic pathway rapidly produces low terminal pH values in plaque. Oral streptococci convert pyruvate to lactic acid (catalysed by lactate dehydrogenase) when sugars are in excess, while formate, acetate and ethanol are the products of metabolism by mutans streptococci under carbohydrate limitation (Carlsson and Hamilton, 1994). Lactate and protons are transported across the cell membrane of *S. mutans* as lactic acid in a carrier-mediated process (Marsh and Martin, 1999). Addition of a fermentable substrate to cells leads to an accumulation of lactate and protons are pumped out by an ATP synthase. A transmembrane pH gradient is generated which can then be used as the driving force to transport the lactate as lactic acid out of the cell. Mutants deficient in lactate dehydrogenase activity do not produce any lactic acid, which has been shown to have a reduced effect on the cariogenicity of the mutant strain (Marsh and Martin, 1999).

1.6.5.3 Acid Tolerance

As mentioned earlier (*see* Section 1.4), the survival of a bacterial species under acidic conditions depends on its ability to maintain intracellular pH homeostasis. *S. mutans* achieves this by proton extrusion via an ATP-synthase (H^+/ATPase) and acid end product efflux (Carlsson and Hamilton, 1994, Svensäter *et al.*, 1997). These mechanisms ensure that the pH inside the cell remains higher than that of the external environment when the pH falls due to the formation of acid end products. It has been shown that *S. mutans* undergoes a specific alteration in its physiology in order to survive

in an acidic environment (Dashper and Reynolds, 1992; Bowden and Hamilton, 1998). The factors involved in this acid tolerance response, as summarised by Marsh and Martin (1999), are:

1. An increase in glycolytic activity
2. A shift to lower pH optimum for glucose transport, glycolysis and proton impermeability (Burne, 1998)
3. Decrease in activity of specific components of the PTS sugar system
4. Increased activity of the H^+/ATP synthase
5. Increased capacity to maintain transmembrane gradients at lower pH values
6. Shift to homo-fermentative metabolism
7. Synthesis of stress response proteins

This metabolic strategy gives *S. mutans* a competitive advantage at low pH over organisms associated with sound enamel, such as *S sanguis*, that lack this response. Mutants which have had some aspect of this aciduricity decreased have shown a reduced tolerance to acid environments and thus have displayed a reduced cariogenic effect (Marsh and Martin, 1999).

1.6.5.4 EPS Synthesis

Extracellular polysaccharides contribute to the plaque matrix, consolidate attachment of cells and may localise acidic fermentation products (Carlsson and Sundström, 1968; McNee *et al.*, 1982; Van Houte *et al.*, 1989). As mentioned in earlier sections, (1.2 and

1.5), *S. mutans* degrades sucrose into glucose and fructose and possesses two specific enzymes for this function. Glucosyltransferases (GTFs) catalyse the synthesis into soluble and insoluble glucans from sucrose (with the release of fructose), which are important in plaque formation and in the consolidation of bacterial attachment to teeth (Koo *et al.*, 2003). Fructosyltransferases (FTFs) produce fructans from sucrose (and liberate glucose), which are frequently labile and can be used by other plaque micro-organisms (Kuramitsu, 1974; Tanzer *et al* 1974; Marsh and Martin, 1999). The importance of polysaccharide production in colonisation and cariogenesis by *S. mutans* has been demonstrated by animal-model experiments utilising isogenic mutants. Mutants of *S. mutans* disrupted in the *gtf*B and *gtf*C genes, normally encoding the enzymes that synthesise water insoluble glucans, are much less cariogenic than the wild type (Munro, *et al.*, 1991; Yamashita *et al.*, 1993; Jenkinson and Lamont, 1997).

1.6.5.5 IPS Production

Intracellular polysaccharides in oral bacteria are usually glycogen-like in structure and are formed in the presence of high concentrations of sugars. Any sugar can act as a substrate provided it can be converted into glucose-1-phosphate. There has been much interest in the possible role of IPS in the pathogenesis of dental caries. During spells of dietary sugar 'famine', stored IPS is converted to energy for acid production (Bowden and Edwardsson, 1994; Carlsson and Hamilton, 1994; Marsh and Martin, 1999). Investigations into both chemically induced and defined mutants defective in IPS synthesis have produced fewer carious lesions than parent strains when inoculated in

pure cultures in gnotobiotic rodents (Loesche, 1986; Lang *et al* 1987; Kuramitsu, 1993; Russell, 1994).

Sections 1.2 and 1.6 both describe other cariogenic bacteria important in the accumulation of dental plaque and the aetiology of dental caries. *S. mutans* is the strongest acid producer, although lactobacilli are regarded as the most acid tolerant bacteria.

1.7 *LACTOBACILLUS* SPP.

1.7.1 Introduction

Lactobacilli comprise part of the indigenous microflora of the mouth, gastrointestinal tract and female genital tract. When encountered in the GI tract they are rarely, if ever, associated with disease. They are also the dominant micro-organism of the vagina from menarche to menopause, where they exert a certain amount of control over their environment. Lactobacilli have usually been considered to be non-pathogenic to man and have in recent years been actively investigated for their potentially beneficial effects (Harty *et al.*, 1994). There is much interest in the use of lactobacilli as probiotics against human gastrointestinal disorders (Song *et al.*, 2000). However there have been an increasing number of reports of their association with human infections, although the number is still small. These include associations with septicaemia, rheumatic vascular disease, meningitis, lung abscesses and infective endocarditis (Sharpe *et al.*, 1973; Biocca and Seppilli, 1974; Davies *et al.*, 1986; Sussman *et al.*, 1986; Nuadé *et al.*, 1988;

Struve *et al.*, 1988). Tenenbaum and Warner (1975) suggested that infective endocarditis cases involving lactobacilli had a predisposing event such as poor dentition or recent dental manipulation. These associations led to the observation by Maskell and Pead (1992) that lactobacilli should be considered as emerging pathogens.

Most *Lactobacillus* species recovered from clinical specimens are microaerophilic, but occasionally an obligately anaerobic isolate can be recovered. The identification of anaerobic lactobacilli to the species level is extremely difficult and, given the low pathogenicity of the organism, is rarely indicated (Hillier and Moncla, 1991). *Lactobacillus* spp. can usually be identified on the basis of Gram stain morphology, a negative catalase test, and gas chromatography showing a major lactic acid peak from glucose. There are commercially available identification systems available, although studies have shown that these tend to be unreliable (Hillier and Moncla, 1991).

1.7.2 Oral Lactobacilli

Lactobacilli are commonly isolated from the oral cavity although they have been reported to comprise less that 1% of the total cultivable microflora (Van Houte, *et al.*, 1981; Marsh and Martin, 1999). Little is known about the preferred habitat of these species in the normal mouth and most studies still refer to them as simply 'lactobacilli' or *Lactobacillus* spp. Edwardsson (1974) and Van Houte (1994) found them to be associated more with carious dentine and the advancing front of the carious lesions than with the initiation of the disease. Lactobacilli have been used as indicator organisms of caries susceptibility since 1941 (Arnold and McClure, 1941; Becks *et al.*, 1944) and a

definite relationship between the incidence of caries and lactobacilli has been indicated in more recent reports (Minah, *et al.*, 1985; Krasse, 1988; Bjarnason, 1989; Alaluusua *et al.*, 1989). Less care has been taken in speciating the lactobacilli from carious lesions compared to *Streptococcus* species and a close association of one or more species of *Lactobacillus* with caries cannot be made. However, Boyar and Bowden (1985) suggested that *L. casei, L. fermentum, L. plantarum* and *L. acidophilus* could be isolated from carious dentin and early caries lesions. More recently Botha and colleagues (1998) reported that *L. paracasei* and *L. rhamnosus* were the predominant species present in caries lesions and their control group.

Simple tests with selective media have been designed for estimating the numbers of lactobacilli in patients' saliva to give an indication of the cariogenic potential of a mouth. Studies have shown that the levels of lactobacilli correlate well with the intake of dietary carbohydrate (Rodriguez, 1930; Hadley, 1933; Jay *et al.*, 1936) and are therefore a useful tool to monitor the dietary behaviour of a patient.

1.7.3 Classification of Oral *Lactobacillus* spp.

Enright *et al.* (1932) recorded evidence that indicated the importance of oral lactobacilli in the causation of tooth decay and advised that taxonomic studies of oral lactobacilli should be undertaken for the purpose of selecting the best name for these tooth strains. According to Williams and Franck (1957), the lactobacilli that were isolated from human saliva or from tooth and other surfaces in the oral cavity were classified into homofermentative and heterofermentative groups based on the relative amount of lactic

acid among the by-products. The homofermenters (mostly *L. casei* strains) produce only trace quantities of by-products other than lactic acid, whereas heterofermenters (*L. brevis, L. buchneri, L. fermentum*) produce considerable amounts of other by-products, such as carbon dioxide, alcohols and acetic acid (Williams and Franck, 1957). However, more recently *L. casei* strains have been re-classified as facultative heterofermenters as they are able to generate lactic, acetic and formic acids (Carlsson and Hamilton, 1994).

Sharpe (1973) used serological characteristics to classify lactobacilli. These were based on the procedure originally devised by Lancefield for determining pathogenic streptococci. Serological group A contained a number of species including *L. helveticus*, which were defined on their physical properties, and groups B and C comprised strains of *L. casei* that were distinguished on the basis of different cell wall antigens. Group D included many *L. plantarum* strains, Group E antigens were isolated from *L. buchneri* and group F was represented by *L. fermentum*. Strains of *L. salivarius* were placed in another group, designated G (Knox and Wicken, 1976).

Rogosa and co-workers (1953) identified lactobacilli in the oral cavity and reported that *L. casei*, *L. fermentum* and *L. acidophilus* were the predominant species isolated from the saliva of schoolchildren, representing 59%, 45% and 22% of the total oral lactobacilli respectively. However, in 1989, Collins *et al.* found that *L. casei* was inadequately defined on the basis of phenotypic criteria which resulted in the recognition of five subspecies; namely: *L. casei* subsp *rhamnosus*, *L. casei* subsp *casei*, *L. casei* subsp *alactosus*, *L. casei* subsp *tolerans* and *L. casei* subsp *pseudoplantarum*. These subspecies displayed considerable heterogeneity and in view of this uncertainty, Collins,

et al. (1989) suggested a new classification based on genetic evaluation. The new classification suggested that the subspecies should be changed as follows:

1. *L. casei* subsp *casei* changed to species *L. casei*
2. *L.casei* subsp *rhamnosus* changed to species *L. rhamnosus*
3. *L. casei* subsp *alactosus*, *L. casei* subsp *tolerans* and *L. casei* subsp *pseudoplantarum* changed to species *L. paracasei*

Apart from a few exceptions, *L. casei* has been classically accepted as the predominant *Lactobacillus* species in dental plaque and saliva (Shovell and Gillis, 1972; Hahn *et al.*, 1989). However, some reports have suggested that *L. casei, L. fermentum* and *L. acidophilus* are unable to form plaque without the participation of *Streptococcus mutans* or *Streptococcus sanguis* (Russel and Ahmed, 1978).

1.7.4 Association of Oral Lactobacilli with Dental Caries

It was Kligler in 1915 that showed the increased presence of *B. acidophilus* in the mouths of individuals with caries (Snyder, 1938) and this was later confirmed and extended by Rodriguez (1930). Hadley (1933) discussed the need for a quantitative method for estimating amounts of *Bacillus acidophilus* in saliva due to previous workers identifying its presence solely by qualitative means (Bunting and Palmerlee, 1925; Enright *et al.*, 1932). These studies revealed differences between caries-susceptible and caries-free subjects and therefore Hadleys' reasoning was to develop a solid medium on which the enumeration of *B. acidophilus* could be carried out and then to undertake a

comparative study of caries-free and caries-susceptible children. The outcome of this study was the observation that "*B. acidophilus* is invariably present, and usually in great numbers, in the mouths of caries-susceptible subjects, in caries-free individuals its presence is variable and usually marked".

Much of the research carried out at that time was undertaken at the University of Michigan Dental School by Bunting and colleagues, where they concluded that a lactobacillus, which they specifically called *Lactobacillus acidophilus*, was the organism to be associated with tooth decay. This was contradicted by other workers who were unable to confirm the correlation of lactobacilli with dental caries or who insisted that other organisms must be considered (Hadley, 1933). However, Snyder (1938) published results on the occurrence of lactobacilli and other acidogenic bacteria in the saliva of selected caries-free children. The conclusions from this study were that the children who had no lactobacilli in their saliva at time of collection remained free from caries, and those who had significantly appreciable numbers of lactobacilli in the saliva showed varying amounts of decay. They also concluded that other aciduric organisms were isolated and that only the yeasts seemed to have any relation to the caries observed and this role was secondary to that played by the lactobacilli.

Further studies carried out on oral lactobacilli focused on defining a selective medium for the isolation and enumeration of these micro-organisms (Rogosa *et al.*, 1951; De Man *et al.*, 1960). More recent studies have concentrated on developing rapid methods for identification of *Lactobacillus* spp., including the Dentocult system and Snyder dip slide test.

1.7.5 Cariogenicity of Lactobacilli

A number of early studies used gnotobiotic rats infected with *Lactobacillus casei* (Rosen *et al.*, 1968) and *L. acidophilus* (Fitzgerald, 1966; Hammond, 1971). Later work employed conventional hamsters infected with *L. salivarius* and *L. fermentum* (Fitzgerald *et al.*, 1980). These experts showed that lactobacilli, in conjunction with diets high in fermentable carbohydrates, resulted in extensive cavitation of the molar teeth and coronal caries. One of the major differences between the two animal models used is that there is the absence of a competing oral flora in the gnotobiotic rat. This induction of caries in the hamsters harbouring a mixed oral flora was therefore regarded as significant because this property has rarely been observed for other plaque micro-organisms, with the exception of *S. mutans* (Fitzgerald *et al.*, 1980).

Duchin and Van Houte (1978) carried out a cross-sectional study into the relationship of *S. mutans* and lactobacilli to smooth surface caries in man and demonstrated that *S. mutans* was more closely associated than *Lactobacillus* spp. with initial lesions. Van Houte *et al.* (1981) carried out a study into the presence of lactobacilli on sound teeth under oral conditions resembling those normally associated with significant caries activity. Lactobacilli were isolated from half of the plaques obtained from their subjects but they were not isolated from about a third of all caries-associated plaques. Their findings support the idea that lactobacilli may contribute to the initiation of some, but not all, caries lesions.

1.7.5.1 EPS and IPS Synthesis

Lactobacilli do not possess the same number of cariogenic traits as *S. mutans*, but they still feature enough to implicate them as causative agents of dental caries. As mentioned earlier (*see* Sections 1.5.4 and 1.6.5.4), the ability to synthesise EPS conveys cariogenicity to *S. mutans*. However, Fitzgerald *et al.* (1980) reported that the strains of *L. salivarius* and *L. fermentum* that induced coronal caries in hamsters did not form measurable amounts of insoluble extracellular polysaccharides. Some lactobacilli have the ability to synthesise glucans as well as heteropolysaccharides (Colby and Russell, 1997).

Although the formation of intracellular polysaccharides is considered to be one of the determinants of virulence in *S. mutans*, the ability to form IPS is a variable trait in lactobacilli. Kobayashi and Takei (1972) reported that the ratio of IPS-producing lactobacilli to total lactobacilli was 48% in plaque and 8.2% in carious dentine of caries-active individuals, but found no IPS-storing lactobacilli in the plaque of caries-inactive persons. *L. salivarius* was found to be the most active IPS former and accounted for 86% of these types in caries-active plaque. Heterofermentative lactobacilli, on the other hand, rarely produced IPS regardless of their source. Fitzgerald *et al.* (1981) reported that out of 32 *Lactobacillus* strains only 7 produced IPS, six of which were associated with moderate to severe caries activity. A majority of strains which were moderately to highly cariogenic were IPS-negative, suggesting that the degree of caries activity was not directly influenced by IPS production.

1.7.5.2 Sugar Transport and Fermentation

Lactobacillus spp. transport sugars by the phosphotransferase system then generate energy via the glycolytic pathway or the pentose phosphate pathway. *L. acidophilus* and *L. salivarius* (homofermenters) utilise the glycolytic pathway and the main end product of metabolism is lactic acid. Other lactobacilli such as *L. fermentum* and *L. brevis* (heterofermenters) degrade glucose by the pentose phosphate pathway, resulting in the formation of lactic and acetic acids and carbon dioxide. *L. casei* and *L. plantarum* are equipped with both of these pathways and are called 'facultative heterofermenters' generating lactic, acetic and formic acids, and ethanol (Carlsson and Hamilton, 1994). The most caries-active strains are among the homofermentative lactobacilli, which as a group attain lower pH levels in carbohydrate fermentations than the heterofermenters (Fitzgerald *et al.*, 1981).

There has been much interest focused on the various sucrose substitutes for sweets and beverages in an attempt to reduce dental caries, therefore many sugar alcohol substitutes such as xylitol and sorbitol are already used. However an early study by Rogosa *et al.* (1953) shows that *L. casei* and *L. salivarius* are able to produce acids from sorbitol. Further studies have also found that *Lactobacillus* spp. are capable of producing acids from sorbitol as well as xylitol (Kalfas *et al.*, 1990 a and b; Badet *et al.*, 2001). However, the threat of caries from the fermentation of sorbitol is considered to be small in people with a low salivary flow and unlikely to increase any cariogenic risk in normal people (Hogg and Rugg-Gunn, 1991).

1.7.5.3 Acid Tolerance

One of the characteristics of lactobacilli is their acid tolerance which enables them to survive and reproduce at low pH (Badet *et al.*, 2001). Svensäter *et al.* (1997) demonstrated that the pH threshold which will result in death for *L. casei* strains varies between 4.5 and 2.3. The aciduriry of *L. casei* has been reported as not only being due to their ability to initiate and maintain growth at low pH levels but also in the ability to produce lactic acid in lactate buffers below pH 5.0 (Harper and Loesche, 1994). Their ability to maintain intracellular pH homeostasis is much the same as that of *S. mutans* (*see* Section 1.6.5.3). They have higher levels of ATP-synthase activity and the pH optimum for activity is much lower than for less tolerant species such as *S. sanguis* or *A. naeslundii* (Marsh and Martin, 1999). However, less is known about the capacity of *Lactobacillus* than *S. mutans* to tolerate acid over long periods of time. Nevertheless they must have the capacity to withstand the continual cycles of short-term acid shock that results from the exposure to dietary carbohydrates and accumulation of acid by-products (Svensäter *et al.*, 1997).

1.8 FLUORIDE

1.8.1 Introduction

When fluoride is considered biochemically, it is usually classed with the trace elements. This is reasonable since fluoride can occur biologically in very small amounts, and concentrations in biological materials generally are in the parts per million (ppm) range

or less. The term "trace" element, however, does not accurately describe the extent to which fluoride is present in the environment (Smith and Ekstrand, 1996). The fluoride concentration in soils varies enormously, the published figures ranging from 10 to 1070 ppm, with average values between 200 and 300 ppm (Murray *et al.*, 1991). Fluoride also occurs in sea water and is present in nearly all fresh ground waters ranging from trace quantities to over 25 mg/l (MRC report, 2002). Additional fluorides are widely distributed in the atmosphere, originating from the dusts of fluoride-containing soils, from gaseous industrial wastes, from the burning of coal fires in populated areas and from gases emitted in areas of volcanic activity. Thus fluoride, in varying concentrations, is freely available in nature (Murray *et al.*, 1991).

1.8.1.1 Fluoride Intake

Water is the predominant source of fluoride for most individuals living in communities in which the fluoride concentration of the water supply is 0.7 mg/L or more. Individuals living in communities with low concentrations of fluoride in the drinking water may still obtain a substantial intake of fluoride from beverages and other foods commercially prepared in locations with fluoridated water (Fomon and Ekstrand, 1996). In 1985, Singer and colleagues estimated that water and non-dairy beverages accounted for 75% of the total fluoride intake of adults in the United States. Grain and cereal products accounted for 7%, meat, fish and poultry provided 6% and all other foods contributed 12%. Pang *et al.* (1992) recorded that fluoride intake in preschool children (1-6 years of age) from North Carolina is predominantly from beverages rather than food. Intake is low from children who consume mainly milk but is considerably higher when there is

greater consumption of fruit juices, fruit-flavoured drinks and carbonated beverages. Much of an infant's diet consists of human milk or milk of other mammals. Infant formulae provide variable amounts of fluoride, the amount depending on the fluoride content of the water used as a diluent (Fomon and Ekstrand, 1996).

There is also no doubt that toothpastes containing fluoride contribute to the total fluoride intake as this has been demonstrated by measurements of elevated fluoride concentrations in plasma and urine after toothpaste use (Fejerskov *et al.*, 1996).

1.8.1.2 Fluoride Excretion

The principal route of fluoride excretion is via the urine and the urinary fluoride level is widely regarded as one of the best indices of fluoride intake. In infants, as much as 90% of fluoride intake may be retained (Bergmann and Bergmann, 1995). This proportion decreases with age (Ekstrand *et al.*, 1984). In older children and adults, more than 90% of fluoride that is ingested is excreted via the urine and only a minor proportion is retained in the skeleton (Bergmann and Bergmann, 1995).

1.8.1.3 The Fluoride Debate

Tooth changes caused by fluoride were first reported in 1916 when Black and McKay described 'mottled enamel' and it was almost thirty years later before it was suggested that it could be related to the naturally fluoridated water supply in the endemic areas (Murray *et al.*, 1991). During the 1930's, Dean and his co-workers expanded on these

observations and established relationships between mottled enamel (chronic fluorosis) and the level of water fluoridation. They also suggested a classification system depending on the degree of enamel changes. In 1933, Ainsworth contributed statistical evidence showing that caries experience in a fluoride area was lower than average. When it became evident that low concentrations of fluoride in the water supplies were also associated with lower-than-expected caries experience (Dean *et al.*, 1939), this classification system became an important tool in the development of strategies for reduction of caries experience without causing unacceptable dental fluorosis (Fejerskov *et al.*, 1996). By 1945, research carried out in the United States had established that apart from dental fluorosis, there was no other association of general health problems found among people who had been drinking waters containing up to 8 mg F/L for long periods of time (Burt and Fejerskov, 1996). Consequently, dental fluorosis became considered as a public health problem only if it was aesthetically unacceptable. However, the opinions of fluoridation and particularly fluoridated water have remained a contentious issue with many reviews carried out over the past fifty years.

Recently, in the U.K., the Department of Health commissioned the National Health Service Centre for Reviews and Dissemination (NHSCRD) at the University of York to produce an 'up to date expert review of fluoride and health'. The York review (www.york.ac.uk/inst/crd/fluorid.htm) confirmed that water fluoridation did exert a beneficial effect on dental caries, but also highlighted the increased prevalence of dental fluorosis associated with fluoridation. The review considered experimental evidence on fluoride associated with bone fracture and bone development problems and concluded that there was no clear association. This was also found between water fluoridation and

incidence or mortality of bone cancers, thyroid cancers or all cancers. Studies examining other possible negative effects were included in the review, but they provide insufficient evidence on any particular outcome to permit confident conclusions. The review recommends further research of a much higher quality in these areas.

1.8.1.4 Fluoride Toxicity

Despite the worldwide availability of fluoride in water, foods, toothpastes, mouthwashes, supplements and various topical applications for teeth, fluoride is also a toxic substance. Its ingestion in large quantities may be followed by rapidly developing signs and symptoms which may result in death (Whitford, 1996). Dental fluorosis occurs when relatively small amounts are ingested during the period of tooth development. Ingestion of larger amounts causes changes in the quality and quantity of the skeleton. This is the basis of the use of fluoride for the treatment of osteoporosis, but the skeletal changes can become so severe as to be classified as crippling skeletal fluorosis.

Fluoride poisoning has also been reported, although most of the cases have occurred accidentally due to mistaking the identity of sodium fluoride for sodium bicarbonate, flour or magnesium sulphate (Epsom salt) (Whitford, 1996). It is essential that the fluoride concentrations in dental products be known by the persons who use them. One problem is that young children occasionally drink mouthrinses or eat dentrifices or too many fluoride tablets. Therefore, recommendations suggest that they should not be used

by young persons without adult supervision and that they should be kept out of the reach of young children.

1.8.2 Mechanisms of Action of Fluoride

The fact that the cariostatic effect of fluoride was first discovered in relation to the natural fluoride content of drinking water was interpreted as meaning that the effect was due to the systemic, pre-eruptive incorporation of fluoride into the tooth. It had already been established that acids initiated dental decay; therefore, the effect of fluoride was expected to be related to reducing the solubility of tooth enamel. It is now believed that the effectiveness of fluoride is due to its presence in the aqueous phase during enamel dissolution and that it acts in three ways:

1. The systemic effect (pre-eruptive): ingested fluoride is incorporated into developing enamel as fluorapatite which reduces its solubility in acid and promotes its remineralisation (Groeneveld, *et al.*, 1990). Fluoride is present in some water supplies (naturally or artificially at 1 part per million), certain foods, tablets or added to milk.
2. The topical effect (post-eruptive): the surface layer of enamel is converted into fluorapatite which reduces its solubility in acid and promotes remineralisation (Groeneveld, *et al.*, 1990). This is achieved through the use of fluoride-containing toothpastes, gels, certain filling materials, e.g. glass ionomers, water, salt and milk fluoridation.

3. The antimicrobial effect: fluoride inhibits plaque metabolism and is concentrated within plaque (Hamilton, 1990). Activity increases at pH values less than 5, especially in the case of *S. mutans.* Its inhibition mechanisms include reducing glycolysis, inhibiting sugar transport indirectly, interfering with bacterial permeability, inhibiting the synthesis of glycogen for intracellular storage, and altering the structural integrity of plaque biofilms.

1.8.2.1 Effect on Enamel

The process of dental decay is shown in Figure 1.6 and can be described briefly as follows. Plaque bacteria metabolise fermentable carbohydrates, producing organic acid such as lactic, acetic and propionic acids. These acids can diffuse through the plaque (A) into the enamel and dissolve minerals (calcium, phosphate and fluoride) wherever there is a susceptible site (B). If this mineral diffuses out of the tooth and into the oral environment, then demineralisation occurs (C). If this process is reversed, the mineral is reabsorbed into the tooth and the damaged crystals are rebuilt, a process called remineralisation (D). Fluoride acts by inhibiting mineral loss at the crystal surfaces and by enhancing this rebuilding or remineralisation of calcium and phosphate in a form more resistant to subsequent acid attack (ten Cate and Featherstone, 1996).

Figure 1.6 The effect of fluoride on enamel demineralisation and remineralisation

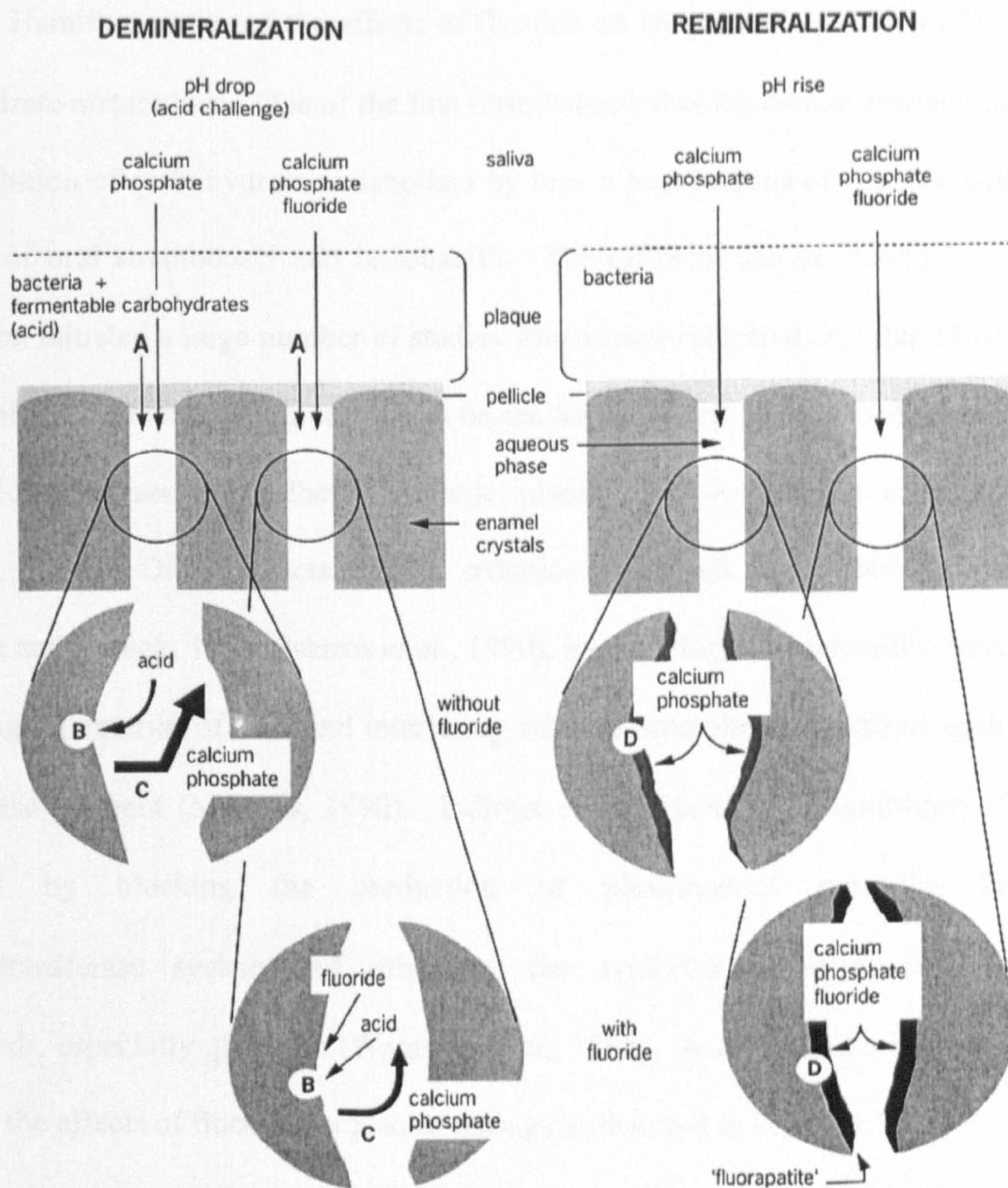


Figure 1.6 Diagram illustrating the demineralisation and remineralisation of enamel when exposed to acid challenge (left) and subsequent pH-rise (right).Step: A. diffusion of acid into the tooth; B. demineralisation at the enamel crystal surface; C. diffusion of mineral components outwards; D. remineralisation at the enamel crystal surface. (ten Cate and Featherstone, 1996).

1.8.2.2 Effect on Oral Bacteria

In 1977, Hamilton reviewed the effects of fluoride on enzymatic regulation of bacterial carbohydrate metabolism. One of the first observations was the demonstration that there was inhibition of carbohydrate metabolism by low concentrations of fluoride with pure cultures of oral streptococci and lactobacilli. The efficient use of fluorides in caries prevention initiated a large number of studies which have indicated that fluoride exerts a vast number of direct and indirect effects on the bacterial cell, some of which may have a significant influence on the acidogenic plaque micro-organisms (Hamilton and Bowden, 1996). Direct effects include reducing glycolysis by inhibition of enolase (Kashket and Bunick, 1978; Psarros *et al.*, 1990), inactivating key metabolic enzymes by acidifying the interior of cells and interfering with transmembrane gradients such as the H^+/ATPase gradient (Marquis, 1990). Indirect effects include the inhibition of sugar transport by blocking the production of phosphoenol pyruvate for the phosphotransferase system and inhibiting the synthesis of intracellular storage compounds, especially glycogen (Wegman *et al.*, 1984). A simplified schematic model showing the effects of fluoride on plaque ecology is featured in Figure 1.7.

Fluoride may affect the development of biofilms by modifying the selective deposition of salivary macromolecules on enamel (Hamilton, 1990; Hamilton and Bowden, 1996). Studies testing oral micro-organisms' susceptibility to fluorides showed that the bactericidal effect was dependent on the concentration of fluorides, on different pH values and on various fluoride salts used (Hamilton, 1990; Maltz and Emilson, 1982).

Figure 1.7 The effects of fluoride on plaque ecology

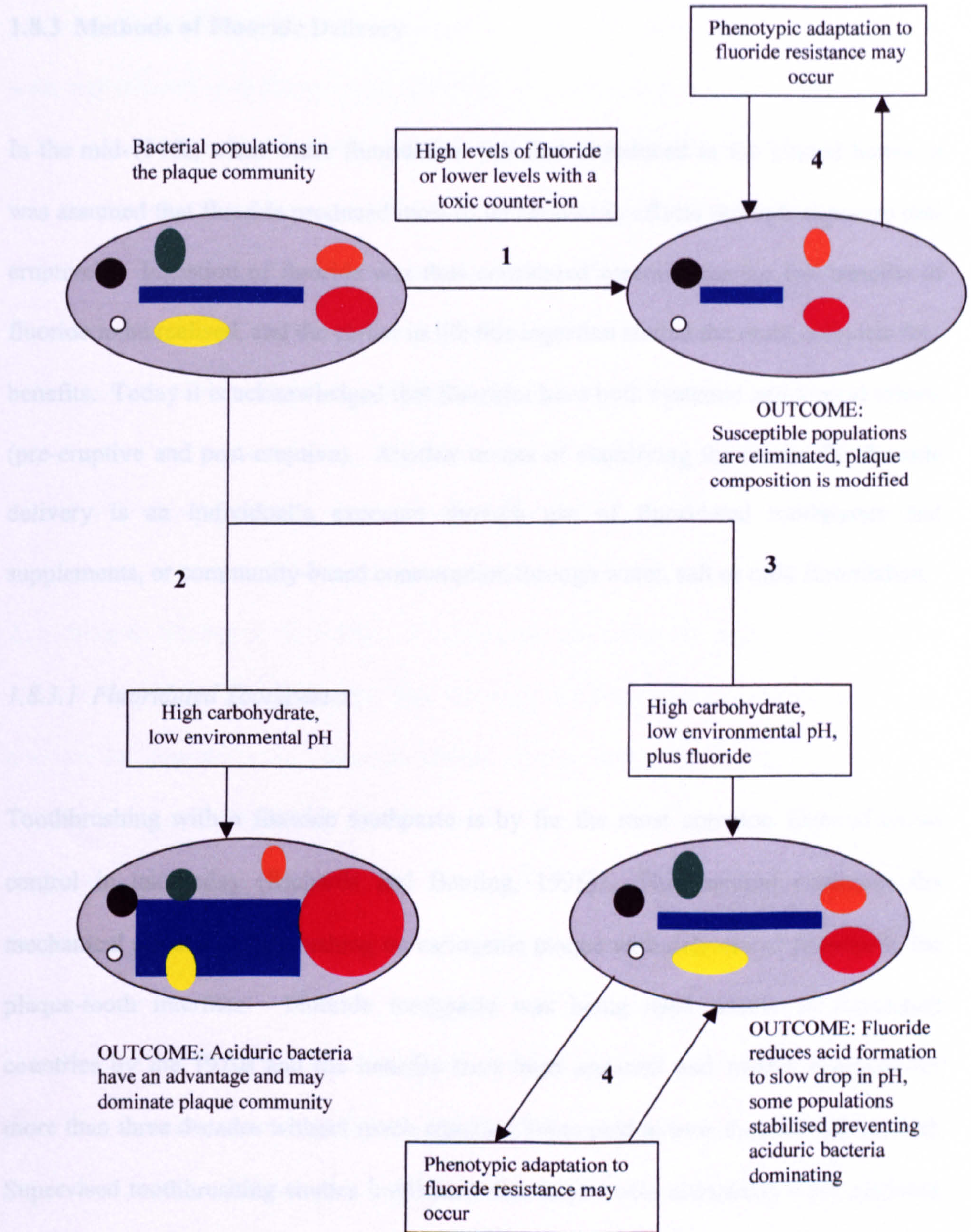


Figure 1.7 A model representing the effects of fluoride on plaque ecology. (Adapted from Hamilton and Bowden, 1996).

1.8.3 Methods of Fluoride Delivery

In the mid-1940s, when water fluoridation was first introduced in the United States, it was assumed that fluoride produced most of its cariostatic effects through exposure pre-eruptively. Ingestion of fluoride was thus considered essential for the full benefits of fluoride to be realised, and the earlier in life this ingestion started the more complete the benefits. Today it is acknowledged that fluorides have both systemic and topical effects (pre-eruptive and post-eruptive). Another means of classifying the modes for fluoride delivery is an individual's exposure through use of fluoridated toothpastes and supplements, or community-based consumption through water, salt or milk fluoridation.

1.8.3.1 Fluoridated Toothpastes

Toothbrushing with a fluoride toothpaste is by far the most common form of caries control in use today (Richards and Banting, 1996). This method combines the mechanical effect of toothbrushing on cariogenic plaque with delivery of fluoride to the plaque-tooth interface. Fluoride toothpaste was being used widely in developed countries by the 1970s and the benefits have been apparent and widely accepted for more than three decades without much attention being paid to how they should be used. Supervised toothbrushing studies implicated that large caries reductions were achieved due to the increased frequency of toothpaste use (Bruun *et al.*, 1982).

Fluoridated toothpastes have been accepted as a primary preventative strategy for caries because of their ease of use, ability to produce significant reductions in dental decay and

low cost. An observed increase in the milder forms of dental fluorosis, in areas both with and without community water fluoridation, have raised concerns about the total daily intake of fluoride of young children. This has prompted recommendations to use less dentifrice, supervise the toothbrushing activity and discourage swallowing of the toothpaste by children under six years of age (Richards and Banting, 1996). Unfortunately, not all children have their teeth brushed regularly and are therefore less able to benefit from this method of delivering fluoride (NHS Scotland, 2002).

1.8.3.2 Fluoride Supplements

According to Murray *et al.* (1991), fluoride-containing pills for dental purposes were first developed in the 1890's but then the aspect of fluoride and dental health lay dormant for over 40 years. Fluoride dietary supplements were introduced in the late 1940's, and were intended as a substitute for fluoridated water for children in non-fluoridated communities (Burt and Marthaler, 1996). They are manufactured in various forms such as tablets to be swallowed, tablets for chewing, lozenges for dissolving slowly in the mouth (Gedalia, 1967) or as drops for young children and so the term "supplements" is used to refer to all forms. Supplements were introduced at a time when it was assumed that the effects of fluoride were mainly pre-eruptive, although this rationale is no longer accepted. Well-conducted trials demonstrated that fluoride supplements provide a posteruptive anti-caries effect in school-age children (DePaola and Lax, 1968; Driscoll *et al.*, 1978, 1981; Stephen and Campbell, 1978). There have also been clinical trials in which fluoride supplements were tested in conjunction with other fluoride therapies in school-based studies (Petersson *et al.*, 1985; Stephen *et al.*,

1990; Driscoll *et al.*, 1992). There was either only a slightly positive caries effect or no effect at all observed in these studies.

The association of fluoride supplements with fluorosis has been the major concern with this vehicle of fluoride delivery. There is also the problem that fluoride is ingested from other sources. Fluoride supplements predated some artificial water fluoridation schemes, fluoride toothpastes, mouthrinses and the widescale use of high-concentration gels. They also preceded the presence of fluoride in processed food and beverages in highly fluoridated countries. Fluoride supplementation may be considered appropriate for high-risk children if the dentist regards compliance as suitable. Compliance of fluoride tablet/drop regimens in the home setting has been found to be a major problem and therefore very limited over the long term (NHS Scotland, 2002).

1.8.3.3 Water Fluoridation

Fluoridation is the controlled addition of a fluoride compound to a public water supply in order to raise the fluoride concentration to a predetermined level for the purpose of preventing dental caries (Burt and Fejerskov, 1996). Most of the studies carried out on the anti-cariogenic effects of water fluoridation have concentrated on the effects on children, but longer-term evidence is also available. The York report (2000) found that the best available evidence from the initiation and discontinuation of water fluoridation suggests that fluoridation does reduce caries prevalence, both as measured by the proportion of children who are caries-free and by the mean decayed, missing and filled deciduous/permanent teeth (dmft/DMFT) scores. They also report that the best available

evidence on stopping water fluoridation indicates that when fluoridation is discontinued, caries prevalence appears to increase in the area that had been fluoridated compared to the control.

The advantages of water fluoridation over other methods of delivery are its cost-effectiveness and the fact that it can benefit the hardest to reach members of a community through other public health programmes (Burt and Fejerskov, 1996). However, issues preventing the widespread fluoridation of public water supplies are the loss of an individual's freedom of choice, conflicting opinions over the efficacy of fluoride and the contradictory nature of fluoridation studies.

1.8.3.4 Salt Fluoridation

Salt fluoridation is the controlled addition of fluoride, usually as potassium fluoride, during the manufacture of salt for human consumption (Burt and Marthaler, 1996). Using salt as a vehicle for other trace elements is not a new concept, since it is a major route for iodine supplementation and in China it is also a vehicle for selenium (Bergmann and Bergmann, 1995). Fluoridated salt controls caries through helping to maintain a constant, low level of fluoride in the intraoral environment. The sale of fluoridated domestic salt was authorised in France in 1987 (Fabien *et al.*, 1996) as well as in Costa Rica and Jamaica (Estupinan-Day, *et al.*, 2001), the State of Mexico in 1988 (Irigoyen and Sanchez-Hinojosa, 2000) and Germany in 1991 (Bergmann and Bergmann, 1995). Salt fluoridation programmes have also been available since the 1950s and studied extensively in Switzerland and Hungary (Toth, 1969; 1976; Marthaler

et al., 1978; Stephen *et al.*, 1999). The outcomes of these studies have been in favour of the fluoridation of salt with regard to the positive effect on dental caries.

Salt fluoridation is beneficial in terms of consumer choice, but it weakens the caries-preventive impact across a whole population, which is a benefit of water fluoridation. The more favourable attitude of European governments towards salt rather than water fluoridation is largely based on the availability of choice (Burt and Marthaler, 1996). Other advantages for salt fluoridation are that salt itself is a supplement, no attention needs to be paid to lifelong daily compliance, it can be taken in small amounts throughout the day and the cost is very low. However, high consumption of sodium is a risk factor for hypertension (Macpherson and Stephen, 2001), and although fluoride is added to salt as potassium fluoride there is a perception by some that its availibility may promote salt intake. Clearly a careful balance needs to be struck here, but the rationale is it could be marketed on the basis, not of encouraging salt intake but simply ensuring that where salt is purchased, the product is available with or without fluoride and it is clearly labelled. Benefits could be enhanced by use in commercial settings such as restaurants or by use in prepared foods and drinks (NHS Scotland, 2002).

1.8.4 Milk Fluoridation

Milk is one of the most complete single food items in the diet, although in the adult diet milk and dairy products are rarely used as single food items, but in combination with other food components. Thus milk has to be considered as a supplement of nutrients from the weaning period up to old age (Benbouzid and Ramanathan, 1996). Among the

many benefits of the consumption of milk and milk products are the teeth and bone-building and strengthening characteristics of certain nutrients. Milk is a source of energy, protein, calcium, lipids and carbohydrates such as lactose (Benbouzid and Ramanathan, 1996). Calcium is a macronutrient that is found in high concentrations in milk and is commonly associated with strong teeth and bones (White, 1987). The slowly fermentable lactose is less cariogenic than sucrose (Rusoff, 1975; Birkhed *et al.*, 1981), and the proteins and fats contained in milk may have a cariostatic effect (McBean and Speakman, 1974).

Milk fluoridation is the addition of a measured quantity of fluoride to bottled or packaged milk to be drunk primarily by children (Burt and Marthaler, 1996). Many researchers have suggested that when a water supply is not available, milk should rank among the first alternative fluoridation vehicles (Ericsson, 1958; Poulsen *et al.*, 1976; White, 1987; Jones *et al.*, 1992). Having both fluoridated and non-fluoridated milk available also means consumer choice and can confine its consumption to those groups needing it most (Borrow and Davis, 1975; Rusoff, 1975). The use of fluoridated milk as a possible dental caries prevention medium was first proposed by a Swiss paediatrician Ziegler in 1953. In a review, Ziegler (1956) reported that fluoridation of milk provided the best means of dosage for all age groups to meet prophylactic requirements. Since then, the caries-inhibiting characteristics of fluoridated milk have been investigated in several studies.

1.8.4.1 Bioavailability of Fluoride in Milk

Bánóczy and co-authors (1996) reviewed the literature concerning the bioavailability of fluoride administered through fluoridated milk. They concluded that there was a moderate caries-preventative effect which did not depend on the fat content of milk. Fluoride concentrations of 5-15 ppm fluoride as calcium fluoride, sodium fluoride, sodium monofluorophosphate or sodium silicofluoride caused a significant caries reduction of 40-50% and did not depend on the compound or concentration used. The bioavailability of fluoride was not reduced by milk, and low accumulation of fluoride was measured in the enamel. In conclusion, it was stated that fluoridated milk keeps a permanently low level of ionised fluoride within the oral cavity, promoting mineralisation and it is likely that this topical mechanism contributes to the caries-preventative effect of fluoridated milk. Fluoride uptake from milk into the developing enamel and dentine of sheep incisors supports also a systemic effect. Human fluoridated milk enamel biopsy and amniotic fluid studies have confirmed fluoride's dual mode of action, i.e. topical and systemic.

1.8.4.2 Clinical Trials and Community Schemes

A major change in interest and promotion of the widespread use of fluoridated milk for children's caries prevention occurred in 1971. Edgar Wilfred Borrow established a charitable foundation in the U.K. for the above purpose. The main purpose of the Borrow Dental Milk Foundation (BDMF) was to promote the study of and research into the fluoridation of milk for human consumption and to help its implementation by

grants, equipment, lectures, scientific papers and every possible means. The support from the BDMF for clinical studies and data from early clinical milk fluoridation schemes seemed to justify further investigation of this means of providing community-based fluoridation for children (Bánóczy *et al*, 1996).

Fluoridated milk schemes have been carried out in Scotland, Hungary and Bulgaria (Stephen, 1984; Bánóczy *et al.*, 1983; Bánóczy, 1985; Ivanova, 1995; Pakhomov, 1995). Overall, the conclusions drawn from these studies showed that the caries-preventative effect of fluoridated milk was greater the earlier in the child's life the consumption commenced. The caries-reducing effect of fluoridated milk appears to be comparable to that of other community-based fluoride vehicles.

Bánóczy and co-authors (1996) reviewed these community-based studies and observed that there were still a number of unanswered questions regarding milk fluoridation and that additional studies should be performed to determine;

1. the age at which it is best to start drinking fluoridated milk;
2. for how many years should it continue;
3. the frequency of consumption;
4. the optimum concentration of fluoride to be added;
5. the anti-caries effect of milk and milk products alone.

1.8.4.3 'In vitro' Studies

Recent interest in the *in vitro* effect of fluoridated milk has included research into its effect on bacterial biofilms and cariogenic oral micro-organisms (Pratten *et al.*, 2000; Kamotsay *et al.*, 2002). Also of interest is characterising the remineralising effect that fluoride has on dental enamel (Toth *et al.*, 1997). Fluoride concentrations in whole saliva and separate gland secretions (Twetman, 1998) and in plaque after either a single intake or repeated intakes of fluoridated milk (Engström, 2002) have also been studied. For the purpose of this thesis, I will focus on the anti-bacterial effect that fluoridated milk exerts.

Pratten (2000) concluded that fluoridated milk was able to increase the pH of an oral bacterial biofilm by decreasing the proportion of streptococci, in particular *S. mutans*, in regions closest to the enamel surface and increasing the proportions of *Veillonella* spp. which convert lactate to propionic acid (which is a weaker acid). Kamotsay and colleagues (2002) studied the effect of both sodium fluoride (NaF) and sodium monofluorophosphate (MFP) at different concentrations on *S. mutans*, *L. acidophilus* and *Candida albicans*. They observed that fluoride in either form did not exert a prompt effect on the viable counts of any of the strains in either phosphate buffered saline or milk. They suggested that repeated prolonged exposure of the micro-organisms in the oral cavity to fluoride may result in a decrease in the number and ratio of the main cariogenic micro-organisms.

CHAPTER 2: GENERAL MATERIALS AND METHODS

The materials and methods described on the following pages are those that were routinely employed throughout the experiments documented in this thesis. The methods which were specifically related to work in individual chapters are dealt with in detail in the Materials and Methods section of that specific chapter.

2.1 Materials

2.1.1 Microbiological Resources

Fastidious Anaerobe Agar, Anaerobe Broth: BioConnections, Leeds, U.K.

Rogosa SL Agar, Mitis Salivarius Agar, Purple Broth Base: Becton Dickinson, Crawley, U.K.

Sterile Defibrinated Horse Blood: E & O Laboratories, Bonnybridge, U.K.

Glucose: Merck, Poole, U.K.

Mannitol, Sorbitol: Sigma Aldrich, Poole, U.K.

Phosphate Buffered Saline: OXOID Ltd, Basingstoke, England.

Protect: Technical Service Consultants, Ltd., Lancashire, U.K.

Rapid ID 32 Strep: bioMerieux U.K. Limited, Basingstoke, U.K.

Type Strains: National Centre for Type Cultures, Public Health Laboratory Service, London, U.K./American Type Culture Collection, LGC Promochem, Middlesex, U.K.

2.1.2 Molecular Biology Resources

Agarose, *Taq* DNA Polymerase, PCR buffer with $MgCl_2$, *Hin*d III, *Cfo* I: Promega, Southampton, U.K.

Ultrapure dNTP Set, 100 bp DNA Marker, Nylon positive charged hybridisation membrane: Amersham Pharmacia Biotech, St Albans, U.K

PCR Primers 27F/1492R, LF-1A/LF-2: MWG Biotech, Milton Keynes, U.K.

Gram positive DNA Isolation Kit (Gentra), DynaWax: Novara Flowgen, Lichfield, U.K.

Achromopeptidase, 100 bp DNA Marker: Sigma Aldrich, Poole, U.K.

Mnl I: Helena Biosciences, Sunderland, U.K.

3MM chromatography paper: Whatman International Limited, Maidstone, England.

DIG-labelled DNA molecular weight marker, DIG Hybridisation and Labelling Kit: Roche Diagnostics, Lewes, U.K.

All other materials were obtained from Merck, Poole, U.K., Fisher Scientific, Leistershire, U.K., or Sigma Aldrich, Poole, U.K.

2.2 Methods

2.2.1 Growth and Storage of Bacteria

Each clinical isolate and type strain was subcultured as a lawn on 3 to 6 blood agar or fastidious anaerobe blood agar plates and incubated under CO_2 or 85% N_2, 10% CO_2 and 5% H_2 in an anaerobic chamber (DonWhitley Scientific, Shipley, U.K.) at 37°C for 2 to 3 days. Bacteria were collected from each of the plates using a sterile swab and pooled in one vial of Protect containing enriched medium and plastic beads. Once the beads were coated in the bacterial suspension, a sterile plastic pipette was used to remove and discard the majority of the suspension. The vials were labelled appropriately and stored in boxes at -80°C until required. Reinstating the bacteria was achieved by aseptically removing 1 to 2 beads from the vials with sterile forceps and gently rubbing the beads over the entire surface of a blood agar plate followed by incubation at 37°C under the appropriate conditions.

2.2.2 Gram Stain

On a cleaned glass microscope slide, a bacterial smear was created using sterile water and a loop of the bacteria to be stained. This suspension was allowed to dry and then heat-fixed by passing through a bunsen flame. The smear was first stained with basic crystal violet stain for 1 minute, followed by treatment for another minute with iodine. The smear was then decolourised by washing briefly (2 to 3 secs) with acetone. Finally,

the smear was counterstained with carbol fuschin. Gram stains were viewed under the light microscope with x1000 magnification under oil immersion.

2.2.3 Sugar Fermentation Test

Isolates displaying morphological characteristics similar to *S. mutans* were subjected to a further biochemical test for provisional identification. Bacterial suspensions of the isolates to be tested were prepared in 0.5 ml aliquots of purple broth base indicator. Three drops of each of the 20% sugar broth preparations required (glucose, mannitol and sorbitol) were inoculated into separate microtitre plate wells. Each carbohydrate broth was then inoculated with one drop of the bacterial suspension. The microtitre plates were incubated aerobically at 37°C overnight. Identification of *S. mutans* isolates was confirmed by a colour change from purple to yellow with all three sugars. This test is based on the principle utilised by the API Rapid ID 32 Strep test which suggests that 99% of *S. mutans* will ferment mannitol and 80% ferment sorbitol whereas for the closest relative, *S. sobrinus*, 99% will ferment mannitol and only 1% will ferment sorbitol.

2.2.4 DNA Extraction with Puregene Kit (Novara Flowgen)

Bacterial suspensions of each isolate were prepared in 600 µl cell suspension solution. Three microlitres of lytic enzyme was added and the suspensions were incubated in a 37°C waterbath for 30 minutes. The suspensions were then centrifuged for 1 minute at 13,000 to 16,000 x g, the supernatant removed and 600 µl cell lysis solution was added

and the pellet resuspended. Samples were incubated at 80°C for 5 minutes to complete cell lysis. The samples were allowed to cool slightly before adding 3 µl RNase A (4 mg/ml) and incubating in a 37°C waterbath for 1 hour. The samples were cooled to room temperature and 200 µl protein precipitation solution was added and vortex-mixed for 20 seconds. The samples were then centrifuged for 3 minutes at 13,000 to 16,000 xg. The supernatants were then poured into 1.5 ml microcentrifuge tubes containing 600 µl 100% isopropanol and mixed by inverting. The samples were centrifuged again for one minute and the supernatants removed. The DNA pellets were allowed to air-dry before rehydrating in 100 µl sterile molecular-biology-grade water overnight at room temperature. DNA was stored at 4°C prior to use.

2.2.5 Restriction Endonuclease Digestion of DNA

Typically, 0.5 to 3 µg of DNA (genomic, plasmid or PCR product) was added to 0.1 volume of the appropriate 10x restriction endonuclease buffer (Promega). Sterile molecular-biology-grade water was added to bring the buffer to the working concentration and either spermidine (1mM final concentration) or bovine serum albumin (10 µg/µl) was added. The appropriate restriction endonuclease was added (5 units/µg of DNA), the contents mixed gently and incubated at 37°C for 3 hours or overnight. Digestion was terminated by the addition of 0.1 volume of sample loading dye (50% w/v glycerol, 0.1% w/v bromophenol blue).

2.2.6 Agarose Gel Electrophoresis

Agarose (0.8 g, 1 g or 2 g) was dissolved to a final concentration of 0.8 %, 1 % or 2 %, respectively, in 100 ml 1x TBE electrophoresis buffer (Table 2.1) by heating in a microwave oven. After cooling to 50°C, 30 μl ethidium bromide (3 mg/ml) was added and mixed by swirling. The molten agarose was poured into the gel tank with the well-forming comb in place. Once the gel had solidified, the comb was removed and the gel immersed in 1x TBE buffer. Samples were loaded into the wells and gels run at a constant voltage of about 70V for 1 hour. DNA was visualised with an ImageMaster ® Video Documentation System (Amersham Pharmacia Biotech), and a permanent record of the image obtained by printing to VDS (TFF) film.

TABLE 2.1 General Stock Solutions and Buffers

Fastidious Anaerobe Broth

29.7 g powder dissolved in 1 litre dH_2O by boiling and frequent mixing. Dispense into containers as required and sterilise by autoclaving.

Phosphate Buffered Saline Rinse

Dissolve one tablet in 100 ml dH_2O. Sterilise by autoclaving.

Fastidious Anaerobe Blood Agar

46 g powder/litre of agar dissolved in dH_2O by boiling and frequent mixing. Sterilise by autoclaving. Cool to 50°C and add 7.5% defibrinated horse blood.

Rogosa SL Agar

75 g powder/litre of agar dissolved in dH_2O by boiling and frequent mixing. Remove from heat and add 1.32 ml glacial acetic acid. Mix well and boil for a further 2 to 3 minutes. Do not autoclave.

Mitis Salivarius-Bacitracin Agar

90 g powder/litre of agar dissolved in dH_2O by boiling and frequent mixing. Sterilise by autoclaving. Cool to 50°C and add 10 ml 0.1% potassium tellurite and 10 ml Bacitracin (20 Units/ml).

Columbia Blood Agar

40 g powder/litre of agar dissolved in dH_2O by boiling and frequent mixing. Sterilise by autoclaving. Cool to 50°C and add 7.5% defibrinated horse blood.

Purple Broth Base Indicator

15 g powder dissolved in 1 litre dH_2O. Dispense as required. Sterilise by autoclaving.

20% Sugar Broth Solutions

10 g carbohydrate (glucose, mannitol or sorbitol) dissolved in 200 ml purple broth base. Dispense as required. Sterilise by filtration. Store at 4°C.

L Broth Medium

tryptone	10 g
yeast extract	5 g
sodium chloride	10 g
dH_2O	to 1 litre

Sterilise by autoclaving

L Agar (1.5%)

L broth	200 ml
agar	3 g

Sterilise by autoclaving

Achromopeptidase

Achromopeptidase at 20 U/µl in 10 mM Tris, 1 mM EDTA, pH 8.0.

10x TBE

Trizma base	108 g
Boric Acid	55 g
0.2 M EDTA (pH 8.0)	50 ml
dH_2O	to 1 litre

Use at final concentration of 1x.

10x TN Buffer

0.1 M Trizma Base	12.1 g
0.1 M NaCl	5.84 g
dH_2O	1 litre

Use at final concentration of 1x.

3 M Sodium Acetate

sodium acetate trihydrate	40.81 g

Dissolve in 80 ml dH_2O. Make up to pH 6.0 with glacial acetic acid. Make up volume to 100 ml with dH_2O. Sterilise by autoclaving.

0.2 M EDTA (pH 8.0)

EDTA	74.4 g
dH_2O	800 ml

Adjust to required pH with NaOH. Make up volume to 1 litre with dH_2O. Sterilise by autoclaving.

5M NaCl

NaCl	292.2 g
dH_2O	to 1 litre

Sterilise by autoclaving.

5M NaOH

NaOH	200 g
dH_2O	to 1 litre
Sterilise by filtration.	

20 % SDS

sodium dodecyl sulphate	200 g
dH_2O	to 1 litre

Ethidium bromide (3 mg/ml)

ethidium bromide	0.3 g
dH_2O	to 1 litre
Store away from light.	

CHAPTER 3: AN *IN SITU* INVESTIGATION INTO THE EFFECT OF FLUORIDATED MILK ON THE PROPORTIONS OF POTENTIALLY CARIOGENIC MICRO-ORGANISMS IN DENTAL PLAQUE.

3.1 Introduction

Dental caries has been described as the single most common chronic disease of childhood (Becker *et al.*, 2002). Evidence generated through extensive studies suggests that caries is the result of the metabolism of acids by particular acidogenic and aciduric bacteria (Loesche, 1976; Emilson and Krasse, 1985). Streptococci and lactobacilli make up the bulk of the acidogenic flora associated with dental plaque and caries. Lactobacilli have long been considered important in caries because of their acidogenic and aciduric properties and because their numbers could be shown to correlate with the level of disease (Jordan, 1986). Jay *et al.* (1936) demonstrated that lactobacilli show a direct response to dietary sugar intake and therefore they have also been used as an indicator of patient co-operation in maintaining a low cariogenic diet. Thus, effective dietary counselling, together with patient motivation, can result in reduced lactobacillus counts and reduced initiation and progression of dental caries. Streptococci are now considered to represent the predominant odontolytic component of the oral flora (Gibbons, 1972; Loesche and Straffon, 1979), thus dental caries exhibits a degree of microbial specificity which makes it possible to express the microbiological risk factor in terms of particular organisms (Jordan, 1986).

One of the first selective media for the identification of lactobacilli was tomato-juice agar described by Hadley (1933). Although all aciduric organisms were cultured from saliva, most of these were lactobacilli and could be easily recognised. This allowed dental researchers to monitor a particular segment of acidogenic oral flora and therefore it was possible to show that the populations of lactobacilli generally reflected the level of dental caries. This technique was widely used until 1951, when Rogosa and colleagues developed a more selective medium for culturing oral lactobacilli. The selectivity of this medium is based on a low pH of 5.4 with a high acetate content, and Tween 80 as a surfactant. This medium is still the preferred choice for the primary culture of lactobacilli in oral samples (van Houte *et al.*, 1981).

The development of specialised culture techniques for monitoring the oral streptococci did not receive much attention until Fitzgerald and co-workers (1960 a, b) demonstrated that certain specific streptococci were highly cariogenic in experimental animals. In 1944, Chapman had recorded the use of mitis-salivarius agar for the isolation of streptococci in mixed cultures. This medium permitted the culturing of viridans streptococci and the distinctive appearance of *S. mutans* allowed populations of this organism in oral samples to be monitored. In 1968, Krasse and colleagues were able to demonstrate a correlation between dental caries and a particular segment of the oral streptococci – the *S. mutans* group.

The development of selective media greatly simplified the detection and assessment of *S. mutans* in oral samples (Carlsson, 1967; Woods, 1971). In 1972, Ikeda and Sandham added 40% sucrose to mitis-salivarius agar and reported that it provided a selective

medium for *S. mutans,* although there was some inhibition evident. Gold and colleagues (1973) reported on a selective medium that was based on the combined selective activity of sucrose at 20% and bacitracin at 0.2 units/ml in mitis-salivarius agar. This selective agar has been shown to support the growth of *S. mutans* serotypes c, e and f, although others have reported that it does not permit isolation of serotypes a and b (not found in humans), and the d/g serotypes (Emilson and Bratthall, 1976; Little *et al.* 1977). Other investigators have reported on various selective media which allow improved recovery of all the major subgroups of *S. mutans* (van Palenstein-Helderman *et al.* 1983; Tanzer *et al*, 1984; Wade *et al.*, 1986; Svanberg and Krasse, 1990).

Saliva also plays many important roles with regard to protecting tooth surfaces from caries. Marked differences in salivary film velocities and clearance rates have been shown to persist in different regions of the mouth, even during chewing (Dawes and Macpherson, 1993; Macpherson and Dawes, 1994). The findings from artificial plaque models (Macpherson and Dawes, 1991) suggest the velocity at which a thin film of saliva passes over the surface of dental plaque may influence the cariogenicity of the underlying plaque. The slow clearance of fermentable carbohydrates and bacterially-produced acids from plaque sites with slow salivary film velocity has been predicted to increase the susceptibility of the enamel to demineralisation and cause a shift in the plaque community with an increase in aciduric bacteria. However, slow diffusion of calcium and phosphate ions from plaques exposed to a low salivary film velocity may result in increased buffering at these sites. Additionally, the retention of fluoride will also tend to have the opposite effect at these locations. The presence of fluoride in plaques for prolonged periods of time may have a direct effect on enamel de- and

remineralisation, and reduce plaque acidogenicity by inhibiting bacterial glycolysis (Oliveby *et al.*, 1990).

As mentioned earlier (*see* Section 1.8.4), the effect of fluoridated milk on oral bacteria implicated in dental caries has been investigated in numerous clinical trials, *in vitro* and *in vivo* studies with favourable conclusions. The main reasoning behind choosing milk as the vehicle for delivery stems from the arguments that fluoridated water does not permit consumer choice and that milk also provides necessary vitamins and minerals for those in the population that need it most, namely young children. The slowly fermentable lactose in milk is less cariogenic than sucrose (Rusoff, 1975; Birkhed *et al.*, 1981) and the proteins and fats contained in milk may have a cariostatic effect (McBean and Speakman, 1974). Experimental studies are designed specifically to test hypotheses under controlled conditions where specific risk factors are manipulated in order to assess their effect on disease. The degree of control of risk factors involved will vary depending on the conditions under which the study is carried out. Clinical trials are usually carried out in highly selected groups of individuals, often selected for having certain predefined characteristics. Such studies are usually undertaken to test possible effects (efficacy) of therapeutic or preventative interventions (Manji and Fejerskov, 1994).

The efficacy of fluoridated milk has been recorded through many studies designed primarily to investigate the influence of fluoride on decayed, missing and filled (DMF) values of teeth from susceptible children (Bánóczy *et al.*, 1983; Stephen *et al.*, 1984; Legett *et al.*, 1987; Gyrukovics *et al.*, 1992; Pakhomov *et al.*, 1995; Mariño *et al.*, 2001).

Although these studies vary in design, they agree that fluoride is an effective vehicle for reduction in DMF values. *In vivo* studies of the effect of fluoridated milk on experimental caries in rats record reduced levels of caries (Poulsen *et al.*, 1976; Stösser *et al.*, 1995). In 1995, Chandler and co-workers described the use of intra-oral appliances in human volunteers to test the caries-protective effect of fluoridated milk on bovine dental enamel. Although the results were not significant in terms of the change in microhardness of dental enamel when exposed to fluoride in milk, the principal concept of the study design was successful.

Although many milk fluoridation programmes have involved the consumption of milk by children, once daily, during school hours, very little clinical work has been carried out to determine the effect of frequency of fluoridated milk intake on caries dynamics. The effect of consuming one high concentration of fluoride per day or staggering consumption to three lower doses is also an area of interest. Additionally, intra-oral studies are required to determine the effect, with and without added fluoride on the de-/re-mineralisation process and plaque composition at sites with differing film velocities. In 1990, Macpherson *et al.* demonstrated that the use of *in situ* models, with enamel blocks mounted on dental appliances, allows the intra-oral study of effects of various environmental conditions and applied substances on plaque composition and metabolism. As part of a larger project, the present study aimed to use *in situ* denture models to provide plaque samples for analysis by microbiological and molecular biological techniques.

The main questions to be answered from this study are:

1. What are the effects of supplementing normal diets with a] no beverage (negative control), b] regular milk and c] fluoridated milk on plaque bacterial counts, particularly *S. mutans* and *Lactobacillus* spp.?
2. Does fluoride exert a specific effect on numbers of *S. mutans* and *Lactobacillus* spp. over any particular experimental protocol?
3. Is there an added protective effect when fluoridated milk is consumed in conjunction with the use of a fluoridated dentifrice?
4. What is the effect of intra-oral test site location on bacterial counts, particularly with regard to proportions of *S. mutans* and *Lactobacillus* spp. within the overall dental plaque bacteria?

3.2 Materials and Methods

3.2.1 Overall Plan of Investigation

A panel of thirty "healthy", complete upper denture wearers were recruited as part of a large fluoridated milk study. For each subject, a copy denture was constructed with test site recesses created in the following positions: upper anterior labial, upper anterior palatal, upper posterior buccal (left and right), and upper posterior palatal (left and right), i.e. sites with varying salivary film velocities (Dawes and Macpherson, 1993). These sites are shown in Figures 3.2.1 (a) and (b).

For each experiment, two enamel blocks were inserted into each test site. One block had an artificial-caries lesion created by the Carbopol method (White, 1987) and half of each lesion was covered in acid-resistant varnish or fissure sealant before the specimens were temporarily mounted. The second block was smaller in size and mounted permanently in the acrylic of the denture for collection of plaque for microbiological analyses. Prior to mounting, the enamel slabs for microbiological purposes were measured with a pair of callipers (Rabone) so that analysis could be extended to Counts/Area. The subject wore the denture continuously for a period of six weeks. During this time, daily removal of the denture for cleaning of the fitting surface was permitted, but the tooth specimens were not brushed. Each subject completed five, six-week experiments, with the order assigned in a random manner. Normal diets were supplemented with either:

Figure 3.2.1 a) Palatal view of enamel slabs on upper denture

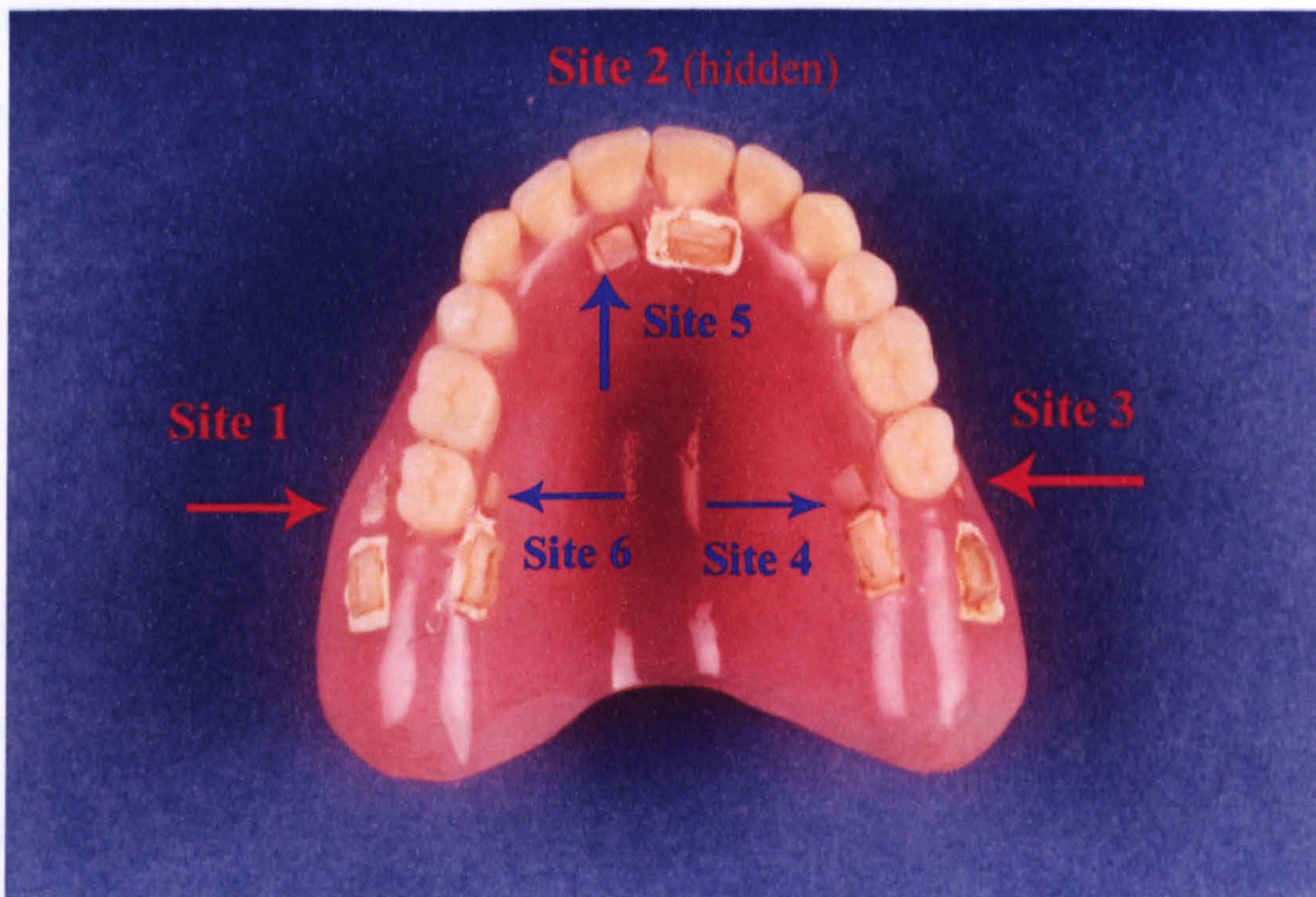


Figure 3.2.1 a) Positions of five enamel slabs from the upper denture, palatal view.

Figure 3.2.1 b) Buccal and labial view of enamel slabs

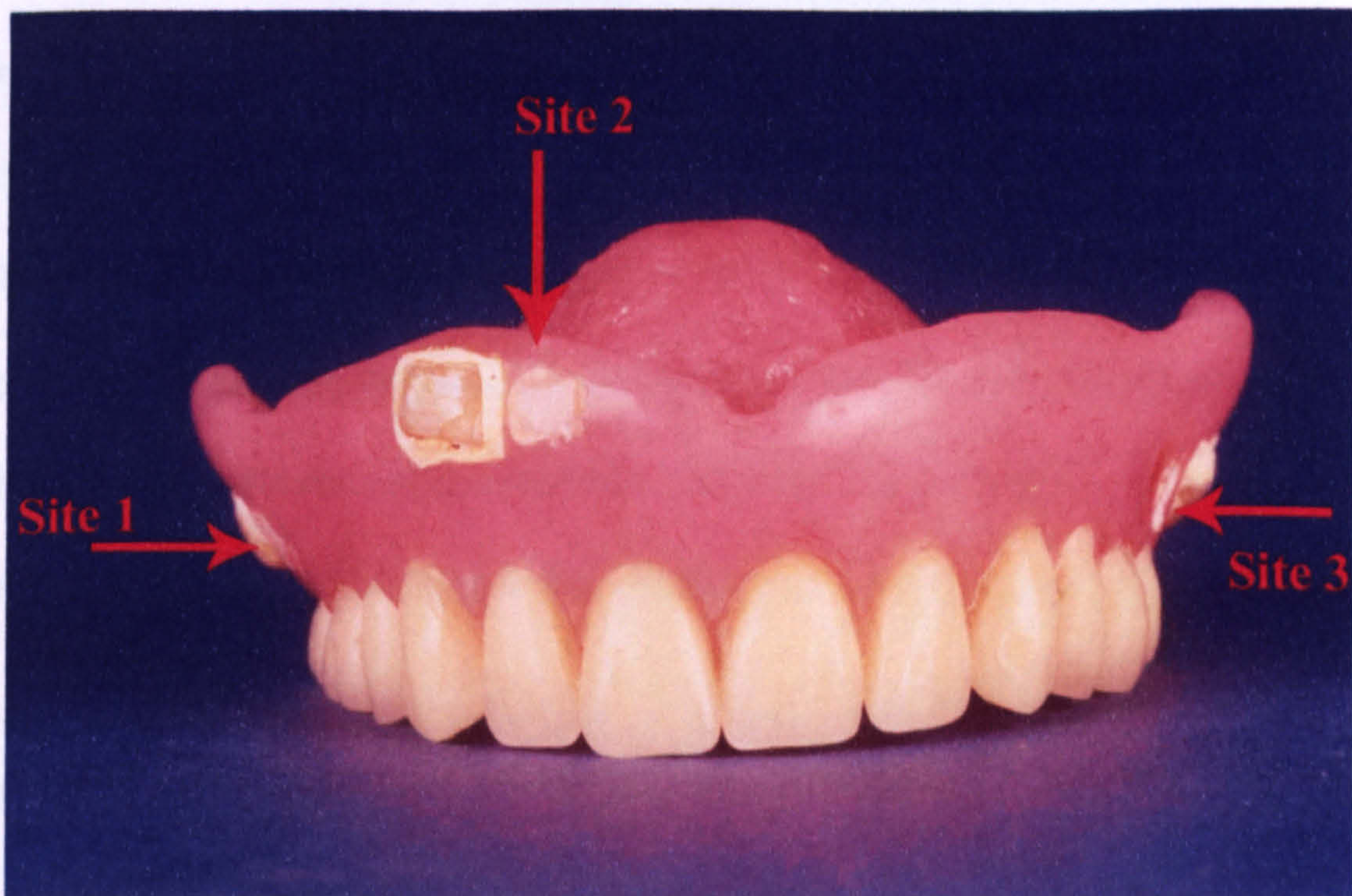


Figure 3.2.1 b) View of three enamel slabs positioned on the upper denture, buccal and labial view.

1. no beverage (negative control)
2. 200 ml milk (x1/day)
3. 200 ml milk (x3/day)
4. 1.5 mg Fluoride in 200 ml milk i.e. 7.5 ppm F (x1/day)
5. 0.5 mg Fluoride in 200 ml milk i.e. 2.5 ppm F (x3/day)

The thirty subjects were divided randomly into two groups with, for each of the five experiments, one group of 15 carrying out the procedures described above, while the other group additionally rinsed twice daily for 2 minutes with a slurry of 1 g dentifrice (1100 ppmF) to 3 g of water (i.e. the dentifrice industry-accepted *in vitro* simulation of toothbrushing twice daily with a fluoridated dentifrice).

During each experiment, the subjects returned at two-weekly intervals for:

- measurement of pH response of plaque overlying the large slab, to 10% sucrose challenge, using the method described by Macpherson *et al*. (1991)
- removal and pooling of plaque from all large slabs for the measurement of plaque fluoride concentration using standard F electrode methodologies
- assessment of degree of enamel demineralisation using light fluorescence.

At the end of each 6-week period of denture wear, the following procedures were carried out:

- measurement of plaque pH response to sucrose using plaque from one half of each large slab per site, followed by pooling of plaque from all the large slabs for fluoride analysis

- removal of plaque from the second, smaller slab for enumeration of total bacterial counts, and identification and enumeration of *S. mutans* and *Lactobacillus* species
- assessment of degree of enamel slab demineralisation using light fluorescence
- recovery of specimens from the denture, removal of varnish, sectioning of the same lesion (i.e. covered and uncovered areas), and assessment of enamel demineralisation using transverse quantitative microradiography.

For each subject, there was at least a three-week wash-out period between each phase of the study. Sodium fluoride solutions of appropriate concentrations were prepared by the Pharmacy Production Department at the Western Infirmary, Glasgow where they were filter-sterilised and 2 ml volumes aliquoted into sterile plastic bijou bottles. These bottles were attached adhesively to the side of the milk containers at the time of product distribution. The subjects were instructed to empty the contents of the bijou bottle into the milk container and to stir immediately prior to drinking the beverage.

For the purpose of investigating the effect of fluoridated milk on cariogenic micro-organisms, fourteen subjects were selected from the total of thirty patients involved in the overall project. These subjects were chosen simply on the basis that they were the first group of individuals ready to start the experiments, and their details can be found in Table 3.2.1.

Table 3.2.1 Patient Details

Patient Number	Sex	Date of Birth	Start Date	End Date	Treatment Group
1	F	04/02/1927	11/10/2000	07/12/2001	T + D
2	F	25/02/1922	24/01/2001	22/02/2002	T + D
3	M	25/02/1936	17/01/2001	20/02/2002	T Only
4	M	09/05/1942	13/10/2000	26/10/2001	T Only
5	M	28/02/1918	26/10/2000	08/03/2002	T + D
6	M	18/06/1933	24/01/2001	18/02/2002	T Only
7	M	13/05/1928	18/10/2000	19/12/2001	T Only
8	F	13/11/1931	02/02/2001	22/02/2002	T Only
9	F	30/06/1939	19/01/2001	18/02/2002	T + D
10	M	28/11/1931	14/01/2001	18/02/2002	T Only
11	M	13/11/1925	01/11/2000	10/12/2001	T + D
12	F	14/08/1925	19/02/2001	22/05/2002	T + D
13	F	10/07/1928	18/10/2000	27/05/2002	T Only
14	F	05/06/1949	01/11/2000	06/03/2002	T + D

Table 3.2.1 Details of fourteen patients recruited for microbiological analysis. Start date corresponds to the first day of Experiment 1 and the end date corresponds to the last day of Experiment 5. The Treatment Groups were assigned in a double-blind random manner.

3.2.2 Subject Screening and Recruitment

Prior to the commencement of this study, ethical approval was obtained from the Greater Glasgow Dental Ethics Committee. Subjects participating in the study were informed of the protocol and written consent was obtained. All participants were free to withdraw from the study at any time. Seven males and 7 females, all full denture wearers, were recruited for the purpose of this study and all had to fit ideal requirements in the prepared selection criteria (*see* Appendix to Chapter 3). Recruitment was either from past attendees of the Prosthodontics Department in Glasgow Dental Hospital, referrals from local general dental practitioners or through opportunistic recruitment of individuals known to the study researchers.

An initial bacteriological screening test was carried out with an unstimulated saliva sample and a phosphate-buffered saline (PBS) oral rinse (Samaranayake, *et al.*, 1986). This was to determine the presence or absence of *S. mutans* and *Lactobacillus* spp. Patients were recruited if they carried both lactobacilli and *S. mutans* or lactobacilli alone.

3.2.3 Plaque Sampling

At the start of each experimental protocol, the subjects refrained from oral hygiene for three days prior to their agreed appointment, then plaque was collected. This was repeated at the end of each six-week experimental period. The plaque which had accumulated on each of the six enamel blocks was collected using a sterile dental probe

and dispersed into 1 ml Fastidious Anaerobe Broth (FAB) base supplied in a sterile plastic disposable bijou bottle. These were then transferred to the microbiology laboratory for immediate processing.

3.2.4 Microbiological Analysis

The plaque samples in FAB were vortex mixed for 30 seconds and ten-fold dilutions were prepared in PBS. Aliquots of 50 μl of 10^{-3} and 10^{-4} dilutions were spiral plated onto Fastidious Anaerobic Agar (FAA) (BioConnections, U. K.) supplemented with defibrinated horse blood (7.5%, v/v) for enumeration of total bacterial counts. Aliquots (50μl) of neat, 10^{-1} and 10^{-2} dilutions were spiral plated onto mitis-salivarius agar (Difco) supplemented with bacitracin (MSB). Neat and 10^{-1} dilutions were spiral plated onto Rogosa SL agar (Difco). All MSB plates were incubated at 37°C for 2/3 days in 95% air and 5% CO_2. The FAA and Rogosa plates were incubated at 37°C for 2/3 days under anaerobic conditions in an atmosphere of 85% N_2, 10% CO_2 and 5% H_2.

On MSB agar, all colonies with morphology characteristic for *S. mutans* were counted (Emilson, 1983). Colonies were then isolated on blood agar and incubated at 37°C in 95% air and 5% CO_2. All colonies growing on Rogosa SL medium were considered to be lactobacilli. Morphologies of different colonial types were recorded and enumerated. Colonies were then isolated on blood agar and incubated at 37°C under anaerobic conditions. Figures 3.2.2 and 3.2.3 show colony morphologies on MSB and Rogosa agars, respectively, after 2-3 days incubation at 37°C.

Figure 3.2.2 Morphological characteristics of oral streptococci grown on MSB agar

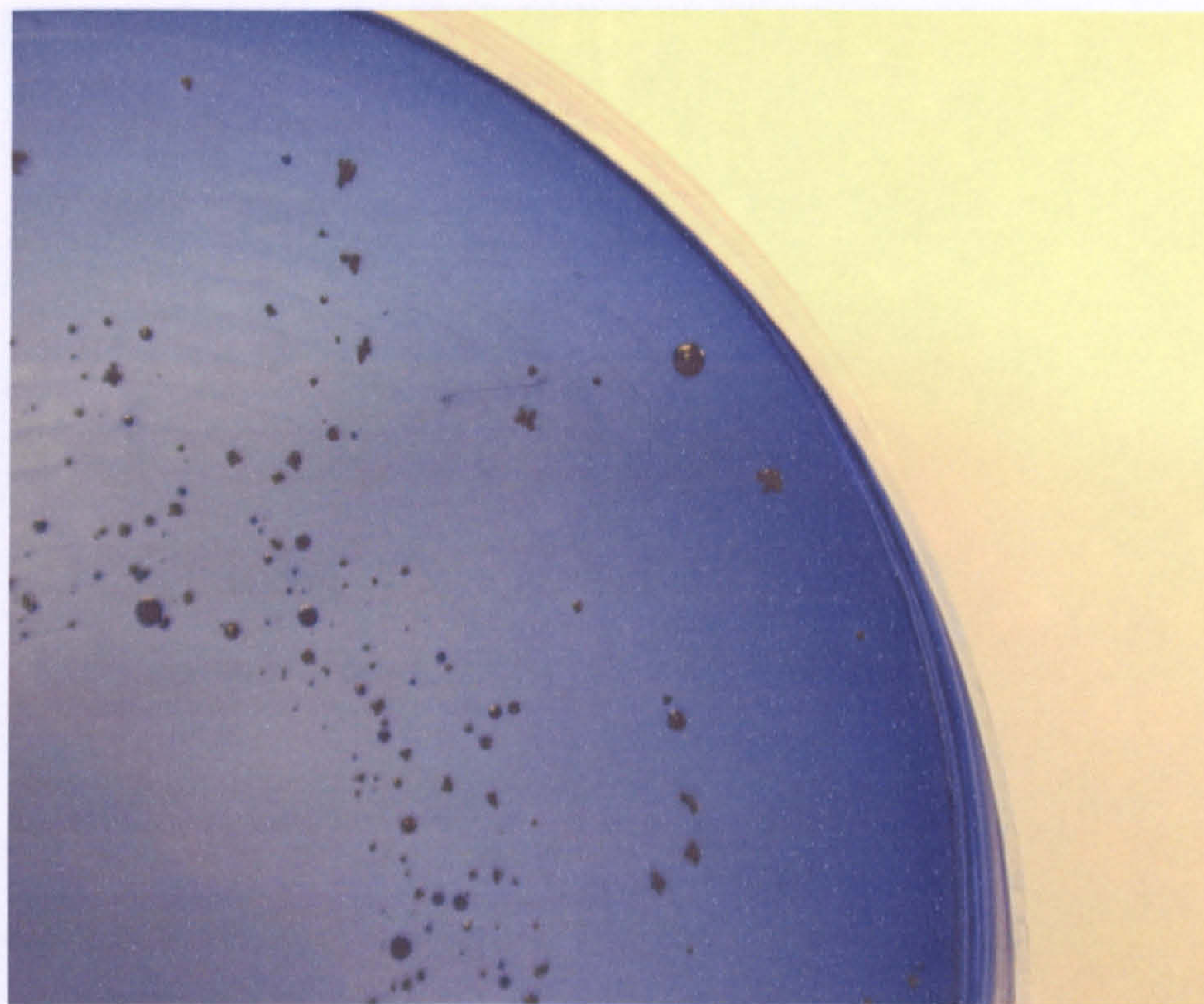


Figure 3.2.2 The differing morphological characteristics of oral streptococci grown on Mitis-Salivarius Bacitracin (MSB) agar.

Figure 3.2.3 Colony morphology of *Lactobacillus* spp. grown on Rogosa agar

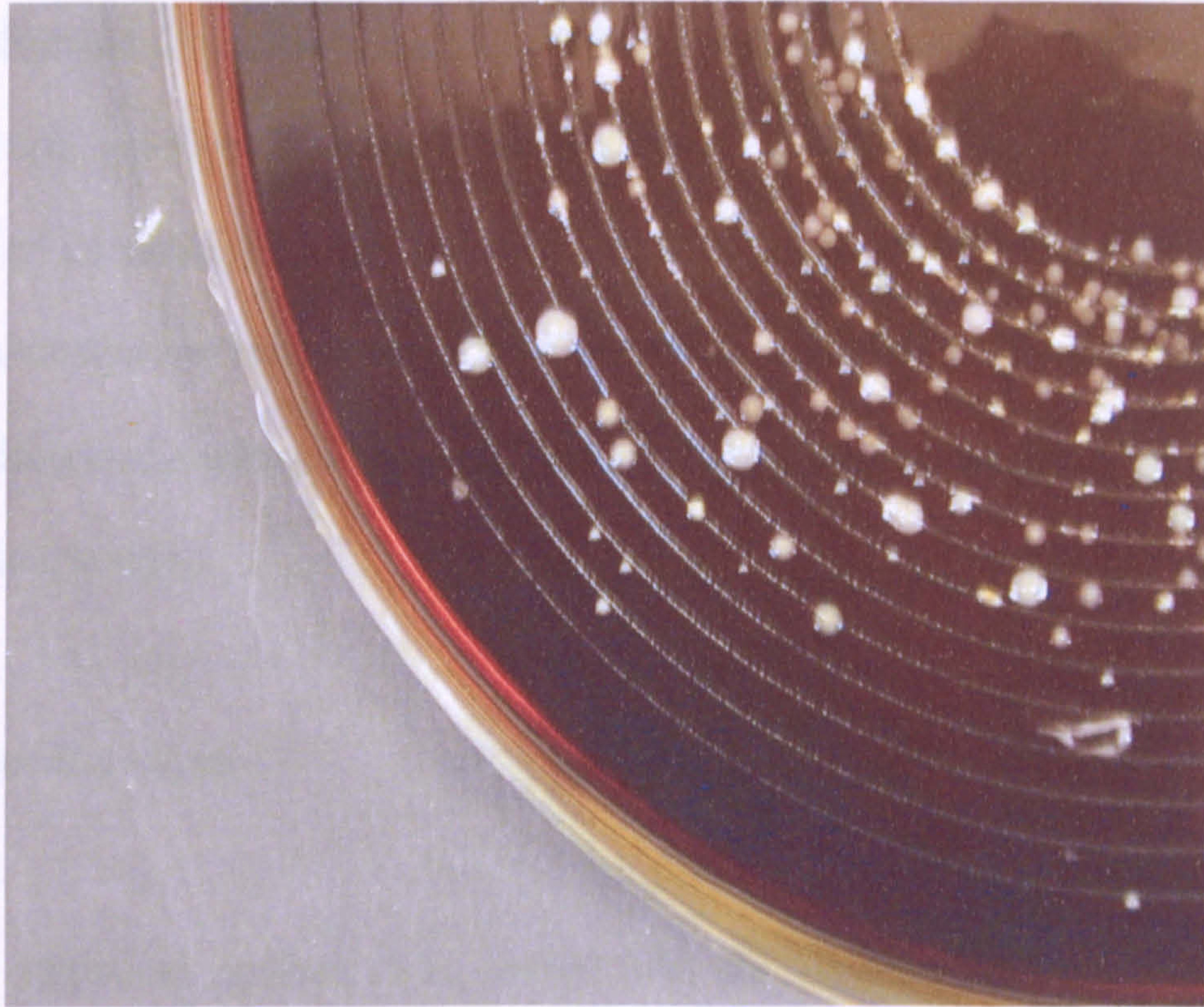


Figure 3.2.3 The differing morphological characteristics of oral *Lactobacillus* spp. spiral-plated on Rogosa agar.

Identification of *Lactobacillus* spp. was confirmed by a positive Gram staining reaction with the characteristics of growth recorded. All isolates were then stored in Protect (Technical Service Consultants Ltd) at -80°C for future studies. Gram staining was carried out on putative *S. mutans* isolates, with a positive staining reaction and appearance of coccobacilli being indicative of *S. mutans.* Further confirmation of *S. mutans* was carried out with a series of biochemical tests utilising sugar fermentation profiles as described earlier. Positive *S. mutans* isolates were then stored in Protect at -80°C for future studies.

3.2.5 Statistical Analysis

All of the statistical analysis was carried out by Siobhan McHugh, a Research Statistician at Glasgow Dental Hospital and School. In this study, data were collected from 14 patients, seven patients in Treatment Only Group and seven patients in Treatment + Dentifrice Group. Each patient had six sites measured at Week 1 and Week 6, for each of five Treatment protocols. The objectives of this study in relation to the larger project were to analyse four particular microbiological outcome measures. These outcome measures (all based on the Count/Area) were:

- Total Counts
- Total Lactobacillus Counts
- Proportion of Lactobacilli in Total Counts
- Total *S. mutans* Counts

There were several factors that could have influenced these outcomes:

- Group: Treatment Only (T Only) or Treatment + Dentifrice (T + D)
- Treatment: Control, Milk (x1/day), Milk (x3/day), Milk + Fluoride (x1/day), Milk + Fluoride (x3/day)
- Site: 1 (upper posterior buccal, left), 2 (upper anterior labial), 3 (upper posterior buccal, right), 4 (upper posterior palatal, right), 5 (upper anterior palatal), 6 (upper posterior palatal, left)
- Week: Weeks 1 and 6 of each treatment protocol
- Subject: 7 Subjects in each Group

Two problems were encountered whilst analysing the raw data. Firstly there were a number of sites where the enamel slab had fallen out, which resulted in 'missing' data. Secondly, due to a relatively high number of zero counts, the distributions of the three count measurements were highly skewed. In order to overcome these problems and allow statistical analysis, the counts were analysed on the $\log_{10}$ scale, with a 'small' constant (K) added to all counts, to address the problem of $\log_{10}(0)$. After examining various plots and statistical models, the constant of K=100 was used for the Total Counts (TC) data, whilst K=10 was used for the Lactobacillus Counts (LC) data. Within the proportions of lactobacilli in the total counts, two conditions were applied. Where LC was greater than TC, this was resolved by making TC=LC, i.e. 100%. Where the lactobacilli counts and total counts were zero, this resulted in 'missing data'. The number of zero counts and 'missing' data within the *S. mutans* data was so great that full

statistical analysis comparing the levels of *S. mutans* across the various factors of interest could not be carried out and so the data is summarised as simply the presence or absence of the organism in a subject.

For analysis of the Total Counts, summary statistics were carried out to determine the medians, with ranges, excluding zero counts and were transformed to the $\log_{10}$ scale. Boxplots were generated to visualise any noticeable trends. Formal analysis was then carried out using a general linear model of $\log_{10}$(Total Counts + 100) on the potential factors of interest to reveal any significant effects.

The same statistical approach was used in analysing the Lactobacillus Counts with the formal analysis of $\log_{10}$(Lactobacillus Counts + 10). Summary statistics were generated for the percentage of Lactobacillus Counts within the Total Counts. This was limited to Sites 1, 2 and 3 due to the high numbers of zero counts in the palatal sites (4, 5 and 6). When the simple percentage of Lactobacillus Counts in the Total Counts was modelled on the various factors, there was a suggestion of some interactions between the factors of interest. However, due to the relatively high number of 0%'s at one end and 100%'s at the other end of the scale, the fit of the statistical model was highly dubious. Therefore an alternative model was looked at by consideration of three components, namely total counts (TC), lactobacillus counts (LC) and non-lactobacillus counts (NLC), where:

$$TC = LC + NLC$$

Therefore, an alternative to the simple ratio of LC/TC is:

$$\frac{LC}{NLC} = \frac{LC}{TC-LC}$$

And for a better statistical fit to the data, the formal analysis was carried out using a general linear model of $\text{Log}_e \left\{ \frac{\text{LC}}{\text{NLC}} \right\}$ on the potential factors of interest.

Where 'effects' are stated in Section 3.3 (Results), there was evidence from the general linear model to suggest that the stated factor was having a statistically significant influence on the relevant outcome of interest.

Occasionally 'interactions' are referred to; these are two-way interactions, between two of the factors of interest, for example the interaction between Week and Site. Where the interaction is mentioned, there was evidence to suggest that the combined effect of two factors was having a statistically significant influence on the relevant outcome of interest.

3.2.6 Hypothesis

The aspect of greatest interest was the effect of Site on the Total Counts and proportions of lactobacilli within these counts. In theory, buccal sites within the mouth (1 and 3) should have a higher salivary flow rate compared to the palatal sites (4, 5 and 6). Additionally, buccal sites should have a higher salivary flow than labial sites. Therefore, although there may be less overall plaque/plaque bacteria at Site 2, the environment may be more acidic and so the proportions of aciduric bacteria (lactobacilli) within plaque from this site should be higher.

The next area of interest, microbiologically, was the effect of treatment on the outcome measures. In order to identify the differences within this factor, further analyses were performed using Bonferroni-adjusted multiple comparisons. It would be expected that the conditions that give optimum results of a decrease in overall counts and a decrease in aciduric bacteria within the total counts would be the consumption of fluoridated milk three times daily and including an additional rinse with a fluoridated dentifrice. The hypothesis is that these conditions would result in lower proportions of lactobacilli within the total counts than the consumption with milk alone with no fluoride.

3.3 Results

All the tables and figures relating to this section are found in the Appendix at the end of this chapter.

3.3.1 Total Counts

Table 3.3.1 a) contains the numbers of slabs with zero counts and the number of missing slabs. Both of these were relatively small at Sites 1-3 but increased at Site 4, especially at Week 6 where 16 slabs were missing. At Sites 5 and 6 the percentage of slabs with zero counts, at Weeks 1 and 6, were high. Site 5 had 52% and 41% zero counts at Week 1 and Week 6, respectively, while Site 6 had 45% and 29% at Week 1 and Week 6, respectively. Table 3.3.1 b) contains the summary statistics of Total Counts, namely the median (excluding zero counts), together with the range of Total Counts (excluding zero counts) separately for Weeks 1 and 6. Table 3.3.1 c) contains the same data as 3.3.1 b), but on the $\log_{10}$ scale.

The summary statistics shown in Tables 3.3.1 b) and c) and Figure 3.3.1 a), suggest that there was an effect of Site on the Total Counts. Sites 1, 2, and 3 appear to have higher numbers than Sites 4, 5, and 6. Figure 3.3.1 a) shows that Sites 1, 2, and 3 appear to cluster together on the grid, as do Sites 4, 5, and 6. Sites 1, 2 and 3 have greater counts and the clustering appears to be more noticeable at Week 6 with Sites 4, 5 and 6 grouping slightly further apart from Sites 1, 2, and 3. Formal analysis using a general linear model of $\log_{10}$(Total Counts + 100) on the potential factors of interest suggests

that there were significant effects of Site and Week only. Overall analysis of the combined Week 1 and Week 6 data revealed that Sites 1, 2 and 3 were significantly higher than Sites 4, 5 and 6, yet Site 1 was also significantly higher than Site 2. Therefore, as expected, Site 1 had higher counts than Site 2, but there was no suggestion of a difference between Sites 2 and 3. There was also the observation that Site 4 had higher counts than Sites 5 and 6. Table 3.3.1 d) shows the average counts per Site, ranked in order of highest to the lowest, for Week 1 and Week 6. This is the average across all the observations.

3.3.2 Lactobacillus Counts

Table 3.3.2 a) contains the number of slabs with zero counts and the number of missing slabs. It is evident that across all Sites, there were a fairly high percentage of zero counts, in particular at Week 1, with 70% at Site 5 and 42% at Site 6. The number of missing slabs was relatively small for Sites 1-3, but increased at Sites 4-6. Table 3.3.2 b) contains the summary statistics of Lactobacilli Counts, namely the median (excluding zero counts), together with the range of Lactobacilli Counts (excluding zero counts), again separate for Weeks 1 and 6. Table 3.3.2 c) contains the same data as 3.3.2 b), but on the $\log_{10}$ scale.

The summary statistics shown in Tables 3.3.2 b) and c) and Figure 3.3.2 a), suggests that there was an effect of Site on the Lactobacillus Counts. Sites 1, 2 and 3 appeared to have higher counts than Sites 4, 5 and 6. Figure 3.3.2 a) shows that Sites 1, 2 and 3 are clustering together on the grid, as do Sites 4, 5, and 6. Sites 1, 2 and 3 have greater

counts and the clustering appears to be more noticeable at Week 6 with Sites 4, 5 and 6 grouping slightly further apart from 1, 2, and 3.

Formal analysis using a general linear model of $\log_{10}$(Lacto Counts + 10) on the potential factors of interest revealed that there were several interactions involving Group (Treatment Only or Treatment + Dentifrice). These interactions meant that the treatment groups had to be analysed separately.

3.3.2.1 Treatment Only

Within this group there was an effect of treatment as well as an interaction between Week and Site. Due to the interaction between Week and Site, the effect of Site had to be analysed separately for Week 1 and Week 6.

Treatment Effect

The control treatment (no beverage) and milk with fluoride consumed three times daily had the lowest Lactobacillus Counts, with the only difference being that the fluoride treatment had a slightly greater range. Milk consumed alone and with fluoride once daily and alone three times a day all had greater counts, although for the consumption of milk three times daily the counts were slightly less. The boxplots shown in Figure 3.3.2 b) show the effects of these different treatments on the Lactobacillus Counts in the Treatment Only Group.

Site Effect, Week 1

At Week 1, Sites 1 and 3 have greater counts than Sites 4, 5 and 6 but Site 2 had greater counts than only Site 5.

Site Effect, Week 6

At Week 6 a different pattern across all the Sites was observed compared with Week 1. Sites 1, 2 and 3 all had greater counts than 4, 5 and 6 and Site 4 also had higher counts than Site 5.

3.3.2.2 Treatment + Dentifrice

Analysis of this Group did not reveal a treatment effect but there was a significant interaction between Week and Site, therefore the Site effect had to be analysed separately for each week.

Site Effect, Week 1

At Week 1, Sites 1 and 2 had higher counts than Sites 4, 5 and 6, and Site 3 had higher counts than 5 and 6.

Site Effect, Week 6

At Week 6, these differences changed so that Sites 1, 2 and 3 were all higher than Sites 4, 5 and 6.

3.3.3 Proportions of Lactobacilli in Total Counts

Table 3.3.3 a) shows the summary statistics for the percentage proportion of Lactobacillus Counts within the Total Counts (% LC/TC). These summary statistics show that there are still a number of slabs with 'missing' counts and with zero percent LC/TC. From a total of 105 slabs, at Week 1 the total number of slabs with missing counts is 12 for the Treatment Only Group and 6 missing counts for the Treatment + Dentifrice Group. The total number of slabs with 0% LC/TC is 13 in the Treatment Only Group and 22 in the Treatment + Dentifrice Group. At Week 6, the total number of slabs with missing counts and the number with 0% LC/TC is 4 in the Treatment Only Group and 2 in the Treatment + Dentifrice Group. When these two parameters are taken together, along with a number of instances of 100% LC/TC, it is the accumulative effect that influences the data set, causing the simple modelling of % LC/TC to be highly dubious.

Table 3.3.3 a) also contains the median % LC/TC, presented with either the zero counts included or excluded. Some of the differences are negligible or very small but there are many cases where this results in large differences. For example, in the Treatment Only Group at Week 1 of the Control, Sites 1, 2 and 3 have medians of 4.21%, 0% and 3.38%,

respectively, with the 0 counts included. However, when the 0 counts are excluded, the medians become 100%, 16.59% and 0.14% for Sites 1, 2 and 3, respectively. This problem seems to be more apparent for the data obtained at Week 1 for both study Groups. However, it still occurs at Week 6, although it appears to be confined mainly to Site 2, with the exception of Site 1 during the Control in the Treatment Only Group.

Using the alternative model described in *Section 3.2.5*, a main effect of Site is observed together with interactions of Group with Treatment and Group with Week. Follow-up multiple comparisons of the main effect of Site show that in terms of this alternative model, there is no evidence of a difference between Sites 1 and 3, as well as insufficient evidence of a significant difference between Sites 1 and 2. However, there is a significant difference between Sites 2 and 3, with Site 2 being much greater than Site 3.

Follow-up multiple comparisons carried out on the interactions of Group with Treatment show that there are no differences between treatments within the Treatment + Dentifrice group, but there is a suggestion (although not significantly) of differences between treatments for the Treatment Only group. Analysis of the interactions of Group with Week, using the alternative model previously described, showed that there is no difference between weeks for the Treatment Only group but there is a significant difference between weeks for the Treatment + Dentifrice group, where Week 6 is much greater than Week 1.

3.3.4 S. mutans Counts

Due to the high number of zero counts, the amount of information that can be analysed from the data collected on *S. mutans* is very limited. The only analysis that can be made is simply the presence or absence of *S. mutans*. This data is shown in Tables 3.3.4 a) and b). The number of slabs with *S. mutans* recorded ranges from either 1/64 to 11/69. The greatest number of patients that carry *S. mutans* at one particular site is four, which occurs at Site 3. The medians (excluding zero counts) show a general increase in *S. mutans* from Week 1 to Week 6, although the overall numbers are generally very low. The observation that all the patients carrying *S. mutans* have fallen into the Treatment + Dentifrice category is purely coincidental and means that no comparisons can be made between the two groups. Comparisons between Sites cannot be made due to the inconsistency of the isolation of *S. mutans*.

3.4 Discussion

Of the infectious diseases that affect humans, dental caries may be the most prevalent (Caufield and Griffin, 2000). Treatment for children with caries can be expensive, often requiring extensive restorative treatment under general anaesthesia (Becker *et al.*, 2002). In 1994, Van Houte reported that *S. mutans* had considerable epidemiological evidence to link it to caries and numerous laboratory investigations have demonstrated the ability of strains of this species to produce the lactic acid which causes dental caries. Van Houte also reported that various *Lactobacillus* species have been consistently associated with caries and are thought to be important secondary pathogens in dental caries.

Fluoridated milk has been suggested as an alternative to water fluoridation for the prevention of dental caries in children. The advantages of milk over water are the retention of consumer choice and also the added nutritional benefits from milk. A number of studies (Toth *et al.*, 1997; Al-Khateeb *et al.*, 1998; Kahama *et al.*, 1998) have investigated the effect of fluoridated milk using *in vitro* enamel demineralising and remineralising models, and *in vivo* studies have measured the fluoride of enamel following prolonged fluoridated milk intake (Toth *et al.*, 1987; 1989). There has been a lack of work carried out in the oral environment measuring changes in mineral content of enamel associated with the consumption of fluoridated milk. Factors such as plaque composition and intra-oral location may all influence the de- and remineralisation effects of fluoridated milk in the oral cavity.

Due to the nature of the overall study design, with regard to the demineralisation and remineralisation of enamel at different sites, the complete denture model was considered to be the most appropriate. However, it is also accepted that there are limitations associated with this model, especially when investigating the microbiological impact. Theilade *et al.* (1983) reported that *S. mutans* and *Lactobacillus* species were regularly identified from denture plaque, although the variation of carriage between subjects was very wide. In the study by Theilade *et al.* (1983), four of the eight subjects studied were negative for *S. mutans.* This observation within edentulous individuals was confirmed in the baseline screening results of the present study. Recruitment of subjects was therefore altered to the preferential carriage of both *S. mutans* and *Lactobacillus* spp., and also the carriage of lactobacilli alone. The underlying principle was the possibility that *S. mutans* may have increased in numbers when tooth specimens were introduced into the oral environment, but no real change was observed. However, missing data and a high percentage of zero counts had a detrimental effect on the overall outcomes of the investigation and influenced the statistical analyses to a great extent, limiting many of the observations.

Another limiting factor was the study numbers. While the main study involved 30 subjects, allocated in a random manner to either of the treatment groups, the volume of work generated during the microbiological analysis was too great to continue with the full number of subjects. It was therefore only possible to use a subset of the volunteers for this aspect of the study. These were the first fourteen subjects processed and started on the experimental treatments, with 50% in the Treatment Only group and 50% in the Treatment + Dentifrice group. Subject compliance was monitored through the return of

fluoride bottles and an end-of-study questionnaire, which revealed that compliance was good and no problems were encountered. It is also recognised that the subjects' diets may have an impact on the findings in addition to the treatment regimes. However it is assumed that the diets are relatively stable within individuals during each of the experiments.

The treatments chosen for this study were designed to answer questions regarding the influence of fluoridated milk on plaque bacterial counts and to determine whether frequency of intake was an influential component. The hypothesis was that milk consumed three times a day with a low fluoride dose would have a more positive anti-bacterial effect compared to no fluoride intake. On top of this rationale was the question of an additional benefit of rinsing with a fluoridated dentifrice to mimic toothbrushing with a fluoridated toothpaste. The main effect of treatment was observed within the treatment only study group for lactobacillus counts where it was shown that the control (no beverage) and milk with fluoride consumed three times a day had the lowest counts. This latter result was expected, although the consumption of milk once a day with the higher dose of fluoride resulted in greater counts than the other treatments. Due to the small number of subjects participating in this study group, this observation cannot be truly interpreted. The fact that treatment did not exert an effect in the treatment and dentifrice group is likely to be caused by a masking effect of the overall amount of exposure to fluoride within this group.

The most influential factor throughout the study was the intra-oral test-site location. These positions represented areas with either a good salivary flow rate or a low salivary

flow rate. The upper posterior buccal sites (1 and 3) had good salivary flow rates and therefore were expected to have higher clearance rates of sugars and acidic metabolic products causing lower proportions of aciduric bacteria (lactobacilli) within the total plaque bacterial counts. In contrast, the upper anterior labial site (2) had a poor salivary flow rate and so should have had greater proportions of lactobacilli within the total counts. Three palatal sites (4, 5 and 6) were also chosen. It was recognised that the movement of the tongue would result in continual removal of plaque from the palatal sites and this was confirmed in the present study when analysing the total counts and lactobacillus counts where Sites 1, 2 and 3 were greater than 4, 5 and 6. However, the number of slabs that were dislodged was disappointing and larger than expected. Efforts were made to increase retention by changing the adhesives that were used, eventually resorting to permanently embedding them with acrylic, but this was not always successful.

Of greatest interest was the effect of site on the proportions of lactobacilli within the total plaque bacteria. Statistical analysis was able to show that the buccal sites had similar proportions to each other and that the upper anterior labial site (2) had greater proportions than the left posterior buccal site (3). This was as predicted, but, analysis on the interactions between Group with both Treatment and Week was not able to provide greater insight into any further influences from the treatment protocols or the additional benefit from the fluoridated dentifrice rinse. The differences that were suggested between treatments in the Treatment Only group could be important, but due to the study number, this difference is not significant. The fact that there was no difference between treatments in the Treatment + Dentifrice group is probably again due to a masking effect

of the overall exposure to fluoride within this group. Unfortunately, the reason for the greater proportions at Week 6 in the Treatment + Dentifrice group and not in the Treatment Only group is unclear and cannot be determined from this study.

Changes in enamel mineral content of slabs forms a separate study which is not yet complete. Therefore it is not possible at this stage to compare the microbiological results obtained with the demineralisation/remineralisation changes at the specific sites. The small study number and large number of treatment regimes means that the quality of the data is less than optimal and that only very limited interpretation of the results is possible. However, there was the anticipated effect of site seen on the buccal and lingual aspects and it will be interesting to see if this is mirrored in the enamel light fluorescence and transverse microradiography results.

3.5 Appendix to Chapter 3

This section contains tables and figures to accompany Chapter 3.

3.5.1 Selection Criteria

Ideal requirements

1. Good attender/ Able to attend on a regular basis.

2. Mobile i.e. not requiring ambulance transport services.

3. Good denture wearer who doesn't require complicated treatment.

4. Must be completely edentulous.

5. Should have a "normal diet"

6. Requires to have an adequate salivary flow rate.

7. Needs to have bacteria required: *Streptococcus mutans* and *Lactobacillus* spp.

8. Should ideally be free of oral candidiasis.

9. Medical history should NOT include Sjögren's syndrome

10. Patient should not take antibiotics during study or use chlorhexidine mouthwash

11. Medication should ideally NOT include: antimicrobials
antihypertensives
anticholinergics
tricyclic antidepressants
sedatives/tranquillisers
diuretics
antihistamines

all of which may reduce the salivary flow.

Assessment

1. Full history and examination.

2. Diet diary - 3 days, one of which is on a weekend - to be sent out with screening appointment.

3. Do unstimulated saliva sample first then PBS rinse, with dentures IN.

4. Test salivary flow rate. Average unstimulated salivary flow rate is 0.3ml per minute.

5. Assess ridge form.

Table 3.3.1. a) Numbers of zero counts and missing slabs for total counts

	Site 1		Site 2		Site 3		Site 4		Site 5		Site 6	
	Wk1	Wk6	Wk1	Wk6	Wk1	Wk6	Wk1	Wk6	Wk1	Wk6	Wk1	Wk6
Number of Slabs	70	70	69	68	69	69	63	54	64	56	60	56
Number of '0's'	7	1	13	4	9	2	15	11	33	23	27	16
%of Slabs with 0 Count	10%	1%	19%	6%	13%	3%	24%	20%	52%	41%	45%	29%
Number of Missing Slabs	0	0	1	2	1	1	7	16	6	14	10	14

Table 3.3.1. b) Summary statistics for total counts at *Week 1* – *medians (range); excluding 0 counts*

Group	Treatment	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6	Overall
T Only	Control	$6.23x10^5$ ($4.41x10^4$ – $2.40x10^6$)	$2.63x10^4$ ($8.85x10^3$ – $3.05x10^5$)	$8.39x10^5$ ($3.75x10^5$ – $1.53x10^6$)	$1.68x10^4$ ($1.53x10^3$ – $1.59x10^5$)	$4.41x10^4$ ($1.96x10^3$ – $8.62x10^4$)	$1.48x10^4$ ($4.70x10^3$ – $2.48x10^4$)	$4.93x10^4$ ($1.53x10^3$ – $2.40x10^6$)
	Milk x1/day	$1.15x10^5$ ($7.08x10^3$ – $2.45x10^6$)	$1.41x10^4$ ($3.54x10^3$ – $2.74x10^6$)	$2.48x10^5$ ($3.35x10^3$ – $5.94x10^6$)	$1.59x10^4$ ($4.52x10^3$ – $1.46x10^6$)	$6.21x10^4$ ($8.25x10^3$ – $1.16x10^5$)	$1.77x10^4$ ($4.22x10^3$ – $3.55x10^5$)	$9.82x10^4$ ($3.35x10^3$ – $5.94x10^6$)
	Milk x3/day	$5.54x10^5$ ($3.54x10^3$ – $2.20x10^6$)	$1.35x10^6$ ($1.43x10^3$ – $2.71x10^6$)	$1.13x10^5$ ($2.03x10^3$ – $1.17x10^7$)	$1.84x10^4$ ($1.59x10^3$ – $4.65 x10^6$)	$6.27x10^3$ ($2.75x10^3$ – $9.79x10^3$)	$3.08x10^4$ ($6.21x10^3$ – $7.61x10^5$)	$7.96x10^4$ ($1.43x10^3$ – $1.17x10^7$)
	M + F x1/day	$4.44x10^5$ ($2.10x10^5$ – $2.41x10^6$)	$3.95x10^5$ ($6.52x10^4$ – $3.08x10^6$)	$1.75x10^5$ ($1.91x10^3$ – $8.49x10^5$)	$9.64x10^3$ ($1.77x10^3$ – $1.91x10^5$)	$1.11x10^4$ ($3.92x10^3$ – $4.60x10^4$)	$1.32x10^4$ ($1.84x10^3$ – $1.99x10^5$)	$6.52x10^4$ ($1.77x10^3$ – $3.08x10^6$)
	M + F x3/day	$5.88x10^4$ ($3.68x10^3$ – $2.44x10^6$)	$4.83x10^5$ ($2.96x10^4$ – $1.02x10^6$)	$4.31x10^5$ ($1.68x10^3$ – $3.15x10^6$)	$7.96x10^3$ ($4.52x10^3$ – $5.81x10^4$)	$6.88x10^4$ ($5.87x10^4$ – $1.58x10^5$)	$6.34x10^3$ ($1.88x10^3$ – $7.06x10^3$)	$5.84x10^4$ ($1.68x10^3$ – $3.15x10^6$)
T+D	Control	$2.90x10^5$ ($1.99x10^3$ – $3.37x10^6$)	$6.24x10^5$ ($3.70x10^3$ – $3.20x10^6$)	$5.41x10^5$ ($1.19x10^4$ – $1.31x10^7$)	$3.53x10^3$ ($1.38x10^3$ – $5.52x10^5$)	$1.63x10^4$ ($3.54x10^3$ – $3.72x10^4$)	$3.96x10^4$ ($1.92x10^3$ – $5.19x10^4$)	$8.10x10^4$ ($1.38x10^3$ – $1.31x10^7$)
	Milk x1/day	$4.36x10^5$ ($1.19x10^4$ – $1.99x10^6$)	$8.81x10^5$ ($3.29x10^3$ – $4.12x10^6$)	$6.63x10^5$ ($1.38x10^3$ – $3.50x10^6$)	$2.64x10^4$ ($1.40x10^4$ – $2.40x10^6$)	$5.14x10^3$ ($2.07x10^3$ – $1.46x10^6$)	$2.25x10^4$ ($3.99x10^3$ – $3.91x10^4$)	$5.13x10^4$ ($1.38x10^3$ – $4.12x10^6$)
	Milk x3/day	$1.56x10^5$ ($1.55x10^4$ – $2.01x10^6$)	$6.54x10^4$ ($8.22x10^3$ – $1.87x10^6$)	$5.56x10^5$ ($1.69x10^3$ – $1.74x10^6$)	$7.06x10^3$ ($2.41x10^3$ – $1.36x10^5$)	$1.28x10^4$ ($4.41x10^3$ – $2.23x10^4$)	$4.28x10^4$ ($3.83x10^3$ – $4.87x10^4$)	$4.33x10^4$ ($1.69x10^3$ – $2.01x10^6$)
	M + F x1/day	$1.13x10^5$ ($1.99x10^4$ – $9.16x10^5$)	$4.70x10^4$ ($3.29x10^3$ – $3.86x10^6$)	$1.48x10^5$ ($1.29x10^4$ – $4.68x10^6$)	$2.70x10^4$ ($1.71x10^3$ – $8.72x10^5$)	$1.04x10^4$ ($3.82x10^3$ – $4.86x10^4$)	$1.20x10^4$ $5.62x10^3$ – $2.14x10^4$)	$4.86x10^4$ ($1.71x10^3$ – $4.68x10^6$)
	M + F x3/day	$5.05x10^5$ ($1.12x10^5$ – $4.15x10^6$)	$5.86x10^4$ ($4.93x10^3$ – $4.14x10^5$)	$7.95x10^4$ ($1.64x10^3$ – $5.41x10^5$)	$1.49x10^4$ ($3.12x10^3$ – $4.36x10^4$)	$3.97x10^3$ ($1.71x10^3$ – $7.64x10^3$)	$7.78x10^3$ ($2.25x10^3$ – $2.25x10^4$)	$2.82x10^4$ ($1.64x10^3$ – $4.15x10^6$)

Table 3.3.1. b) Summary statistics for total counts at *Week 6 - medians (range); excluding 0 counts*

Group	Treatment	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6	Overall
T Only	Control	$5.07x10^5$ ($1.77x10^3$ – $9.29x10^5$)	$1.62x10^5$ ($3.67x10^4$ – $1.94x10^6$)	$2.39x10^6$ ($2.03x10^4$ – $1.03x10^7$)	$1.35x10^5$ ($7.93x10^3$ – $3.16x10^5$)	$1.56x10^5$ ($3.92x10^3$ – $3.08x10^5$)	$7.06x10^3$ ($2.11x10^3$ – $9.52x10^5$)	$3.08x10^5$ ($1.77x10^3$ – $1.03x10^7$)
	Milk x1/day	$4.50x10^6$ ($6.19x10^4$ – $6.22x10^6$)	$5.47x10^5$ ($5.08x10^4$ – $1.77x10^7$)	$7.86x10^5$ ($5.03x10^4$ – $5.55x10^6$)	$3.19x10^4$ ($1.59x10^3$ – $1.78x10^6$)	$3.17x10^4$ ($2.51x10^3$ – $1.51x10^5$)	$2.21x10^4$ ($1.69x10^4$ – $2.12x10^5$)	$1.75x10^5$ ($1.59x10^3$ – $1.77x10^7$)
	Milk x3/day	$2.39x10^6$ ($3.05x10^3$ – $5.62x10^7$)	$2.95x10^5$ ($4.07x10^4$ – $2.93x10^7$)	$4.83x10^6$ ($6.08x10^3$ – $1.53x10^7$)	$1.17x10^5$ ($1.43x10^4$ – $1.42 x10^6$)	$3.01x10^4$ ($5.87x10^3$ – $3.62x10^4$)	$6.34x10^3$ ($1.84x10^3$ – $3.47x10^5$)	$2.26x10^5$ ($1.84x10^3$ – $5.62x10^7$)
	M + F x1/day	$2.00x10^6$ ($4.57x10^3$ – $2.74x10^6$)	$1.41x10^6$ ($1.75x10^5$ – $6.92x10^7$)	$1.10x10^6$ ($7.46x10^4$ – $6.10x10^7$)	$8.22x10^4$ ($1.53x10^3$ – $2.23x10^6$)	$1.76x10^4$ ($1.96x10^3$ – $4.80x10^5$)	$5.32x10^3$ ($1.84x10^3$ – $1.99x10^5$)	$1.95x10^5$ ($1.53x10^3$ – $6.92x10^7$)
	M + F x3/day	$1.65x10^6$ ($3.05x10^3$ – $5.62x10^6$)	$1.02x10^5$ ($1.14x10^4$ – $5.91x10^6$)	$1.15x10^6$ ($4.06x10^3$ – $1.06x10^7$)	$1.27x10^4$ ($3.54x10^3$ – $4.01x10^5$)	$1.16x10^4$ ($9.51x10^3$ – $1.37x10^4$)	$3.76x10^3$ ($2.11x10^3$ – $1.38x10^5$)	$2.01x10^5$ ($2.11x10^3$ – $1.06x10^7$)
T+D	Control	$1.34x10^6$ ($8.68x10^3$ – $8.81x10^6$)	$7.42x10^5$ ($4.81x10^4$ – $5.46x10^6$)	$1.94x10^6$ ($3.75x10^3$ – $8.03x10^6$)	$1.84x10^5$ ($6.72x10^4$ – $4.54x10^5$)	$1.02x10^5$ ($1.34x10^4$ – $1.86x10^5$)	$3.92x10^5$ ($3.75x10^3$ – $1.84x10^6$)	$4.64x10^5$ ($3.75x10^3$ – $8.81x10^6$)
	Milk x1/day	$1.97x10^6$ ($5.05x10^4$ – $1.44x10^7$)	$2.88x10^5$ ($6.77x10^3$ – $1.45x10^7$)	$3.02x10^6$ ($4.15x10^3$ – $1.27x10^7$)	$2.05x10^4$ ($3.41x10^3$ – $5.71x10^6$)	$2.87x10^4$ ($1.71x10^3$ – $1.61x10^6$)	$4.19x10^4$ ($2.25x10^4$ – $8.15x10^4$)	$3.25x10^5$ ($1.71x10^3$ – $1.45x10^7$)
	Milk x3/day	$1.22x10^6$ ($2.25x10^5$ – $7.11x10^6$)	$1.20x10^6$ ($3.29x10^4$ – $9.07x10^6$)	$2.17x10^6$ ($1.50x10^4$ – $2.79x10^7$)	$7.24x10^4$ ($3.02x10^4$ – $7.33x10^4$)	$1.18x10^5$ ($2.07x10^3$ – $7.07x10^5$)	$2.30x10^5$ ($3.26x10^4$ – $4.28x10^5$)	$5.08x10^5$ ($2.07x10^3$ – $2.79x10^7$)
	M + F x1/day	$7.37x10^5$ ($1.01x10^5$ – $9.18x10^6$)	$4.90x10^5$ ($4.52x10^3$ – $6.06x10^6$)	$1.69x10^6$ ($1.93x10^4$ – $7.23x10^6$)	$4.12x10^4$ ($1.64x10^3$ – $1.03x10^5$)	$1.53x10^4$ ($2.50x10^3$ – $2.56x10^5$)	$8.62x10^4$ ($1.48x10^3$ – $1.97x10^5$)	$2.56x10^5$ ($1.48x10^3$ – $9.18x10^6$)
	M + F x3/day	$1.07x10^6$ ($2.39x10^5$ – $3.41x10^6$)	$2.67x10^6$ ($5.59x10^4$ – $5.21x10^6$)	$5.44x10^5$ ($9.95x10^4$ – $4.05x10^6$)	$2.97x10^4$ ($8.53x10^3$ – $1.38x10^6$)	$3.32x10^3$ ($2.50x10^3$ – $4.14x10^3$)	$1.89x10^4$ ($1.87x10^3$ – $3.60x10^4$)	$4.09x10^5$ ($1.87x10^3$ – $5.21x10^6$)

Table 3.3.1. c) Summary statistics for $\log_{10}$(total counts + 100) at *Week 1 - medians (range);excluding 0 counts*

Group	Treatment	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6	Overall
T Only	Control	5.70 (4.65 – 6.38)	4.42 (3.95 – 5.48)	5.90 (5.57 – 6.19)	4.23 (3.21 – 5.20)	4.13 (3.31 – 4.94)	4.04 (3.68 – 4.40)	4.69 (3.21 – 6.38)
	Milk x1/day	5.04 (3.86 – 6.39)	4.15 (3.56 – 6.44)	5.40 (3.54 – 6.78)	4.20 (3.67 – 6.16)	4.49 (3.92 – 5.07)	4.25 (3.64 – 5.55)	4.99 (3.54 – 6.77)
	Milk x3/day	5.71 (3.56 – 6.34)	6.13 (3.19 – 6.43)	5.05 (3.33 – 7.07)	4.27 (3.23 – 6.67)	3.73 (3.46 – 4.00)	4.33 (3.80 – 5.88)	4.90 (3.19 – 7.07)
	M + F x1/day	5.65 (5.32 – 6.38)	5.60 (4.82 – 6.49)	5.16 (3.30 – 5.93)	3.86 (3.27 – 5.28)	4.05 (3.60 – 4.66)	4.10 (3.29 – 5.30)	4.82 (3.27 – 6.49)
	M + F x3/day	4.77 (3.58 – 6.39)	5.31 (4.47 – 6.01)	5.62 (3.25 – 6.50)	3.91 (3.67 – 4.77)	4.84 (4.77 – 5.20)	3.81 (3.30 – 3.86)	4.77 (3.25 – 6.50)
T+D	Control	5.46 (3.32 – 6.53)	5.68 (3.58 – 6.51)	5.52 (4.08 – 7.12)	3.56 (3.17 – 5.74)	4.03 (3.56 – 4.57)	4.59 (3.31 – 4.72)	4.88 (3.17 – 7.12)
	Milk x1/day	5.63 (4.08 – 6.30)	5.95 (3.53 – 6.62)	5.76 (3.17 – 6.54)	4.42 (4.15 – 6.38)	3.72 (3.34 – 6.16)	4.35 (3.61 – 4.59)	4.71 (3.17 – 6.62)
	Milk x3/day	5.19 (4.19 – 6.30)	4.81 (3.92 – 6.27)	5.55 (3.25 – 6.24)	3.86 (3.40 – 5.13)	4.03 (3.63 – 4.35)	4.63 (3.59 – 4.69)	4.64 3.25 – 6.30)
	M + F x1/day	5.05 (4.30 – 5.96)	4.67 (3.53 – 6.59)	5.14 (4.11 – 6.67)	4.39 (3.26 – 5.94)	4.02 (3.59 – 4.69)	4.08 (3.76 – 4.33)	4.69 (3.26 – 6.67)
	M + F x3/day	5.70 (5.05 – 6.62)	4.72 (3.70 – 5.62)	4.75 (3.24 – 5.73)	4.10 (3.51 – 4.64)	3.61 (3.26 – 3.89)	3.89 (3.37 – 4.35)	4.44 (3.24 – 6.62)

Table 3.3.1. c) Summary statistics for log_{10}(total counts + 100) – at *Week 6 - medians (range); excluding 0 counts*

Group	Treatment	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6	Overall
T Only	Control	5.71 (3.27 – 5.97)	5.16 (4.57 – 6.29)	6.36 (4.31 – 7.01)	5.13 (3.91 – 5.50)	4.55 (3.60 – 5.49)	3.86 (3.34 – 5.98)	5.49 (3.27 – 7.01)
	Milk x1/day	6.65 (4.79 – 6.79)	5.74 (4.71 – 7.25)	5.71 (4.70 – 6.74)	4.51 (3.23 – 6.25)	4.50 (3.42 – 5.18)	4.35 (4.23 – 5.33)	5.24 (3.23 – 7.25)
	Milk x3/day	6.38 (3.50 – 7.75)	5.46 (4.61 – 7.47)	6.68 (3.79 – 7.19)	5.07 (4.16 – 6.15)	4.48 (3.78 – 4.56)	3.81 (3.29 – 5.54)	5.35 (3.29 – 7.75)
	M + F x1/day	6.30 (3.67 – 6.44)	5.90 (5.24 – 7.84)	6.04 (4.87 – 7.79)	4.67 (3.21 – 6.35)	4.25 (3.31 – 5.68)	3.73 (3.29 – 5.30)	5.29 (3.21 – 7.84)
	M + F x3/day	6.22 (3.50 – 6.75)	5.01 (4.06 – 6.77)	6.06 (3.62 – 7.03)	4.11 (3.56 – 5.60)	4.06 (3.98 – 4.14)	3.59 (3.34 – 5.14)	5.30 (3.34 – 7.03)
T+D	Control	6.13 (3.94 – 6.95)	5.87 (4.68 – 6.74)	6.14 (3.59 – 6.91)	5.27 (4.83 – 5.67)	4.83 (4.13 – 5.27)	5.27 (3.59 – 6.27)	5.67 (3.59 – 6.95)
	Milk x1/day	6.30 (4.70 – 7.16)	5.39 (3.84 – 7.16)	6.48 (3.63 – 7.10)	4.31 (3.55 – 6.76)	4.46 (3.26 – 6.21)	4.14 (3.37 – 4.91)	5.48 (3.26 – 7.16)
	Milk x3/day	6.09 (5.35 – 6.85)	6.08 (4.52 – 6.96)	6.34 (4.18 – 7.45)	4.86 (4.48 – 4.47)	4.79 (3.34 – 5.85)	5.07 (4.52 – 5.63)	5.70 (3.34 – 7.45)
	M + F x1/day	5.87 (5.01 – 6.96)	5.69 (3.67 – 6.78)	6.23 (4.29 – 6.86)	4.63 (3.24 – 5.01)	4.19 (3.42 – 5.41)	4.94 (3.20 – 5.30)	5.41 (3.20 – 6.96)
	M + F x3/day	6.03 (5.38 – 6.53)	6.29 (4.75 – 6.72)	5.74 (5.00 – 6.61)	4.47 (3.94 – 6.14)	3.52 (3.42 – 3.63)	3.93 (3.30 – 4.56)	5.61 (3.29 – 6.72)

Table 3.3.1 d) Average of the medians of log_{10} (total counts + 100) *(range)* across all observations, for Week 1 and Week 6

WEEK	SITE 1	SITE 2	SITE 3	SITE 4	SITE 5	SITE 6
WEEK 1	5.33 *(2.00-6.62)*	4.62 *(2.00-6.62)*	5.13 *(2.00-7.13)*	3.86 *(2.00-6.67)*	2.00 *(2.00-6.16)*	3.34 *(2.00-5.88)*
WEEK 6	6.03 *(2.00-7.75)*	5.60 *(2.00-7.84)*	6.23 *(2.00-7.79)*	4.47 *(2.00-6.76)*	3.42 *(2.00-6.21)*	3.61 *(2.00-6.27)*

Table 3.3.1 d) Average of the medians of log_{10} (total counts + 100) *(range)* across all observations, for Week 1 and Week 6.

Colour coded from highest - Red
Pink
Green
Blue
Brown
lowest - Grey

Table 3.3.1 d) Average of the medians of log_{10} (total counts + 100) *(range)* across all observations, for Week 1 and Week 6

WEEK	SITE 1	SITE 2	SITE 3	SITE 4	SITE 5	SITE 6
WEEK 1	5.33 *(2.00-6.62)*	4.62 *(2.00-6.62)*	5.13 *(2.00-7.13)*	3.86 *(2.00-6.67)*	2.00 *(2.00-6.16)*	3.34 *(2.00-5.88)*
WEEK 6	6.03 *(2.00-7.75)*	5.60 *(2.00-7.84)*	6.23 *(2.00-7.79)*	4.47 *(2.00-6.76)*	3.42 *(2.00-6.21)*	3.61 *(2.00-6.27)*

Table 3.3.1 d) Average of the medians of log_{10} (total counts + 100) *(range)* across all observations, for Week 1 and Week 6.

Colour coded from highest - Red
Pink
Green
Blue
Brown
lowest - Grey

Figure 3.3.1 Boxplots illustrating site by week effect on total counts

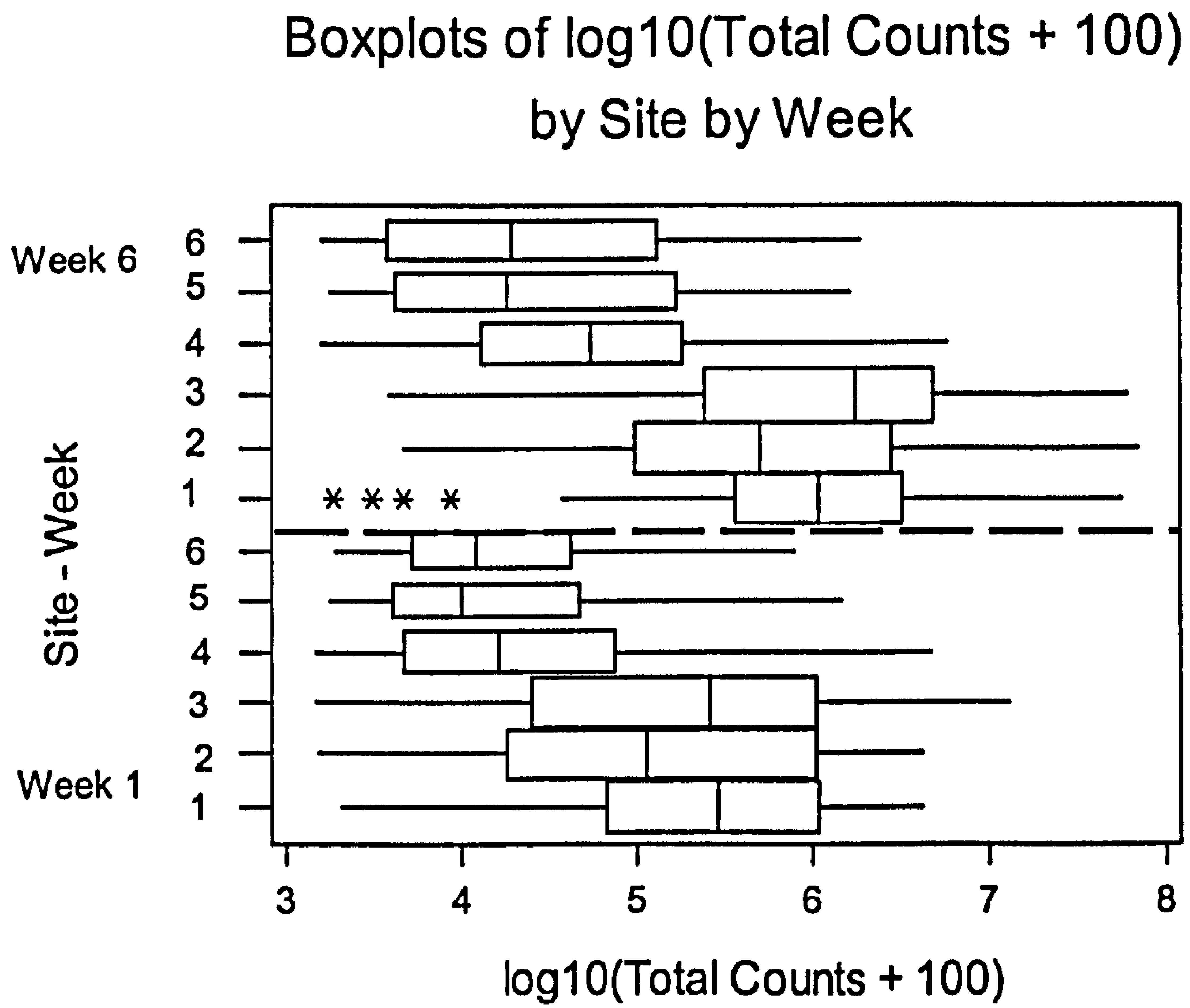


Figure 3.3.1 Boxplots of Log_{10}(Total Counts + 100) by Site By Week

Table 3.3.2. a) Numbers of zero counts and missing slabs for lacto counts

	Site 1		Site 2		Site 3		Site 4		Site 5		Site 6	
	Wk1	Wk6	Wk1	Wk6	Wk1	Wk6	Wk1	Wk6	Wk1	Wk6	Wk1	Wk6
Number of Slabs	70	70	69	68	69	69	63	54	64	56	60	56
Number of '0's'	10	2	25	7	16	0	23	7	45	22	25	22
%of Slabs with 0 Count	14%	3%	36%	10%	23%	0%	37%	13%	70%	39%	42%	9%
Number of Missing Slabs	0	0	1	2	1	1	7	16	6	14	10	14

Table 3.3.2. b) Summary statistics for lacto counts at *Week 1 – medians (range); excluding 0 counts*

Group	Treatment	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6	Overall
T Only	Control	$0.40x10^{1}$ ($0.20x10^{1}$ – $4.17x10^{4}$)	$5.59x10^{3}$ ($1.54x10^{2}$ – $5.06x10^{4}$)	$1.36x10^{4}$ ($0.80x10^{1}$ – $1.88x10^{4}$)	$2.20x10^{1}$ ($0.50x10^{1}$ – $2.17x10^{2}$)	$4.42x10^{2}$ ($4.42x10^{2}$ – $4.42x10^{2}$)	$1.60x10^{1}$ ($0.20x10^{1}$ – $2.81x10^{2}$)	$9.50x10^{1}$ ($0.20x10^{1}$ – $5.06x10^{4}$)
	Milk x1/day	$7.11x10^{3}$ ($0.20x10^{1}$ – $2.72x10^{4}$)	$2.10x10^{2}$ ($0.10x10^{1}$ – $8.16x10^{4}$)	$4.01x10^{2}$ ($1.00x10^{1}$ – $2.59x10^{5}$)	$3.40x10^{1}$ ($0.20x10^{1}$ – $3.50x10^{3}$)	$5.10x10^{1}$ ($1.10x10^{1}$ – $1.06x10^{2}$)	$0.80x10^{1}$ ($0.20x10^{1}$ – $3.76x10^{3}$)	$4.60x10^{1}$ ($0.10x10^{1}$ – $2.59x10^{5}$)
	Milk x3/day	$3.68x10^{2}$ ($0.50x10^{1}$ – $3.96x10^{4}$)	$1.92x10^{3}$ ($1.70x10^{1}$ – $1.10x10^{5}$)	$5.74x10^{3}$ ($5.00x10^{1}$ – $1.04x10^{5}$)	$1.11x10^{2}$ ($0.40x10^{1}$ – $3.00 x10^{3}$)	$1.10x10^{1}$ ($0.80x10^{1}$ – $1.60x10^{1}$)	$1.34x10^{2}$ ($0.20x10^{1}$ – $3.25x10^{2}$)	$1.60x10^{2}$ ($0.20x10^{1}$ – $1.10x10^{5}$)
	M + F x1/day	$4.27x10^{3}$ ($0.50x10^{1}$ – $2.17x10^{5}$)	$7.05x10^{4}$ ($2.07x10^{3}$ – $1.39x10^{5}$)	$1.65x10^{3}$ ($2.42x10^{2}$ – $8.92x10^{4}$)	$3.86x10^{2}$ ($2.80x10^{1}$ – $1.21x10^{4}$)	$1.94x10^{2}$ ($5.00x10^{1}$ – $5.84x10^{2}$)	$2.60x10^{1}$ ($0.20x10^{1}$ – $4.08x10^{4}$)	$8.42x10^{2}$ ($0.20x10^{1}$ – $2.17x10^{5}$)
	M + F x3/day	$5.90x10^{2}$ ($0.70x10^{1}$ – $2.38x10^{4}$)	$1.06x10^{2}$ ($0.80x10^{1}$ – $1.90x10^{5}$)	$6.70x10^{1}$ ($0.20x10^{1}$ – $1.48x10^{4}$)	$2.69x10^{2}$ ($0.30x10^{1}$ – $7.22x10^{2}$)	$1.16x10^{2}$ ($1.16x10^{2}$ – $1.16x10^{2}$)	$2.90x10^{1}$ ($0.90x10^{1}$ – $4.47x10^{2}$)	$6.30x10^{1}$ ($0.20x10^{1}$ – $1.90x10^{5}$)
T+D	Control	$8.50x10^{1}$ ($0.30x10^{1}$ – $5.37x10^{5}$)	$1.59x10^{4}$ ($3.48x10^{3}$ – $1.41x10^{5}$)	$1.58x10^{3}$ ($0.50x10^{1}$ – $8.94x10^{3}$)	$3.70x10^{1}$ ($1.70x10^{1}$ – $4.60x10^{1}$)	$8.06x10^{3}$ ($1.90x10^{1}$ – $1.61x10^{4}$)	$4.20x10^{1}$ ($2.90x10^{1}$ – $6.18x10^{3}$)	$1.07x10^{2}$ ($0.30x10^{1}$ – $5.37x10^{5}$)
	Milk x1/day	$9.70x10^{1}$ ($1.80x10^{1}$ – $1.76x10^{3}$)	$2.95x10^{4}$ ($0.20x10^{1}$ – $1.55x10^{5}$)	$1.90x10^{2}$ ($3.30x10^{1}$ – $3.38x10^{5}$)	$8.20x10^{1}$ ($0.50x10^{1}$ – $3.12x10^{2}$)	$5.50x10^{1}$ ($0.20x10^{1}$ – $1.08x10^{2}$)	$1.50x10^{1}$ ($0.20x10^{1}$ – $2.80x10^{1}$)	$1.08x10^{2}$ ($0.20x10^{1}$ – $3.38x10^{5}$)
	Milk x3/day	$1.83x10^{4}$ ($1.61x10^{2}$ – $4.36x10^{5}$)	$1.64x10^{3}$ ($1.27x10^{2}$ – $1.05x10^{5}$)	$6.93x10^{2}$ ($1.30x10^{1}$ – $3.72x10^{3}$)	$1.12x10^{2}$ ($1.90x10^{1}$ – $1.45x10^{2}$)	$3.85x10^{3}$ ($7.03x10^{2}$ – $7.00x10^{3}$)	$5.01x10^{2}$ ($0.60x10^{1}$ – $9.96x10^{2}$)	$6.98x10^{2}$ ($0.60x10^{1}$ – $4.36x10^{5}$)
	M + F x1/day	$1.66x10^{2}$ ($1.51x10^{2}$ – $7.50x10^{3}$)	$1.38x10^{4}$ ($0.20x10^{1}$ – $2.92x10^{5}$)	$5.06x10^{2}$ ($4.00x10^{1}$ – $2.28x10^{4}$)	$6.00x10^{1}$ ($4.60x10^{1}$ – $7.50x10^{1}$)	$0.60x10^{1}$ ($0.60x10^{1}$ – $0.60x10^{1}$)	$5.61x10^{2}$ $0.20x10^{1}$ – $1.12x10^{3}$)	$1.65x10^{2}$ ($0.20x10^{1}$ – $2.92x10^{5}$)
	M + F x3/day	$1.64x10^{4}$ ($1.65x10^{2}$ – $2.13x10^{4}$)	$6.18x10^{4}$ ($2.03x10^{3}$ – $2.01x10^{5}$)	$8.00x10^{1}$ ($0.20x10^{1}$ – $1.82x10^{2}$)	$8.00x10^{1}$ ($0.30x10^{1}$ – $6.93x10^{3}$)	$2.07x10^{2}$ ($2.07x10^{2}$ – $2.07x10^{2}$)	$1.40x10^{1}$ ($0.20x10^{1}$ – $1.60x10^{1}$)	$1.82x10^{2}$ ($0.20x10^{1}$ – $2.01x10^{5}$)

Table 3.3.2. b) Summary statistics for lacto counts at *Week 6 - medians (range); excluding 0 counts*

Group	Treatment	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6	Overall
T Only	Control	$1.47x10^4$ ($0.20x10^1$ – $1.10x10^5$)	$3.29x10^3$ ($1.40x10^1$ – $3.19x10^5$)	$5.64x10^2$ ($0.30x10^1$ – $2.83x10^5$)	$2.30x10^1$ ($1.10x10^1$ – $2.45x10^2$)	$0.40x10^1$ ($0.40x10^1$ – $0.40x10^1$)	$2.10x10^2$ ($0.60x10^1$ – $2.03x10^3$)	$3.50x10^2$ ($0.20x10^1$ – $3.19x10^5$)
	Milk x1/day	$6.48x10^4$ ($1.40x10^1$ – $1.11x10^6$)	$1.58x10^4$ ($1.60x10^1$ – $3.51x10^5$)	$1.72x10^3$ ($2.60x10^1$ – $1.30x10^5$)	$2.29x10^2$ ($0.70x10^1$ – $1.43x10^4$)	$8.50x10^1$ ($0.60x10^1$ – $1.80x10^4$)	$2.30x10^3$ ($0.20x10^1$ – $7.81x10^4$)	$1.72x10^3$ ($0.20x10^1$ – $1.11x10^6$)
	Milk x3/day	$2.92x10^4$ ($3.34x10^2$ – $3.96x10^5$)	$4.94x10^3$ ($2.62x10^2$ – $9.76x10^4$)	$4.72x10^3$ ($9.10x10^1$ – $4.16x10^5$)	$2.17x10^2$ ($2.70x10^1$ – $1.70x10^4$)	$0.30x10^1$ ($0.20x10^1$ – $1.26x10^3$)	$1.67x10^2$ ($0.20x10^1$ – $4.73x10^2$)	$8.62x10^2$ ($0.20x10^1$ – $4.16x10^5$)
	M + F x1/day	$2.48x10^4$ ($0.60x10^1$ – $2.44x10^5$)	$1.96x10^4$ ($3.42x10^2$ – $1.23x10^5$)	$2.91x10^4$ ($2.60x10^1$ – $1.89x10^5$)	$1.19x10^2$ ($0.20x10^1$ – $5.94x10^4$)	$1.13x10^2$ ($2.40x10^1$ – $6.68x10^3$)	$3.50x10^1$ ($1.10x10^1$ – $6.78x10^4$)	$1.80x10^3$ ($0.20x10^1$ – $2.44x10^5$)
	M + F x3/day	$1.58x10^4$ ($9.10x10^1$ – $1.03x10^5$)	$3.28x10^3$ ($0.10x10^1$ – $2.26x10^5$)	$3.92x10^2$ ($6.50x10^1$ – $1.94x10^5$)	$5.30x10^1$ ($0.20x10^1$ – $3.14x10^2$)	$3.50x10^1$ ($1.10x10^1$ – $3.33x10^2$)	- -	$2.76x10^2$ ($0.20x10^1$ – $2.26x10^5$)
T+D	Control	$2.65x10^4$ ($5.00x10^2$ – $8.07x10^5$)	$1.53x10^5$ ($6.25x10^2$ – $4.39x10^5$)	$2.59x10^4$ ($6.00x10^1$ – $1.20x10^5$)	$6.70x10^2$ ($0.30x10^1$ – $1.68x10^4$)	$1.70x10^1$ ($1.00x10^1$ – $1.50x10^4$)	$9.09x10^4$ ($1.90x10^1$ – $3.18x10^5$)	$1.69x10^4$ ($0.30x10^1$ – $8.07x10^5$)
	Milk x1/day	$3.07x10^4$ ($1.35x10^3$ – $1.90x10^6$)	$9.85x10^3$ ($1.04x10^2$ – $1.03x10^6$)	$6.61x10^3$ ($1.06x10^2$ – $7.34x10^4$)	$4.20x10^1$ ($0.30x10^1$ – $1.75x10^4$)	$5.00x10^1$ ($1.10x10^1$ – $1.13x10^5$)	$6.40x10^1$ ($2.80x10^1$ – $1.05x10^2$)	$1.35x10^3$ ($0.30x10^1$ – $1.90x10^6$)
	Milk x3/day	$7.22x10^3$ ($3.40x10^2$ – $1.24x10^5$)	$4.30x10^4$ ($4.90x10^3$ – $1.23x10^6$)	$4.17x10^4$ ($1.50x10^1$ – $5.80x10^5$)	$7.40x10^1$ ($2.80x10^1$ – $1.00x10^2$)	$4.91x10^2$ ($0.20x10^1$ – $3.01x10^3$)	$1.28x10^2$ ($0.20x10^1$ – $1.46x10^4$)	$3.96x10^3$ ($0.20x10^1$ – $1.23x10^6$)
	M + F x1/day	$6.36x10^3$ ($2.78x10^3$ – $1.42x10^5$)	$5.20x10^4$ ($2.00x10^1$ – $1.75x10^5$)	$1.17x10^4$ ($1.57x10^2$ – $5.75x10^4$)	$2.16x10^2$ ($0.20x10^1$ – $5.21x10^3$)	$1.16x10^3$ ($1.00x10^1$ – $7.07x10^3$)	$2.10x10^1$ $0.20x10^1$ – $4.79x10^3$)	$4.79x10^3$ ($0.20x10^1$ – $1.75x10^5$)
	M + F x3/day	$4.93x10^4$ ($1.36x10^3$ – $8.95x10^5$)	$9.53x10^4$ ($0.30x10^1$ – $1.16x10^6$)	$1.36x10^4$ ($6.32x10^2$ – $1.49x10^5$)	$4.57x10^3$ ($3.20x10^1$ – $2.08x10^4$)	$0.40x10^1$ ($0.20x10^1$ – $0.60x10^1$)	$2.43x10^2$ ($0.70x10^1$ – $1.64x10^4$)	$1.36x10^4$ ($0.20x10^1$ – $1.16x10^6$)

Table 3.3.2. c) Summary statistics for log_{10}(lacto counts + 10) at *Week 1 - medians (range)*; excluding 0 counts

Group	Treatment	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6	Overall
T Only	Control	1.13 (1.06 – 4.62)	3.75 (2.22 – 4.70)	4.13 (1.24 – 4.27)	1.47 (1.19 – 2.36)	2.66 (2.66 – 2.66)	1.36 (1.08– 2.46)	1.94 (1.06 – 4.70)
	Milk x1/day	3.85 (1.06 – 4.44)	1.49 (1.06 – 4.91)	2.61 (1.30 – 5.41)	1.64 (1.06 – 3.55)	1.78 (1.32 – 2.07)	1.25 (1.08 – 3.58)	1.74 (1.06 – 5.41)
	Milk x3/day	2.58 (1.19 – 4.60)	2.56 (1.44 – 5.04)	3.76 (1.78 – 5.02)	1.82 (1.13 – 3.48)	1.32 (1.26 – 1.41)	2.07 (1.07 – 2.53)	2.23 (1.07 – 5.04)
	M + F x1/day	3.57 (1.16 – 5.34)	4.23 (3.32 – 5.14)	3.20 (2.40 – 4.95)	2.60 (1.58 – 4.08)	2.31 (1.78 – 2.77)	1.56 (1.07 – 4.61)	2.91 (1.07 – 5.34)
	M + F x3/day	1.84 (1.24 – 4.38)	2.07 (1.26 – 5.28)	1.89 (1.07 – 4.17)	1.98 (1.12 – 2.87)	2.10 (2.10 – 2.10)	1.56 (1.29– 2.66)	1.86 (1.07 – 5.28)
T+D	Control	1.97 (1.11 – 5.73)	4.15 (3.54 – 5.15)	3.07 (1.17 – 3.95)	1.67 (1.44 – 1.75)	2.83 (1.46 – 4.21)	1.72 (1.59 – 3.79)	2.07 (1.11 – 5.73)
	Milk x1/day	2.03 (1.45 – 3.25)	3.92 (1.09 – 5.19)	2.30 (1.63 – 5.53)	1.96 (1.17 – 2.51)	1.58 (1.08 – 2.07)	1.33 (1.08 – 1.58)	2.07 (1.08 – 5.53)
	Milk x3/day	4.26 (2.23 – 5.64)	3.22 (2.14 – 5.02)	2.85 (1.36 – 3.57)	2.09 (1.47 – 2.19)	3.35 (2.85 – 3.85)	2.10 (1.19 – 3.00)	2.85 (1.19 – 5.64)
	M + F x1/day	2.25 (2.21 – 3.88)	4.14 (1.09 – 5.47)	2.17 (1.70 – 4.36)	1.85 (1.75 – 1.93)	1.20 (1.20 – 1.20)	2.07 (1.08 – 3.05)	2.24 (1.08 – 5.47)
	M + F x3/day	4.22 (2.24 – 4.33)	4.37 (3.31 – 5.30)	1.95 (1.07 – 2.28)	1.95 (1.12– 3.84)	2.34 (2.34 – 2.34)	1.38 (1.08 – 1.41)	2.28 (1.07 – 5.30)

Table 3.3.2. c) Summary statistics for $\log_{10}$(lacto counts + 10) – at *Week 6 - medians (range); excluding 0 counts*

Group	Treatment	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6	Overall
T Only	Control	4.07 (1.07 – 5.04)	3.52 (1.38 – 5.50)	2.76 (1.13 – 5.45)	1.52 (1.33 – 2.41)	1.14 (1.14 – 1.14)	2.28 (1.19 – 3.31)	2.56 (1.07 – 5.50)
	Milk x1/day	4.81 (1.38 – 6.05)	4.19 (1.41 – 5.55)	3.24 (1.56 – 5.11)	2.35 (1.23 – 4.16)	1.98 (1.21 – 4.26)	3.32 (1.07 – 4.89)	3.24 (1.07 – 6.05)
	Milk x3/day	4.35 (2.54 – 5.60)	3.52 (2.44 – 4.99)	3.68 (2.01 – 5.62)	2.36 (1.57 – 4.23)	1.13 (1.07 – 3.10)	2.22 (1.08 – 2.68)	2.90 (1.07 – 5.62)
	M + F x1/day	4.40 (1.21 – 5.39)	4.20 (2.55 – 5.09)	4.46 (1.56 – 5.28)	2.11 (1.06 – 4.77)	2.02 (1.53 – 3.83)	1.66 (1.32 – 4.83)	3.15 (1.06 – 5.39)
	M + F x3/day	4.20 (2.01 – 5.01)	2.98 (1.06 – 5.35)	2.60 (1.88 – 5.29)	1.80 (1.07 – 2.51)	1.66 (1.32 – 2.54)	- -	2.46 (1.06 – 5.35)
T+D	Control	4.42 (2.71 – 5.91)	5.19 (2.80 – 5.64)	4.30 (1.85 – 5.08)	2.83 (1.13 – 4.23)	1.43 (1.29 – 4.18)	4.96 (1.47 – 5.50)	4.23 (1.13 – 5.91)
	Milk x1/day	4.49 (3.13 – 6.28)	3.59 (2.06 – 6.01)	3.82 (2.07 – 4.87)	1.69 (1.10 – 4.24)	1.78 (1.33 – 5.05)	1.87 (1.58 – 2.06)	3.13 (1.01 – 6.28)
	Milk x3/day	3.86 (2.54 – 5.09)	4.58 (3.69 – 6.09)	4.62 (1.40 – 5.76)	1.93 (1.58 – 2.04)	2.70 (1.08 – 3.48)	2.14 (1.08 – 4.17)	3.59 (1.08 – 6.09)
	M + F x1/day	3.80 (3.45 – 5.15)	4.67 (1.48 – 5.24)	4.07 (2.22 – 4.76)	2.35 (1.07– 3.72)	3.07 (1.31 – 3.85)	1.49 (1.08 – 3.68)	3.68 (1.07 – 5.24)
	M + F x3/day	4.69 (3.14 – 5.95)	4.98 (1.12 – 6.07)	4.13 (2.81 – 5.17)	3.66 (1.62 – 4.32)	1.14 (1.08 – 1.21)	2.40 (1.24 – 4.22)	4.13 (1.08 – 6.06)

Table 3.3.2 d) Average of the medians of the $\log_{10}$ (lacto counts + 10) *(range)* across all observations for Week 1 and Week 6, for Treatment Only and Treatment + Dentifrice groups individually

GROUP	WEEK	SITE 1	SITE 2	SITE 3	SITE 4	SITE 5	SITE 6
TREATMENT ONLY	WEEK 1	3.50 *(1.00-6.29)*	2.23 *(1.00-6.31)*	3.44 *(1.00-6.31)*	2.11 *(1.00-5.20)*	1.00 *(1.00-3.82)*	1.77 *(1.00-5.60)*
	WEEK 6	5.24 *(1.00-7.16)*	4.57 *(1.00-6.57)*	4.29 *(1.70-6.70)*	2.79 *(1.00-5.83)*	1.70 *(1.00-5.27)*	2.11 *(1.00-5.82)*
TREATMENT + DENTIFRICE	WEEK 1	3.19 *(1.00-6.80)*	3.98 *(1.00-6.49)*	2.87 *(1.00-6.60)*	1.85 *(1.00-4.73)*	1.00 *(1.00-5.16)*	1.00 *(1.00-4.82)*
	WEEK 6	5.44 *(3.59-7.35)*	5.53 *(1.00-7.13)*	5.06 *(2.23-6.85)*	2.80 *(1.00-5.21)*	1.94 *(1.00-6.04)*	2.32 *(1.00-6.47)*

Table 3.3.2 d) Average of the medians of the $\log_{10}$ (lacto counts + 10) *(range)* across all observations for Week 1 and Week 6, for Treatment Only and Treatment + Dentifrice groups individually.

Colour coded from highest - Red
Pink
Green
Blue
Brown
lowest - Grey

Figure 3.3.2 a) Boxplots illustrating site by week effect on lactobacillus counts

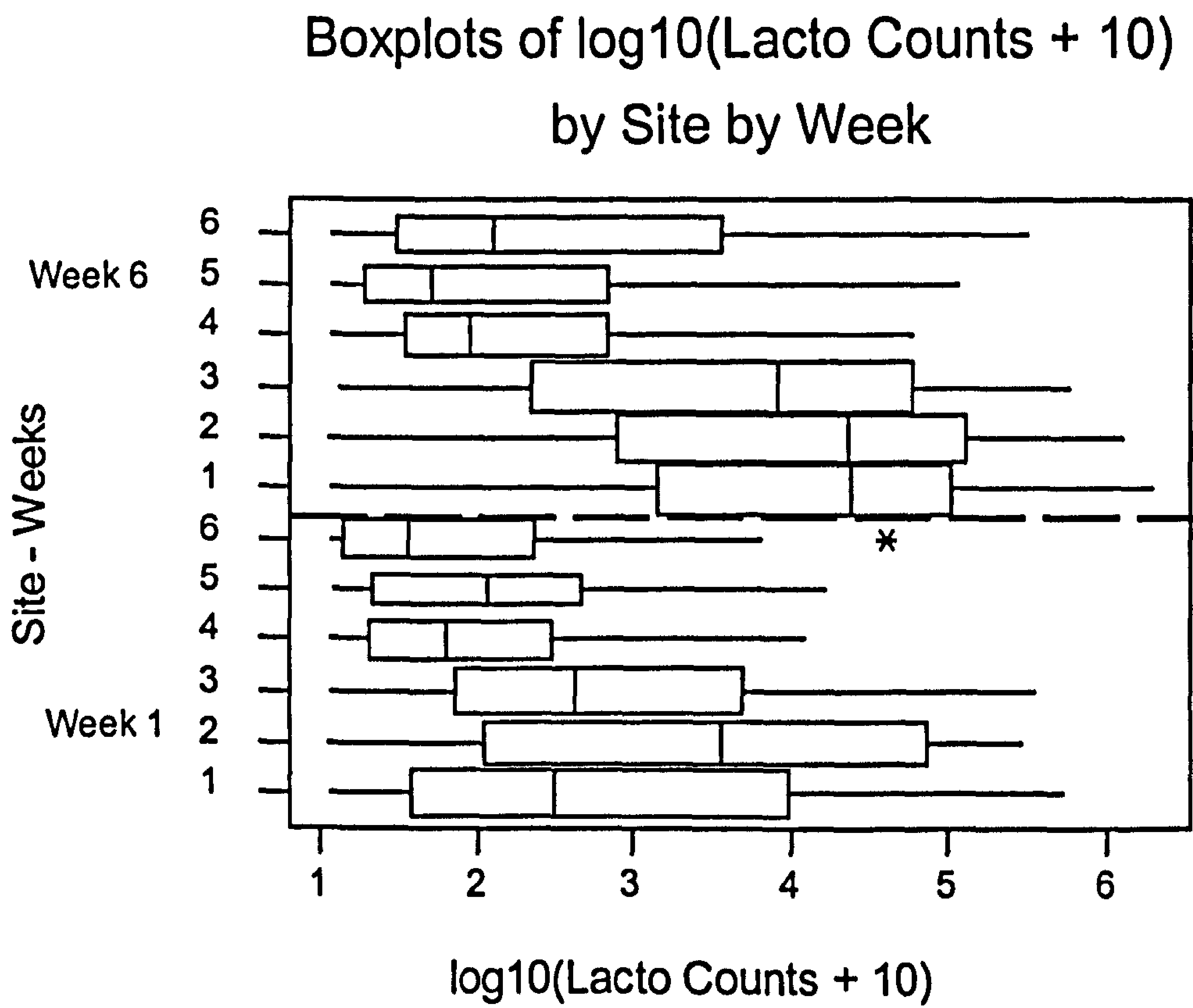


Figure 3.3.2 a) Boxplots of $\log_{10}$(lacto counts + 10) by site by week.

Figure 3.3.2 b) Boxplots illustrating the effect of treatments on Treatment Only Group

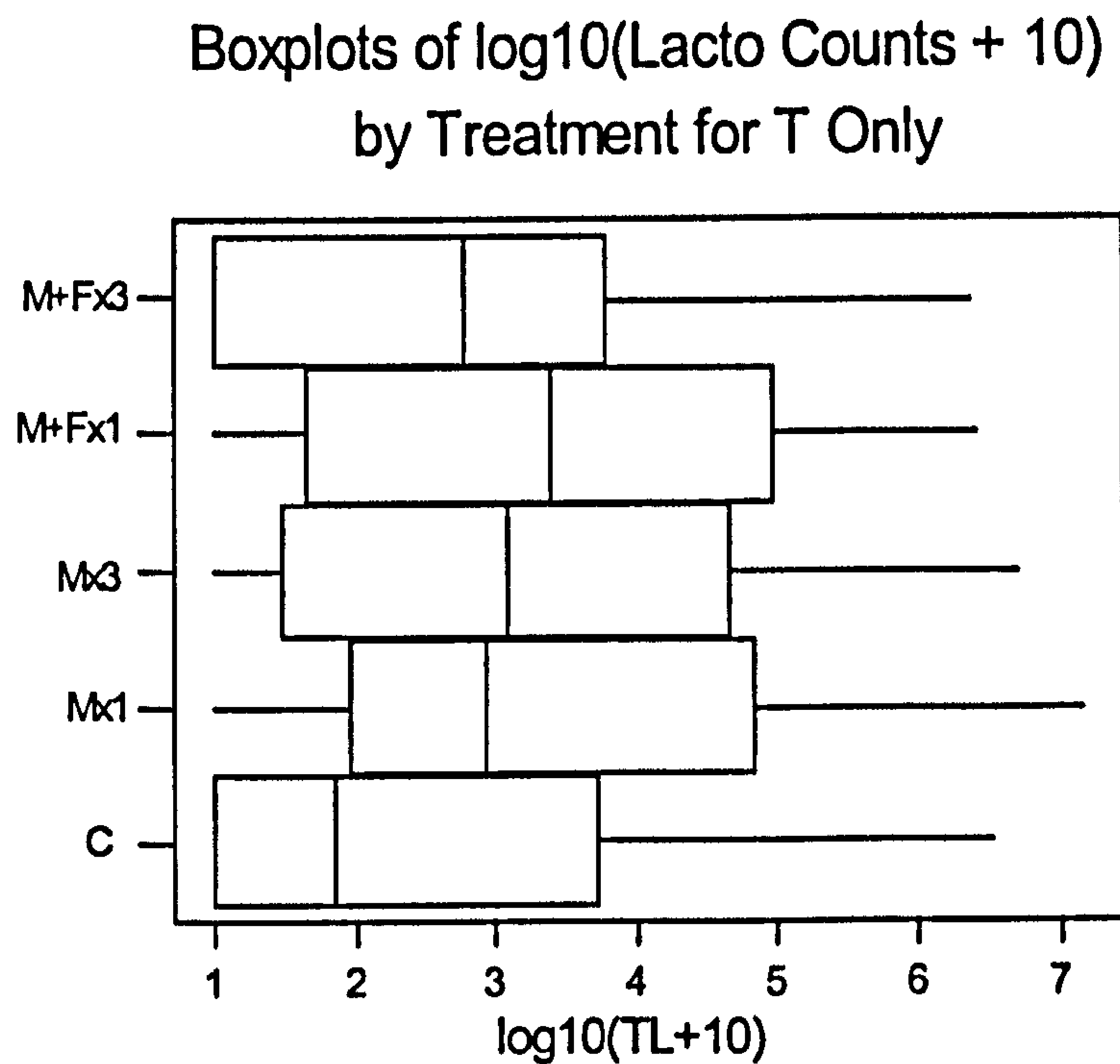


Figure 3.3.2 b) Boxplots of the effect of treatments on the Treatment Only study group. (C), no beverage control; (Mx1), milk consumed once a day; (Mx3), milk consumed three times a day; (M+Fx1), milk with fluoride consumed once a day; (M+Fx3), milk with fluoride consumed three times a day.

Table 3.3.3 Percentage of LC/TC by Site, Group and Treatment for Week 1

		T ONLY				T+D			
Treatment	Site	No. Slabs with Missing Counts	No. Slabs with 0% LC/TC	Median (range) % LC/TC including 0 counts	Median (range) % LC/TC excluding 0 counts	No. Slabs with Missing Counts	No. Slabs with 0% LC/TC	Median (range) % LC/TC including 0 counts	Median (range) % LC/TC excluding 0 counts
Control	1	0	2	4.21% (0.00 – 100.00)%	100.00% (0.73 – 100.00)%	0	0	0.03% (0.00 – 18.02)%	0.03% (0.00 – 18.02)%
	2	0	4	0.00% (0.00 – 21.26)%	16.59% (1.74 – 21.26)%	1	2	0.98% (0.00 – 4.41)%	1.92% (0.35 – 4.41)%
	3	2	2	3.38% (0.00 – 100.00)%	0.14% (0.01 – 0.47)%	1	0	0.14% (0.01 – 0.47)%	0.14% (0.01 – 0.47)%
Milk x1/day	1	0	0	1.11% (0.02 – 100.00)%	1.11% (0.02 – 100.00)%	0	1	0.08% (0.00 – 1.23)%	0.16% (0.00 – 1.23)%
	2	0	1	2.98% (0.00 – 100.00)%	8.50% (0.18 – 100.00)%	0	1	1.41% (0.00 – 54.87)%	2.59% (0.00 – 100.00)%
	3	0	0	0.30% (0.01 – 5.85)%	0.30% (0.01 – 5.85)%	1	2	0.00% (0.00 – 13.57)%	0.32% (0.01 – 13.57)%
Milk x3/day	1	0	0	1.25% (0.00 – 100.00)%	1.25% (0.00 – 100.0)%	0	2	0.54% (0.00 – 41.78)%	21.69% (0.01 – 41.78)%
	2	2	1	0.00% (0.00 – 4.06)%	0.14% (0.00 – 4.06)%	0	2	1.80% (0.00 – 40.37)%	12.18% (0.01 – 40.37)%
	3	0	2	0.09% (0.00 – 7.88)%	0.26% (0.01 – 100.00)%	1	1	0.24% (0.00 – 100.00)%	0.26% (0.00 – 100.00)%
M + F x1/day	1	1	0	3.14% (0.48 – 100.00)%	3.14% (0.48 – 100.00)%	0	2	0.10% (0.00 – 8.15)%	0.15% (0.03 – 8.15)%
	2	4	1	0.07% (0.00 – 35.19)%	17.6% (0.07 – 35.19)%	0	2	0.62% (0.00 – 100.00)%	7.57% (0.34 – 100.00)%
	3	1	0	2.87% (0.26 – 58.12)%	2.87% (0.26 – 58.12)%	0	2	0.07% (0.00 – 100.00)%	0.49% (0.04 – 100.00)%
M + F x3/day	1	0	0	0.25% (0.01 – 2.03)%	0.25% (0.01 – 2.03)%	0	2	0.31% (0.00 – 4.51)%	0.40% (0.08 – 4.51)%
	2	2	0	1.73% (0.03 – 100.00)%	1.73% (0.03 – 100.00)%	1	2	13.30% (0.00 – 100.00)%	34.76% (12.61 – 100.00)%
	3	0	0	0.13% (0.01 – 100.00)%	0.13% (0.01 – 100.00)%	1	1	0.11% (0.00 – 2.00)%	0.12% (0.07 – 2.00)%

Table 3.3.3 Percentage of LC/TC by Site, Group and Treatment for Week 6

		T ONLY				T+D			
Treatment	Site	No. Slabs with Missing Counts	No. Slabs with 0% LC/TC	Median (range) % LC/TC including 0 counts	Median (range) % LC/TC excluding 0 counts	No. Slabs with Missing Counts	No. Slabs with 0% LC/TC	Median (range) % LC/TC including 0 counts	Median (range) % LC/TC excluding 0 counts
Control	1	0	1	0.76% (0.00 – 22.36)%	1.74% (0.09 – 22.36)%	0	0	6.58% (0.02 – 15.51)%	6.58% (0.02 – 15.51)%
	2	1	1	1.93% (0.00 – 16.44)%	3.65% (0.04 – 16.44)%	0	0	11.17% (0.08 – 85.48)%	11.17% (0.08 – 85.48)%
	3	0	0	0.52% (0.00 – 100.00)%	0.52% (0.00 – 100.00)%	1	0	2.18% (0.02 – 7.37)%	2.18% (0.02 – 7.37)%
Milk x1/day	1	0	0	3.43% (0.02 – 100.00)%	3.43% (0.02 – 100.00)%	0	0	2.06% (0.11 – 41.56)%	2.06% (0.11 – 41.56)%
	2	1	0	2.27% (0.02 – 36.61)%	2.27% (0.02 – 36.61)%	1	0	1.91% (0.31 – 29.45)%	1.91% (0.31 – 29.45)%
	3	0	0	0.90% (0.00 – 100.00)%	0.90% (0.00 – 100.00)%	0	0	0.81% (0.00 – 4.43)%	0.81% (0.00 – 4.43)%
Milk x3/day	1	0	1	2.00% (0.00 – 28.53)%	2.09% (0.00 – 28.53)%	0	0	0.59% (0.01 – 30.54)%	0.59% (0.01 – 30.54)%
	2	1	0	4.08% (0.00 – 9.01)%	4.08% (0.00 – 9.01)%	0	1	1.57% (0.00 – 39.95)%	1.74% (0..48 – 39.95)%
	3	0	0	1.50% (0.00 – 8.61)%	1.50% (0.00 – 8.61)%	0	0	1.92% (0.03 – 14.39)%	1.92% (0.03 – 14.39)%
M + F x1/day	1	0	0	4.60% (0.00 – 27.48)%	4.60% (0.00 – 27.48)%	0	0	0.41% (0.26 – 31.91)%	0.41% (0.26 – 31.91)%
	2	1	0	1.03% (0.01 – 13.00)%	1.03% (0.01 – 13.00)%	0	1	0.50% (0.00 – 35.71)%	4.27% (0.10 – 35.71)%
	3	0	0	0.39% (0.01 – 12.82)%	0.39% (0.01 – 12.82)%	0	0	0.80% (0.02 – 60.62)%	0.80% (0.02 – 60.62)%
M + F x3/day	1	0	0	3.00% (0.01 – 3.82)%	3.00% (0.00 – 27.48)%	0	0	4.04% (0.29 – 26.24)%	4.04% (0.29 – 26.24)%
	2	0	1	0.11% (0.00 – 4.25)%	0.45% (0.01 – 4.25)%	0	0	22.27% (0.01 – 100.00)%	22.27% (0.01 – 100.00)%
	3	0	0	0.40% (0.00 – 7.19)%	0.40% (0.00 – 7.19)%	0	0	0.35% (0.25 – 60.62)%	0.35% (0.25 – 60.62)%

Table 3.3.4 a) Numbers of zero counts and missing slabs for *Strep. mutans*

	Site 1		Site 2		Site 3		Site 4		Site 5		Site 6	
	Wk1	Wk6	Wk1	Wk6	Wk1	Wk6	Wk1	Wk6	Wk1	Wk6	Wk1	Wk6
Number of Slabs	70	70	69	68	69	69	63	54	64	56	60	56
Number of Missing Slabs	0	0	1	2	1	1	7	16	6	14	10	14
Number of Slabs with *Strep. Mutans*	8	9	4	4	6	11	4	4	1	2	2	3
Number of Patients with *Strep. Mutans*	3	3	2	2	4	4	1	2	1	1	2	2

Table 3.3.4. b) Summary statistics for *Strep. mutans* at *Week 1 – medians (range); excluding 0 counts*

Group	Treatment	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6	Overall
T Only	Control	-	-	-	-	-	-	-
	Milk x1/day	-	-	-	-	-	-	-
	Milk x3/day	-	-	-	-	-	-	-
	M + F x1/day	-	-	-	-	-	-	-
	M + F x3/day	-	-	-	-	-	-	-
T+D	Control	3170 (2250 - 4090)	-	-	-	-	-	3170 (2250 – 4090)
	Milk x1/day	1590 (1590 – 1590)	865 (865 – 865)	2830 (2830 – 2830)	18.0 (18.0 – 18.0)	61.1 (61.1 – 61.1)	-	865 (18.0 – 2830)
	Milk x3/day	1650 (1650 – 1650)	3170 (3170 – 3170)	1000 (1000 – 1000)	5.5 (5.5 – 5.5)	-	-	1325 (5.5 – 1650)
	M + F x1/day	1709 (68.2 – 3350)	7.5 (7.5 – 7.5)	11.5 (6.6 – 2560)	2.6 (2.6 – 2.6)	-	19.7 (7.5 – 31.9)	11.0 (2.6 – 3350)
	M + F x3/day	121 (1.5 – 241)	201 (201 – 201)	98.6 (98.6 – 98.6)	56.6 (56.6 – 56.6)	-	-	98.6 (1.5 – 241)

Table 3.3.4 b) Summary statistics for *Strep. mutans* at *Week 6 – medians (range); excluding 0 counts*

Group	Treatment	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6	Overall
T Only	Control	-	-	-	-	-	-	-
	Milk x1/day	-	-	-	-	-	-	-
	Milk x3/day	-	-	-	-	-	-	-
	M + F x1/day	-	-	-	-	-	-	-
	M + F x3/day	-	-	-	-	-	-	-
T+D	Control	928 (436 – 1420)	-	12400 (105 – 13900)	13800 (13800 – 13800)	-	419 (419 – 419)	1420 (105 – 13900)
	Milk x1/day	3330 (3330 - 3330)	1510 (1510 - 1510)	2880 (39.0 - 5720)	- -	- -	-	2420 (39.0 – 5720)
	Milk x3/day	2260 (26.0 - 15800)	81.0 (81.0 – 81.0)	1805 (1540 - 2070)	2230 (2230 - 2230)	19.0 (19.0 – 19.0)	14.0 (14.0 – 14.0)	1540 (14.0 – 15800)
	M + F x1/day	7880 (4260 - 11500)	1021 (51.0 - 1990)	50400 (22200 - 59900)	516 (3.0 - 1030)	2.0 (2.0 – 2.0)	333 (333 - 333)	1990 (2.0 – 59900)
	M + F x3/day	827 (827 - 827)	-	1990 (1990 - 1990)	-	-	-	1408 (827 – 1990)

CHAPTER 4: AN INVESTIGATION, USING RIBOTYPING TECHNIQUES, INTO PATIENT STRAIN VARIATION OF *S. MUTANS.*

4.1 Introduction

The mutans streptococci are a phenotypically related group of bacteria which have been implicated as the principal aetiological agents of human dental caries (Igarashi *et al.*, 1996a). This group of organisms has been divided into seven species and eight different serological types (a through h) (Whiley and Beighton, 1998). They consist of *S. cricetus* (serotype a), *S. rattus* (serotype b), *S. mutans* (serotypes c, e and f), *S. sobrinus* (serotypes d and g), *S. downei* (serotype h), *S. ferus* and *S. macacae*. Among the mutans streptococci, *S. mutans* and *S. sobrinus* are the species which have been implicated as the prime causative organisms of human dental caries (Oho *et al.*, 2000). In particular, *S. mutans* has been the most frequently isolated species from human dental plaque (Hamada and Slade, 1980; Loesche, 1986).

A variety of methods, which are reliant on phenotypic characteristics, are available to distinguish *S. mutans* from other mutans streptococci. Like all streptococci, the mutans group are fermentative organisms as they obtain their energy entirely by incomplete oxidation of carbohydrates. As a result they produce lactic acid and small amounts of formate, acetate and ethanol (Carlsson and Hamilton, 1994). *S. mutans* has the ability to ferment the widest range of carbohydrates, in particular mannitol and sorbitol, which other mutans streptococci are unable to ferment. Differences between isolates of mutans

streptococci have been revealed by studies utilising bacteriocin typing (Hamada and Ooshima, 1975; Hamada and Slade, 1980). Alaluusua *et al.* (1991) observed that all isolates from the same serotype from one individual had a very similar bacteriocin pattern, but that there was wide variation in bacteriocin production between individuals.

Genotypic typing methods such as restriction endonuclease analysis (REA) and restriction fragment length polymorphism (RFLP) have been applied to isolates of mutans streptococci (Saarela *et al.*, 1993a). REA is a technique that involves the digestion of bacterial chromosomal DNA with a variety of enzymes called restriction endonucleases (Owen, 1989; Saarela *et al.*, 1993b). Separation of the digested DNA by agarose gel electrophoresis reveals restriction fragment patterns that can distinguish between isolates. Previous application of this technique has revealed considerable genetic heterogeneity among mutans streptococci (Kulkarni *et al.*, 1989; Kozai *et al.*, 1991; Saarela *et al.*, 1993a; Shiroza *et al.*, 1998). This research suggested that epidemiologically unrelated individuals usually have different clones of a single mutans streptococcal species. The application of the technique to *S. mutans* strain delineation has revealed unique RFLP patterns (Caufield and Walker, 1989; Kulkarni *et al.*, 1989; Shiroza *et al.*, 1998). The term 'DNA polymorphism' refers to the change in size of a restriction fragment that can be brought about by mutations in the genome sequence. However, polymorphisms often represent neutral mutations and do not cause any change in the phenotype.

REA and RFLP are limited in their ability to detect strain variation as they can yield highly complex digest patterns, with bands that are closely spaced together, which can

make reading and interpreting the genomic profiles difficult. DNA probes have been used in Southern-blot hybridisation analyses to reduce the large number of bands generated by REA and RFLP. The probes that are used for epidemiological typing include random or specific chromosomal sequences, toxins and antibiotic resistance genes and insertion sequences (Pitt, 1993; Cangelosi *et al.*, 1994). Ribosomal RNA (rRNA) sequences of *Escherichia coli* are the most universally utilised probes. Bacterial ribosomal operons are typically organised in the order 16S, 23S and 5S rRNA genes with intergenic spacers of variable length between the 16S and 23S RNA genes and downstream of the 5S rRNA gene (Novák *et al.*, 1997). rRNA operons occur in multiple copies within bacterial genomes and are essential for the survival of all organisms, and as a result of this evolutionary role they have a well conserved structure (Gürtler and Stanisich, 1996). These properties make them ideal to use as species-specific DNA probes to detect a wide range of bacteria. The variation in the number and size of fragments in bacterial DNA digests which are complementary to the rRNA sequences form the basis of ribotyping (Pitt, 1993). Previous studies have reported that the most suitable restriction endonuclease for optimum discrimination between isolates of *S. mutans* is *Hin*d III (Saarela *et al.*, 1993a; Novák *et al.* 1997).

4.2 Materials and Methods

4.2.1 Bacterial Strains

From a total of thirty subjects who were participating in a fluoridated milk study, only six were identified as being carriers of *S. mutans*. During the course of the study, a total of 105 isolates of *S. mutans* were obtained from plaque sampled from these subjects. The samples were collected and stored at -80°C over a period of two years. Isolates were originally cultured on mitis salivarius-bacitracin agar medium (MSB). They were selected from the primary plates on the basis of colony morphology (Gold *et al.*, 1973), and confirmed as *S. mutans* by sugar fermentation profiles and a positive Gram stain reaction as described previously. Isolates were initiated for chromosomal DNA isolation from frozen stocks.

E. coli DH5α carrying plasmid pKK3535 (kindly provided by S. Alaluusua, Department of Paedodontics and Orthodontics, University of Helsinki, Finland) was grown on solid LB broth supplemented with 100µg/ml ampicillin.

4.2.2 DNA Isolation

The commercially available Puregene DNA isolation kit (Novara Flowgen) was used to extract chromosomal DNA from 102 isolates of *S. mutans* as described previously. Two modifications were made to the standard protocol. 1x TN buffer was used in place of

the cell suspension solution supplied with the kit and the lytic enzyme supplied was replaced with achromopeptidase at 20U/μl.

Approximate DNA yield was ascertained by 0.8% (w/v) agarose gel electrophoresis, using a 100 base pair ladder (Amersham Pharmacia Biotech) as a molecular size marker. Photographs of gels were taken under ultra-violet illumination using an Image Master ® VDS gel documentation system (Amersham Pharmacia Biotech).

4.2.3 Plasmid Purification and Isolation

Plasmid DNA from *E. coli* strain DH5α carrying plasmid pKK3535 was grown in 200 ml LB broth supplemented with 100 μg/ml ampicillin at 37°C overnight with shaking. Plasmid pKK3535 was purified using Wizard™ Plus Minipreps DNA Purification System (Promega) and isolated using the Qiagen Plasmid Isolation Kit. Cells were pelleted by centrifugation at 2,000 x g for 10 minutes and supernatant removed. The pellet was resuspended in 400 μl cell resuspension solution and then transferred to a 1.5 ml microcentrifuge tube. Four hundred microlitres of cell lysis solution was added and mixed by inverting, with 3 to 5 minute incubation for efficient lysis. Four hundred microlitres of neutralisation solution was added and mixed by inverting then centrifuged at 10,000 x g for 5 minutes. For each miniprep, one Wizard™ Minicolumn was prepared by pipetting 1 ml of resuspended resin into a 2 ml syringe barrel attached to a minicolumn. The cleared lysate from the miniprep was transferred into the syringe barrel and slurry gently pushed into minicolumn with syringe plunger, collecting the discard in a 20 ml universal bottle. The plunger was removed and 2 ml column wash

solution with ethanol added was pipetted into the barrel. The plunger was reinserted and wash solution pushed through the minicolumn. The syringe was then discarded and minicolumn transferred to a 1.5 ml microcentrifuge tube and centrifuged for 2 minutes. The minicolumn was transferred to a fresh tube and 50 μl sterile molecular-biology-grade water preheated to 70°C was applied to the minicolumn and left for 1 minute before centrifugation for 20 seconds to elute DNA.

4.2.4 Restriction Endonuclease Digestion of DNA

Chromosomal *S. mutans* DNA (2 to 3 μg) was digested to completion with the restriction endonuclease *Hin*dIII (Promega) to a total volume of 40 μl. Samples were incubated in a 37°C water bath overnight.

4.2.5 Agarose Gel Electrophoresis

The DNA restriction fragments were separated by electrophoresis through 1% agarose gels at 110V for 3 hours. Lambda DNA digested with *Hin*dIII (Roche Diagnostics Ltd) was used as a molecular size marker. The top left-hand corner of each gel was cut off and the gels photographed using the Image Master ® VDS system.

4.2.6 Southern Blotting

Table 4.2.1 contains details of the stock solutions and buffers required for Southern blotting and subsequent ribotyping stages. Gels were sequentially soaked twice for 15

minutes each in 250 ml 0.25 M HCl, 250 ml denaturation solution and 250 ml neutralisation solution. Each step was carried out in a glass dish at room temperature with constant rotational shaking. One piece of positively-charged nylon membrane (Roche) and six pieces of Whatman 3MM chromatography paper were cut to be slightly wider and longer than the gel. The top left corner was cut off the membrane. All were soaked in 1x SSC buffer until saturated. DNA transfer was achieved by the capillary action of the Southern blotting technique, using a fixed wick blotting unit (Anachem, Luton, U.K.). The gel was placed DNA side facing upwards on the wick, removing any bubbles by rolling a 10 ml pipette gently over the surface. On this was placed the membrane, the Whatman paper and a 4" high stack of paper towels. The unit was filled to the level of the wick with transfer buffer (20x SSC). The top of the unit was fitted and evenly weighted with a light weight to ensure complete and even transfer. The membrane was washed briefly in 2x SSC and left to air dry. The transferred DNA was fixed by ultra violet irradiation in a UVC-508 ultraviolet crosslinker (Anachem) at 254 nm for 1 min.

4.2.7 Probe Labelling, Hybridisation and Detection

Fragments were hybridised to the recombinant plasmid pKK3535, which consists of the cloning vector pBR322 and the *rrnB* operon of the *E. coli* chromosome (Brosius *et al.*, 1981). Since plasmid pBR322 alone does not hybridise to the chromosomal DNA of mutans streptococci, the whole pKK3535 plasmid can be used as a probe (Saarela *et al.*, 1993a & b). Random primed labelling of pKK3535, hybridisation of the membranes and subsequent immunodetection were performed with the nonradioactive digoxigenin DNA

labelling and detection kit according to the manufacturer's instructions (Boehringer Mannheim GmbH). pKK3535 (10 ng/μl) was first linearised by digestion with *Hin*dIII (10 U/μl) then denatured for 10 minutes in a boiling water bath. To the denatured DNA was added 2 μl hexanucleotide mixture, 2 μl dNTP labelling mixture, 1 μl Klenow enzyme and sterile molecular-biology-grade water to a total volume of 20μl. This was vortexed briefly, centrifuged then incubated at 37°C in a water bath overnight. The reaction was terminated by adding EDTA to a final concentration of 0.2 M.

Membranes were prehybridised in 20 ml prehybridisation solution for four hours at 68°C in a hybridisation oven (Hybaid) with constant rotation. Prehybridisation solution was removed and replaced with 10 ml hybridisation solution containing freshly heat-denatured DIG-dUTP-labelled probe. Membranes were incubated in a hybridisation oven at 68°C overnight with constant rotational shaking.

Unbound probe was removed by carrying out a number of stringency washes as follows: twice at room temperature with 250 ml 2x SSC/0.1% SDS (w/v) for 5 minutes each and twice at 68°C with 250 ml 0.1x SSC/0.1% SDS (w/v) for 15 minutes each. These were carried out with constant rotational shaking. The immunological detection was carried out according to the manufacturer's instructions. Membranes were blocked for one hour in blocking solution then incubated with 20 ml blocking solution and 4 μl Anti-DIG-AP (150 mU/ml) for 30 minutes. The antibody solution was then discarded and membranes washed twice for 15 minutes in 100 ml maleic acid buffer to remove unbound antibody. Membranes were equilibrated in 20 ml colour detection buffer then approximately 10 ml colour substrate was added. The substrate for alkaline phosphatase in the colour

reaction was nitroblue tetrazolium chloride in combination with 5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) included in the DNA labelling and detection kit. The membranes were incubated in sealed containers in the dark to allow colour development. Once the desired bands were detected, the membranes were washed with water to prevent over-development.

TABLE 4.2.1 Southern Blotting and Ribotyping Stock Solutions and Buffers

Solution / Component	Amount
0.25 M HCl	
concentrated HCl	10 ml
dH_2O	470 ml
Denaturation Solution	
NaCl	87.66 g
NaOH	20 g
dH_2O	to 1 L
Neutralisation Solution	
NaCl	87.66 g
Tris	60.55 g
dH_2O	to 1 L
20 X SSC	
NaCl	175.3 g
Sodium citrate	88.2 g
dH_2O	800 ml
Adjust to pH 7.0 with NaOH. Make up volume to 1 litre with dH_2O and autoclave.	
Maleic Acid Buffer	
Maleic Acid	11.6 g
NaCl	8.76 g
dH_2O	800 ml
Adjust to pH 7.5 with NaOH. Make up volume to 1 litre with dH_2O and autoclave.	
Blocking Solution (10%)	
Maleic Acid Buffer	40 ml
Blocking Reagent	5 g
Dissolve blocking reagent with constant stirring. Make up volume to 50 ml with Maleic Acid Buffer.	
Hybridisation Buffer	
5 X SSC	100 ml
0.1 % (w/v) N-laurylsarcosine	0.1 g
20 % (w/v) SDS	1 ml

Prehybridisation Solution

Hybridisation Buffer	18 ml
10% Blocking Solution	2 ml

Hybridisation Solution

Hybridisation Buffer	10 ml
10% Blocking Solution	9 ml
Denatured probe (5 ng/µl DNA)	20 µl

2 X SSC/0.1% SDS

20 X SSC	100 ml
20% SDS	5 ml
dH_2O	895 ml

0.2 X SSC/0.1% SDS

20 X SSC	10 ml
20% SDS	5 ml
dH_2O	980 ml

Blocking Solution (1%)

Maleic Acid Buffer	90 ml
10% Blocking Solution	10 ml

Blocking Buffer

Maleic Acid Buffer	18 ml
10% Blocking Solution	2 ml
Anti-DIG-AP (150 mU/ml)	4 µl

Colour Detection Buffer

1 M Tris-HCl, pH 8.0	20 ml
NaCl	1.167 g
dH_2O	160 ml

Adjust to pH 9.5 with NaOH. Make up volume to 200 ml with dH_2O.

Colour Substrate Solution

Colour Detection Buffer	10ml
NBT/BCIP	200µl

4.3 Results

Nine of 102 isolates that were analysed did not fully digest with *Hin*d III, therefore no results were obtained from these. Thus, a total of 93 strains of *S. mutans* were analysed. Chromosomal DNA from the clinical *S. mutans* isolates was digested with *Hin*d III and this produced between four and thirteen bands. The band sizes ranged from about 3 kbp to 21kbp and clustered into three distinct size groups upon which the differentiation of genotypes was based.

4.3.1 Designation of Genotypes

In each fingerprint profile generated by ribotyping, the DNA bands appeared to be clustering into three groups, between 21 kbp and 3 kbp, which enabled differences to be observed within each group. The differences within each of these groups then allowed the differentiation between genotypes to be made. Figure 4.3.1 illustrates the banding groups generated by ribotyping and the profiles of two genotypes to demonstrate profile differences. Analysis of the profiling patterns from each of the clinical isolates revealed thirteen different genotypes among the six unrelated subjects. A scheme for genotype designation was devised with the subject prefix in upper case and each individual genotype in lower case. When a particular genotype pattern appeared in an unrelated subject, then the genotype was designated the same as the original isolate, i.e. genotype *Cb* was isolated from subject E, so was therefore recorded as *Cb* rather than *Eb*. Table 4.3.1 shows the bands present in these groups for the genotypes isolated from six

Figure 4.3.1 Genotype banding groups

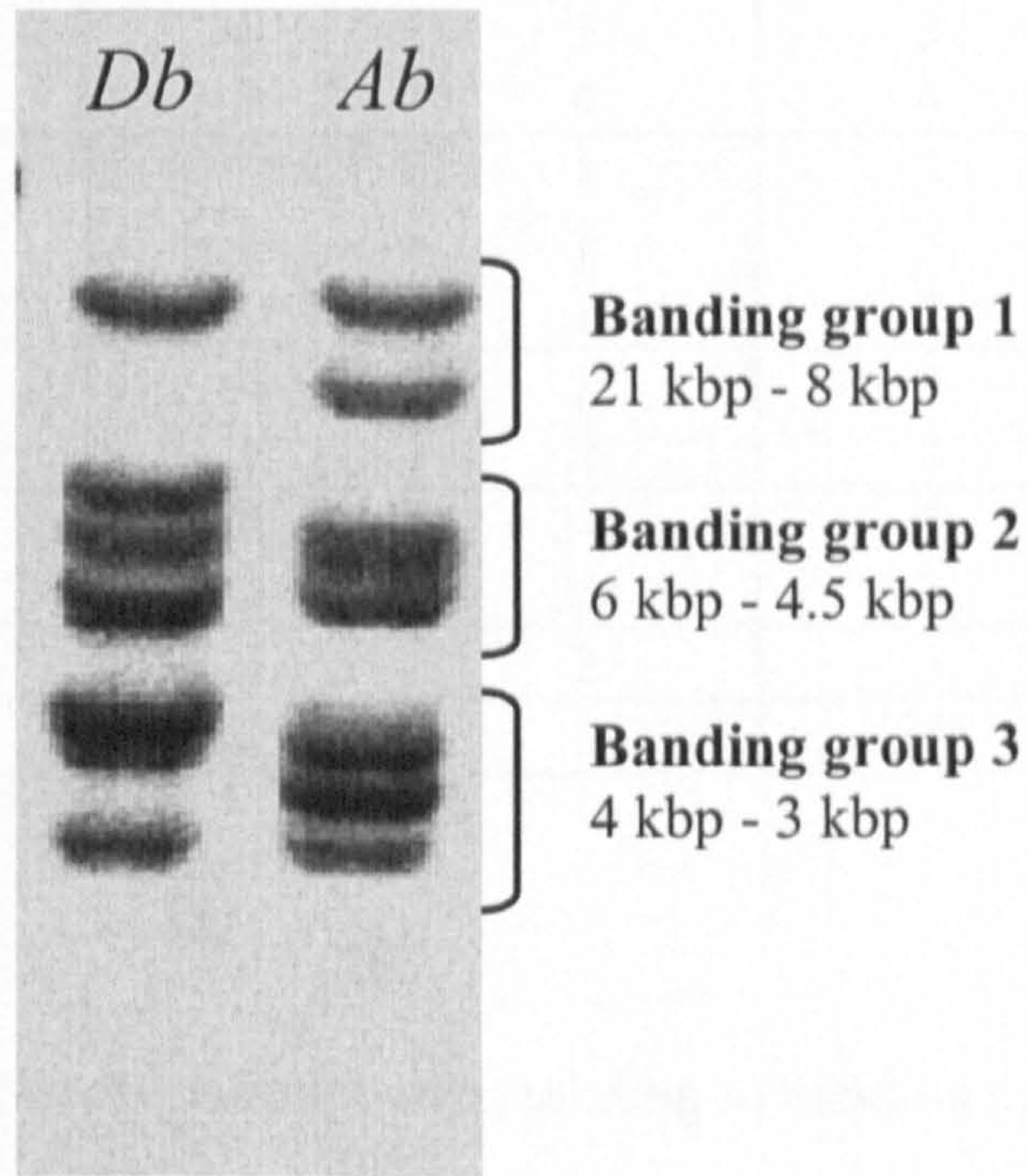


Figure 4.3.1 Identification of banding groups generated by ribotyping of clinical *S. mutans* isolates. Genotype *Db* was isolated from a female subject and genotype *Ab* was isolated from a male subject.

Table 4.3.1. Genotype Profiles

Genotype Designation	No. Bands Group 1	No. Bands Group 2	No. Bands Group 3
Aa	5	3	4
Ab	2	3	3
Ac	5	3	3
Ad	3	4	4
Ba	9	1	3
Bb	3	1	3
Bc	0	1	3
Ca	4	2	3
Cb	1	2	3
Da	6	3/4	3
Db	1	3/4	3
Ea	5	2	3
Fa	4/5	3	3/4

Table 4.3.1. Genotype designation relating to banding profiles

subjects during a two-year fluoridated milk study. Table 4.3.2 shows the distribution and frequency of isolation of each of these *S. mutans* genotypes.

4.3.2 Identification of Genotypes from One Subject (A)

Fifteen individual colonies with morphology and sugar fermentation profiles expected for *S. mutans* were obtained from the plaque of one subject and isolated on one MSB plate. Hybridisation profiles showed that all fifteen isolates were identical except for one, which appears to have an extra band at about 2.8 kbp (Figure 4.3.2, Lane 8). These isolates were analysed alongside *S. mutans* NCTC 10449 type strain, serotype c. Figure 4.3.2 shows the genotypic stability across *S. mutans* isolates from Subject A and band profile for type strain NCTC 10449. This subject also had strains isolated on seven other separate occasions (Table 4.3.1). Throughout the duration of the fluoridated milk study, a total of 25 isolates were collected and hybridisation profiles recognised up to three different genotypes.

4.3.3 Analysis of Genotypes from Six Subjects (A-F)

The six subjects harboured strains representing one, two or three genotypes. Diversity was apparent across the study group as a whole (Figure 4.3.3) but individually there was evidence of greater strain stability. An example of this stability can be seen in Figure 4.3.4, which shows that subject D harbours two distinct *S. mutans* genotypes.

Table 4.3.2 *S. mutans* Genotype Distribution

Subject (M or F)	Exp. No.	Experiment Interval	Sample Collection Date	Genotype Designation	Number Isolated (Site Isolated)
A (M)	R274	Wk 1 Exp 2	19/02/01	*Ad*	5 (1,3,4)
				Aa	1 (2)
	R319	Wk 6 Exp 2	02/04/01	*Ac*	1 (1)
	R343	Wk 1 Exp 3	02/05/01	*Aa*	2 (1,3)
				Ab	1 (4)
	R377	Wk 6 Exp 3	13/06/01	*Aa*	1 (1)
	R439	Wk 1 Exp 4	20/08/01	*Ab*	5 (1,2,3,4)
				Aa	1 (6)
	R471	Wk 6 Exp 4	01/10/01	*Ab*	4 (1,6)
				Aa	2 (3,4)
				Ac	1 (3)
	R486	Wk 1 Exp 5	29/10/01	*Ab*	1 (4)
	R504	Wk 6 Exp 5	10/12/01	*Ab*	15 (3)
B (M)	R207	Wk 6 Exp 1	22/11/01	*Ba*	3 (1,2,4)
	R241	Wk 1 Exp 2	12/01/01	*Ba*	2 (4,6)
				Bb	1 (2)
	R280	Wk 6 Exp 2	23/02/01	*Bb*	4 (1,2,4,6)
				Ba	2 (3)
				Bc	2 (2,5)
C (F)	R282	Baseline	26/02/01	*Ca*	1 (4)
				Cb	1 (1)
	R431	Wk 1 Exp 2	30/07/01	*Cb*	3 (1,2,3)
D (F)	R320	Wk 6 Exp 1	02/04/01	*Da*	5 (1,4,5,6)
				Db	2 (4,6)
	R361	Wk 1 Exp 2	23/05/01	*Db*	1 (1)
	R405	Wk 6 Exp 2	04/07/01	*Da*	1 (1)
				Db	4 (3,4,6)
	R478	Wk 6 Exp 3	15/10/01	*Da*	3 (2,3,5)
				Db	3 (1,3,4)
E (F)	R331	Wk 6 Exp 2	23/04/01	*Ea*	1 (1)
	R422	Wk 1 Exp 3	20/07/01	*Ea*	3 (1,2,3)
				Cb	1 (5)
	R447	Wk 6 Exp 3	31/08/01	*Cb*	3 (1,2,3)
	R511	Wk 6 Exp 4	21/12/01	*Ea*	3 (1,2,3)
				Cb	2 (1,3)
F (M)	R382	Wk 6 Exp 3	15/06/01	*Fa*	1 (3)

Table 4.3.2 Distribution of *S. mutans* genotypes among 6 unrelated subjects.

Figure 4.3.2 Genotypic stability of *S. mutans* isolates identified from Subject (A)

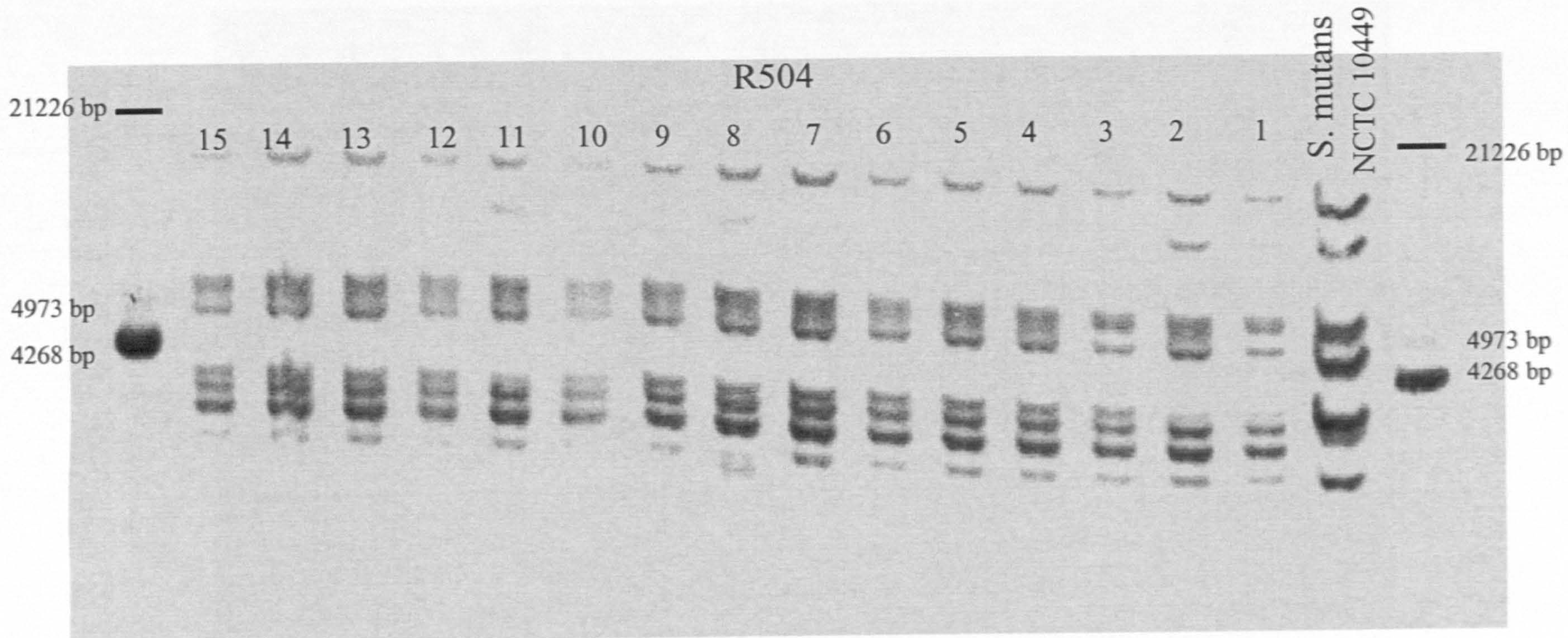


Figure 4.3.2 Genotypic stability demonstrated in the ribotyping profiles generated by the analysis of 15 *S. mutans* isolates identified from one subject (A) at Week 6 Experiment 5. The isolate in Lane 8 appears to have one extra band of 2.8kbp. Reference strain *S. mutans* NCTC 10449 was also analysed with this set of isolates.

Figure 4.3.3 A typical blot demonstrating genotypic diversity of *S. mutans* within unrelated individuals

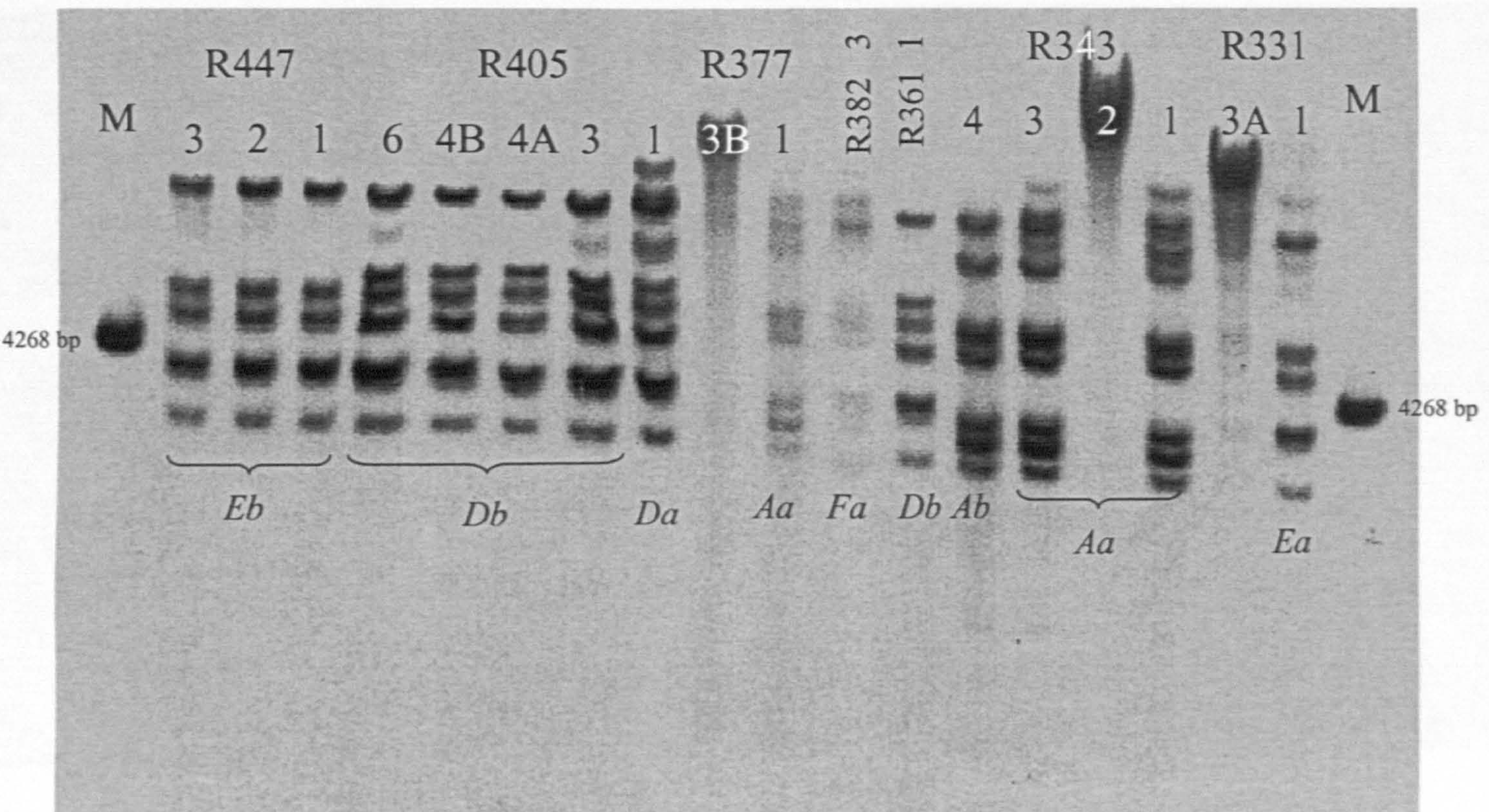


Figure 4.3.3 Typical blot demonstrating the diversity of *S. mutans* genotypes isolated from four unrelated individuals. Three of these isolates (R377 3B, R343 2, R331 3A) did not digest with *Hin*dIII, therefore they were re-analysed at a later stage.

Figure 4.3.4 Ribotyping profiles demonstrating stability of genotypes within an individual over four experiment intervals

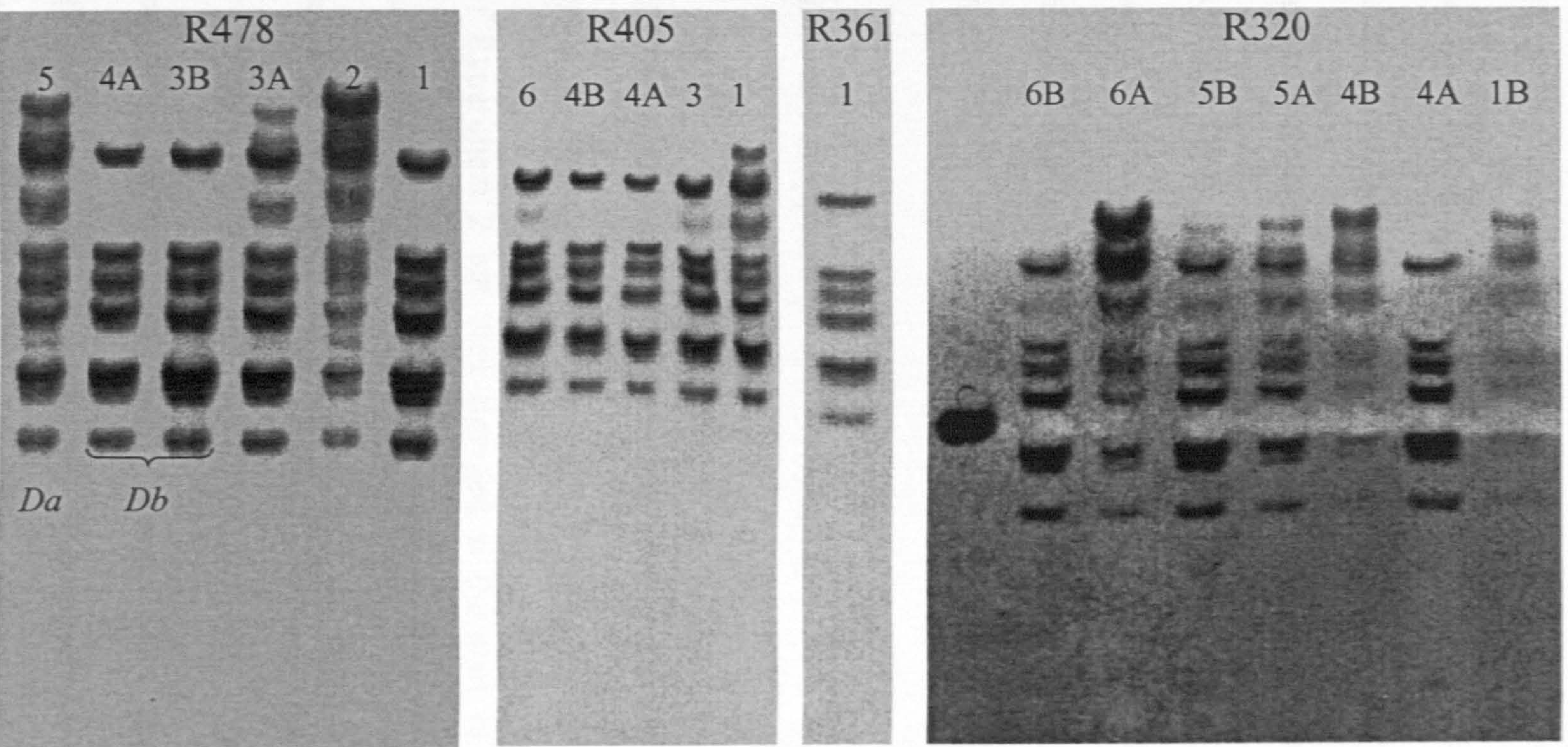


Figure 4.3.4 Ribotyping profiles which demonstrate genotype stability within an individual (D). This subject harbours two *S. mutans* genotypes, *Da* and *Db* marked only on the first three lanes, isolated on four separate occasions.

In Group 3 of the genotype band profiles (Figure 4.3.1), it was apparent that a different pattern appeared in the male subjects compared to the female subjects. In the male subjects, three or four bands tended to emerge close together between 3 kbp and 4 kbp with the middle band (s) at 3.5 kbp being of a slightly stronger intensity, which suggests that there is more than one band of the same size. In the female subjects, the three bands were spaced differently. Two appeared to be closer together (about 4 kbp and 3.9 kbp) with a single band slightly further apart (about 3.2 kbp). Figure 4.3.5 shows the comparison of these two groups between Subject A and Subject D. The strains isolated from female subjects appeared to be closer in profile to the type strain NCTC 10449 (Figure 4.3.2).

4.3.4 Loss and Reappearance of Genotypes

It would appear that genotypes were lost at intervals and then reappeared later at the same tooth site (Table 4.3.2). In Subject A, genotype *Aa* was not isolated prior to the start of Experiment 2 but then emerged at the start of the second experiment. It remained present until the fifth experiment when it was then absent and did not reappear over the remaining period. The same observation was made with genotype *Ab*, which was not present during the second experiment but emerged at site 4 at the start of the third experiment and remained present in the individual throughout the rest of the study, although not always at the same site. This observation was repeated within each individual sampled, for each genotype within an individual.

Figure 4.3.5 Comparisons between male and female genotype profiles

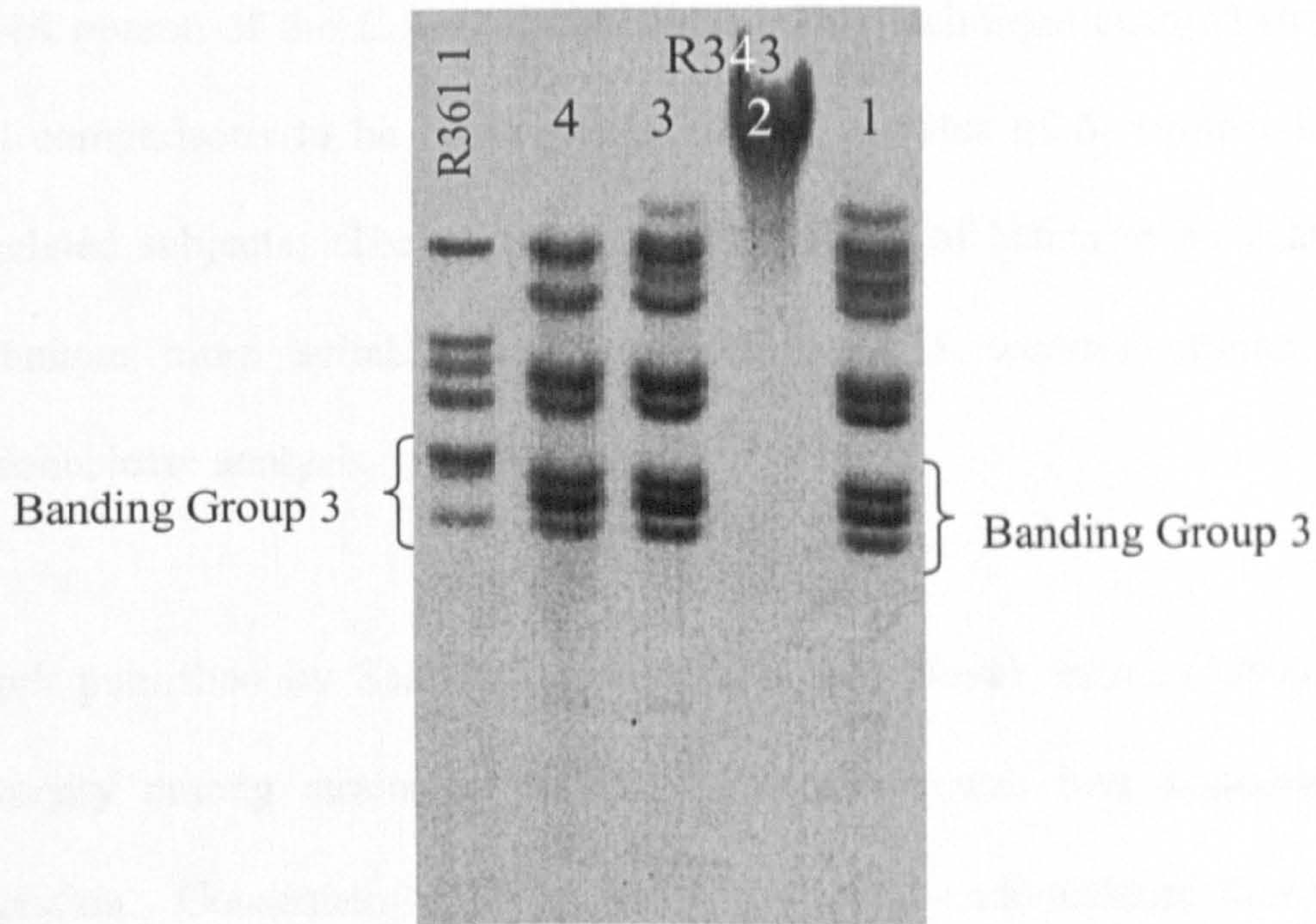


Figure 4.3.5 The comparison between male and female subjects with different banding group 3 profiles. Isolate R361 1 is from female subject (D) and the isolates from R343 are typical of a male band pattern.

4.4 Discussion

In the present study, the restriction fragments of *S. mutans* chromosomal DNA were hybridised to the digoxigenin-labelled plasmid pKK3535, which contains the *rrnB* rRNA operon of the *E. coli* chromosome. This technique enabled strain identification and comparisons to be made within clinical isolates of *S. mutans* harboured by six unrelated subjects. The reduction in the number of bands to be analysed makes this technique more suitable for identification of *S. mutans* strains than restriction endonuclease analysis.

Work published by Saarela *et al.* (1993a) and Novák *et al.* (1997) determined that diversity among strains of mutans streptococci was best visualised after *Hin*dIII digestion. Consequently, this was the restriction endonuclease that was used for the digestion of bacterial DNA prior to hybridisation analysis.

The production of restriction fragment length polymorphisms reflects the amount of genetic diversity within strains of *S. mutans*. A DNA polymorphism is a restricition fragment of changed size, usually as a result of base changes in the genome. These mutations tend to be neutral and cause no change in the phenotype. Sugar fermentation tests are able to confirm this by the identification of these isolates as *S. mutans*, utilising their ability to ferment the sugars mannitol and sorbitol. Serotyping is unsatisfactory for epidemiological purposes, particularly since serotype *c* is by far the most common in both adults and children in the Western World (Berkowitz and Jordan, 1975; Keene *et al.*, 1977). Bacteriocin typing has also been used to show similarities between

phenotypes. Although bacteriocin typing of *S. mutans* isolates is reproducible, it has been shown to be difficult to distinguish between *d/g* strains due to their poor bacteriocin production (Davey and Rodgers, 1984).

Strains that were isolated from individuals tended to have the same banding profiles throughout the course of this two-year study. Although *S. mutans* was not present at every interval, the stability of the strain profiles remained consistent and is therefore in agreement with the work carried out by Redmo-Emanuelsson and Thornqvist (2000). However, in contrast to further findings by Redmo-Emanuelsson *et al.* (2003), any sharing of sites between genotypes was observed infrequently, only occurring once or at most twice within an individual. The results from this study show that five of the six subjects analysed predominantly carried two or three different genotypes representing serotype *c*. The finding that the majority of isolates with different genotypes had several bands of the same size supports the possibility of a mutation occurring in the chromosome of the original strain (Saarela *et al.*, 1993a). The analysis of 15 replicates isolated from one of the primary plates was an attempt to ensure that the initial morphological identification of *S. mutans* was as accurate as possible. Isolation of only one genotype from this experiment shows that the identification of *S. mutans* isolates was consistent and accurate. In an attempt to prevent missing any *S. mutans* genotypes, caution was taken when sub-culturing from the primary plates and any slight morphological differences in colonial type was accounted for and the isolates processed individually. These precautions should mean that no genotype carried by a particular subject would be missed. However, it is possible that genotypes have been missed, especially from the time of sampling. More genotypes may have been identified had the

initial plaque sample been incubated overnight to allow preliminary growth of *S. mutans* before plating onto selective media. Another possibility is that the genotypes were simply missed at the time of sampling. Since only a 50 μl aliquot was taken from 1 ml of plaque suspension to be inoculated onto each MSB plate, it could be possible that the sample was not a true representation of the overall plaque bacteria. Vortex mixing was carried out on each plaque sample to distribute the plaque and avoid this problem.

The greatest degree of polymorphism was observed in the DNA fragments of Banding Group 1, varying in size from 21 kbp to 8 kbp. Li *et al.* (1992) reported similar results and suggested that genetic rearrangements or the integration or deletion of an insertion sequence could lead to size variation. They concluded that genetic rearrangements could account for the wide diversity seen in patterns from non-related as well as related genotypes in mutans streptococci. The majority of research carried out on the heterogeneity of mutans streptococcal strains has focused on carriage within related subjects (Davey and Rodgers, 1984; Li and Caufield, 1995; Kozai *et al.*, 1999). The results from this study suggest that there may be some sharing of genotypes amongst unrelated individuals.

This study has permitted the investigation of *S. mutans* genotypes across a period of two years and allowed observations to be made on individual carriage of the organism. From the data collected, there is the suggestion that genotypes of *S. mutans* can be lost at certain periods of time and then reappear on the same tooth surface. The slight variation in ribotyping profiles of isolates that essentially look identical could possibly have been more certainly identified had the isolates been processed in duplicate or an additional

typing method such as randomly amplified polymorphic DNA (RAPD) analysis could have been applied. This would have allowed confirmation of the profiles and therefore the isolate could have been determined as either being the same genotype or a separate genotype that may have arisen from a mutation within the chromosome of the original strain.

Novák *et al.* (1997) describe the use of an internal fragment of 23S rRNA from the *rrnB* operon of *S. mutans* to analyse various restriction endonuclease digests of chromosomal DNA isolates of mutans streptococci. The novel aspect of this study was the utilisation of a cloned probe originating from *S. mutans* and containing only a defined portion of the *rrn* genes. As genotypic heterogeneity in *rrn* operons has been a useful strain-specific marker in many bacterial species, these researchers examined the heterogeneity of the 3′ end of the *rrnB* operon in various strains of *S. mutans*. They suggested that the diversity in the single locus revealed by RFLP analysis of the PCR-amplified 3′ end of *rrnB* makes this technique a useful alternative to ribotyping or an additional tool for the distinction of strains with similar or identical ribotypes. PCR-RFLP analysis has also been carried out on the glucosytransferase genes *gtfB* and *gtfC* with similar results (Shiroza *et al.*, 1998).

Direct detection of *S. mutans* has been documented using PCR amplification and has been applied in clinical situations. PCR amplification of *S. mutans* prior to sequencing has been successful for diagnosis of recurrent *S. mutans* endocarditis (Gauduchon *et al.*, 2001). Synthetic oligonucleotide primers have been designed to amplify the *spaP* and *dexA* genes of *S. mutans* (Ono *et al.*, 1994; Igarashi *et al.*, 1996 a, b; Yano *et al.*, 2002).

Although Igarashi *et al.* (1996a; b) successfully report the presence of *S. mutans* and Yano *et al.* (2002) were able to quantify its levels with real-time PCR, not all *S. mutans* strains possess the same cariogenic properties (de Soet *et al.*, 1991). It is apparent that each strain should be isolated and its cariogenic potential evaluated based on genetic heterogeneity. More recent work on the identification of mutans streptococci has utilised a PCR-based procedure of DNA fingerprinting, called arbitrarily primed PCR (AP-PCR) or randomly amplified polymorphic DNA (RAPD) analysis (Saarela *et al.*, 1996; Li and Caufield, 1998). Truong *et al.* (2000) published data which indicated that AP-PCR could generate characteristic and unique RAPD fingerprints that were highly specific for *S. mutans* and *S. sobrinus*. However, they acknowledged that the handling of large numbers of polymorphic banding patterns for molecular systematics would benefit from improvements.

Studies that report an association between gender and *S. mutans* genotypes have been carried out within families and therefore the suggestion of an unrelated association could be an important one. Further investigation into the gender-specific genotype observation with a larger study number would be of interest to determine whether this is a universal trend or confined to this study. Another area of interest would be to follow the loss and reappearance of *S. mutans* genotypes to determine whether there are any specific factors which trigger this effect. There may also be a particular time at which this occurs, in which case more regular sampling may be recommended.

CHAPTER 5: STRAIN DETERMINATION, USING POLYMERASE CHAIN REACTION-RESTRICTION FRAGMENT LENGTH POLYMORPHISM ANALYSIS AND DNA SEQUENCING, OF *LACTOBACILLUS* SPECIES FROM CLINICAL SPECIMENS.

5.1 Introduction

Lactobacilli are part of the commensal microflora of the mouth, gastrointestinal tract and female genital tract. They are very rarely associated with disease although they have been implicated in endocarditis (Davies *et al.*, 1986; Sussman *et al.*, 1986), pulmonary infections and bacteraemias (Bayer *et al.*, 1978). *Lactobacillus* species are also used extensively in the food industry and are essential to the dairy industry in cheeses, yoghurt and other products (Drake *et al.*, 1996). Homofermentative lactobacilli such as strains of the *Lactobacillus acidophilus* complex are widely used as starter bacteria of probiotic cultures for the production of fermented milks (Roy *et al.*, 2000).

The genus *Lactobacillus* encompasses a diverse collection of Gram-positive, catalase-negative, non-sporeforming, rod-shaped organisms. These lactic acid bacteria are typically chemoorganotrophic and ferment carbohydrates with lactic acid as a major end product (Roy *et al.*, 2000). Lactobacilli are commonly isolated from the oral cavity although they are reported to comprise less than 1% of the total cultivable microflora (Van Houte *et al.*, 1981; Marsh and Martin, 1999). A definite relationship between the incidence of caries and lactobacilli has been indicated in many studies (Minah *et al.*,

1981; Krasse, 1988; Bjarnson, 1989; Alaluusua *et al.*, 1989). *L. casei, L. fermentum, L. plantarum* and *L. acidophilus* have been isolated from carious dentin and early caries lesions (Boyar and Bowden, 1985). More recently, *L. paracasei, L. rhamnosus* and *L. fermentum* were reported to be the predominant species present in caries lesions (Botha *et al.*, 1998). However, there is little knowledge on the diversity of the species in relation to caries, nor of their role (Bowden, 1997), and there is a lack of studies of the oral *Lactobacillus* species in association with caries that use reliable identification methods (Richard *et al.*, 2001).

At present, the genus *Lactobacillus* consists of more than sixty described species that are genetically quite diverse (Nour, 1998). Traditionally, the identification of lactobacilli has been, and still is, based mainly on morphological, physiological and biochemical markers. In more recent years, research into the genomic structure and phylogenetic relationships between *Lactobacillus* spp. has considerably changed the taxonomy of these organisms (Collins *et al.*, 1991; Nour, 1998). Due to the importance of *Lactobacillus* spp. in the food industry, the majority of research into this group has been carried out in relation to *L. acidophilus, L. casei, L. rhamnosus* and *L. paracasei.*

Although phenotypic methods of identifying *Lactobacillus* spp. are still in use, the identification of some species by biochemical methods alone is not reliable (Nour, 1998; Tynkkynen *et al.*, 1999). Saxelin *et al.* (1996) and Zhong *et al* (1998) both reported difficulties in identifying lactobacilli in clinical samples. There is therefore a need for rapid and reliable species-specific identification using more modern methods, such as PCR. Species-specific PCR primers have been described that amplify sequences within

the 16S-23S rRNA spacer regions of *L. paracasei, L. rhamnosus, L. delbrueckii, L. acidophilus* and *L. helveticus* (Tilsala-Timisjärvi and Alatossava, 1997) and within the 16S rRNA gene of *L. rhamnosus* (Alander *et al.*, 1999). Genotypic methods used for strain typing are typically PCR methods such as randomly amplified polymorphic DNA (RAPD) analysis or variations of restriction enzyme analysis, for example pulsed-field gel electrophoresis (PFGE), restriction fragment length polymorphism (RFLP) analysis and ribotyping. In RAPD analysis, short arbitrary sequences are used as primers in PCR, which yields strain-specific amplification product patterns (Welsh and McClelland, 1990). In PFGE, RFLP and ribotyping analysis, genomic DNA is digested with restriction enzymes. In PFGE (Schwartz and Cantor, 1984) and RFLP, enzymes that infrequently cut bacterial DNA are used and large genomic fragments are separated, while in ribotyping (Saarela *et al.*, 1993b), rRNA genes and/or their spacer regions are used as probes that hybridise with genomic restriction fragments.

The main aim of this study was to determine the viability of the use of PCR-RFLP to discriminate between species of *Lactobacillus*. The bacterial 16S rRNA of clinical isolates, originally identified morphologically as *Lactobacillus* spp., was firstly amplified by PCR, and RFLP analysis was then carried out in an attempt to speciate them definitively. The clinical isolates were obtained from subjects participating in a fluoridated milk study and therefore the possibility of a longitudinal effect of fluoridated milk on strains isolated from one particular individual, over a period of 2 years, was also investigated. Some of the isolates that did not type to any of the reference strains were processed for DNA sequencing to identify them to species level.

5.2 Materials and Methods

5.2.1 Bacterial Strains

A total of 216 isolates of *Lactobacillus* spp. were obtained from thirteen subjects who were participating in the fluoridated milk study described in Chapter 3. The samples were collected and stored at -80°C over a period of two years. Isolates were originally cultured from Rogosa SL agar medium. They were sub-cultured from the primary plates on the basis of colony morphology (Rogosa *et al.*, 1951), and confirmed as *Lactobacillus* spp. by a positive Gram stain reaction as described previously. Isolates were initiated for chromosomal DNA isolation from frozen stocks.

Reference strains of the *Lactobacillus* spp. were obtained from the NCTC (National Centre for Type Cultures, London, U.K.) and ATCC (American Type Culture Collection, London, U.K.). These strains were: *L. fermentum* ATCC 14931, *L. salivarius* ATCC 11741, *L. casei* subsp *casei* ATCC 393, *L. odontolyticus* NCTC 1406, *L. vaginalis* NCTC 12197, *L. casei* var *rhamnosus* (NCTC 6375, 10302) *L. delbrueckii* NCTC 12712 and *L. acidophilus* NCTC 1723. Cultures were reconstituted on fastidious anaerobe agar (BioConnections) supplemented with defibrinated horse blood (7.5%, v/v) at 37°C for 2 days in an anaerobic chamber under an atmosphere of 85% N_2, 10% CO_2 and 5% H_2.

5.2.2 DNA Extraction

Reference strains were harvested from the plates and genomic DNA was extracted using the Puregene DNA isolation kit (Novara Flowgen) as described in Chapter 2. Clinical isolates were harvested from the plates and lysates were prepared by adding achromopeptidase (Sigma) to bacterial suspensions in 10 mM Tris-HCl, 1 mM EDTA, pH7.0. Samples were incubated at 56°C for 30 minutes, boiled for 5 minutes and stored at -70°C until required.

5.2.3 PCR

The primers used for amplification targeted conserved regions of the 16S rRNA gene and were designed to amplify DNA from most bacterial species. The primers used were 5′-AGA GTT TGA TC(A/C) TGG CTC AG-3′ (27F; *Escherichia coli* positions 8 to 27) and 5′-TAC GG(C/T) TAC CTT GTT ACG ACT T-3′ (1492R; *E. coli* positions 1510 to 1492) (MWG Biotech), which give an expected amplification product of approximately 1,500 bp (Lane, 1991). All PCR reactions were carried out in a total volume of 50 µl, comprising 5 µl of extracted bacterial DNA and 45 µl of reaction mixture containing 1x PCR buffer (10mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100), 1.0 unit of *Taq* DNA polymerase (Promega), 0.2 mM of each deoxyribonucleoside triphosphate and each primer at a concentration of 0.2 µM. In order to increase the sensitivity and specificity of the assay, "hot start" PCR was used. The primers were separated from other reaction components by a layer of wax (DyNAwax; Flowgen), which prevented the reaction from starting until the wax melted upon initiation of

thermal cycling. PCR was carried out in an OmniGene thermal cycler (Hybaid Ltd, Teddington, U.K.). After an initial denaturation step at 94°C for 5 minutes, 35 cycles were carried out, each comprising of denaturation at 94°C for 1 minute, annealing at 58°C for 1 minute 30 seconds and extension at 72°C for 1 minute, followed by a final extension step at 72°C for 10 minutes. Five microlitres of each PCR product were electrophoresed on a 2% agarose gel. Reaction products were either processed immediately or stored at -20°C until required.

5.2.4 RFLP Analysis

RFLP analysis was carried out on the PCR products. Aliquots containing 0.5 µg DNA were digested in a total volume of 15µl with 5.0 U *Mnl*I (Helena Biosciences) at 37°C for 3 hours. Restriction fragments generated were visualised by agarose gel electrophoresis. Isolates representing strains of *L. casei* and *L. casei* subsp. *rhamnosus* were further digested with either 5.0 U *Cfo*I, *Hin*fI or *Rsa*I (Promega) at 37°C for 3 hours to allow discrimination between these strains.

5.2.5 Plasmid Ligation and Transformation

PCR products were ligated into the pCR2.1-TOPO vector (Invitrogen, Paisley, U.K.) and transformed into *Escherichia coli* TOP10 cells (Invitrogen) as follows. Briefly, 1 µl salt solution and 1 µl TOPO® vector (10 ng/µl) was added to 4 µl (100 ng) fresh PCR product and incubated on ice for 5 minutes. Two microlitres of the TOPO® reaction mix was added to one vial of One Shot® Chemically Competent *Escherichia coli*, mixed

gently and then incubated on ice for 30 minutes. The cells were heat-shocked at 42°C for 30 seconds then the tubes immediately transferred to ice. Two hundred and fifty microlitres of SOC medium was added and then incubated at 37°C for 1 hour. Fifty microlitres of each transformation was then spread on L-agar plates containing ampicillin (100 μg/ml) and incubated overnight at 37°C.

5.2.6 Plasmid Purification

Plasmid DNA from recombinant clones was purified with the QIAquick® PCR Purification kit (Qiagen Ltd, Crawley, U.K.). To 1 volume of PCR sample was added 5 volumes of Buffer PB. A QIAquick spin column was placed in a 2 ml collection tube and the sample was applied to the column and centrifuged at 13,000 x g for 1 minute. The flow-through was discarded and the column placed back in the same tube. In order to wash the DNA, 0.75 ml Buffer PE was added to the column and centrifuged for 1 minute. The flow-through was again discarded and the column placed back in the tube. This was then centrifuged for a further minute to remove residual ethanol. The column was then placed in a fresh 1.5 ml microcentrifuge tube and 30 μl of molecular-biology-grade water was added. This was allowed to stand for over a minute and then centrifuged for 1 minute to elute the DNA.

5.2.7 DNA Sequencing

Sequencing reactions were performed with Thermo Sequenase Fluorescent Labelled Primer Cycle Sequencing Kit with 7-deaza-dGTP (Amersham Pharmacia Biotech, St

Albans, U.K.). The sequencing reaction was set up with 112 ng plasmid DNA (100 fmol), 1 μl (1.0 pmol/μl) IRD800-labelled M13 universal primer [-21] (5′-TGTAAAACGACGGCCAGT-3′), 7.2 μl 3.5x SequiTherm EXCEL II sequencing buffer, 1 μl (5 U/μl) EXCEL II DNA polymerase and made up to 20 μl with sterile molecular-biology-grade water. For each isolate, 4.5 μl of the sequencing reaction was added to each of 1.5 μl A, C, G and T reagent (primer termination mixes for each dideoxynucleoside triphosphate). One drop of Chill-out 14 wax (Genetic Research Instrumentation, Braintree, U.K.) was laid over the reaction. Sequencing reactions were performed using a Primus 96 DNA thermal cycler (MWG Biotech) using the following cycling parameters: (i) initial denaturation at 95°C for 30s; (ii) 10s at 95°C, 30s at 57°C and 30s at 70°C, for 20 cycles; and (iii) 10s at 95°C and 30s at 70°C for 15 cycles. Six microlitres of formamide loading dye was added to each reaction mixture following thermal cycling. Approximately 1.5 μl of each denatured sequencing reaction mixture was run on a LI-COR Gene ReadIR 4200S automated DNA sequencing system (MWG Biotech) overnight using the following conditions: gel temperature, 50°C; voltage, 1500V; current, 35.0 Ma; power, 31.5 W.

5.2.8 Sequence Analysis

The sequences obtained from the LI-COR BaseImagIR v4.1 image analysis program were converted to FASTA format and the 16S sequences were then compared with 16S rRNA gene sequences from the public sequence databases Genbank and EMBL using the advanced gapped BLAST program, version 2.1. The BLAST program was then run

through the National Centre for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST).

5.3 Results

5.3.1 Discrimination Amongst Reference Strains

The 16S rRNA gene was successfully amplified from all eight reference strains tested, as demonstrated by the appearance of a 1,500 bp PCR product. The expected sizes of the restriction fragments (at least 100 bp long) generated by digestion of PCR products from each of the reference strains with *Mnl*I are shown in Table 5.3.1. Further digestion of reference strains of *L. casei* subsp. *casei* (ATCC 393) and *L. casei* var. *rhamnosus* (NCTC 6375 and 10302) with enzymes *Hinf*I, *Cfo*I and *Rsa*I showed that *Cfo*I could discriminate between these strains. The expected fragment sizes following digestion with *Hinf*I, *Cfo*I and *Rsa*I are also shown in Table 5.3.1. Typical restriction patterns obtained following digestion of reference strains with *Mnl*I and *Cfo*I are shown in Figures 5.3.1 a) and b), respectively. *Mnl*I gave distinct restriction profiles for six of eight *Lactobacillus* reference strains, and further digestion with *Cfo*I gave distinct restriction profiles for *L. casei* subsp. *casei* and *L. casei* var. *rhamnosus*.

5.3.2 Analysis of Clinical Isolates

The 16S rRNA gene was successfully amplified by PCR from all of the *Lactobacillus* isolates tested which had been previously identified by conventional microbiological culture. A total of 216 isolates were analysed by RFLP with 94 isolates identifying as one of the eight reference strains. Figures 5.3.2 a) and b) show examples of the restriction profiles obtained from the clinical isolates after digestion with *Mnl*I and *Cfo*I,

Table 5.3.1 Restriction fragments for *Lactobacillus* type strains

Strain	*L. fermentum*	*L. salivarius*	*L. casei* var *rhamnosus*				*L. odontolyticus*	*L. delbrueckii*	*L. vaginalis*	*L. acidophilus*	*L. casei* subsp *casei*			
	14931	**11741**	**6375/10302**				**1406**	**12712**	**12197**	**1723**	**393**			
Enzyme	*Mn*lI	*Mn*lI	*Mn*lI	*Cfo*I	*Hin*fI	*Rsa*I	*Mn*lI	*Mn*lI	*Mn*lI	*Mn*lI	*Mn*lI	*Cfo*I	*Hin*fI	*Rsa*I
Bands	380	350	350	600	980	900	350	350	390	380	350	500	980	900
	340	300	290	510	390	380	300	300	350	310	290	410		380
	310	250	260	400				260	310	280	260	390		
	250							200	290					
	180													

Table 5.3.1 Expected sizes of restriction fragments generated by digestion of *Lactobacillus* spp. reference strains with restriction enzymes *Mn*lI, *Cfo*I, *Hin*fI and *Rsa*I.

Figure 5.3.1 a) RFLP profiles generated from *Mnl*I digestion of *Lactobacillus* reference strains

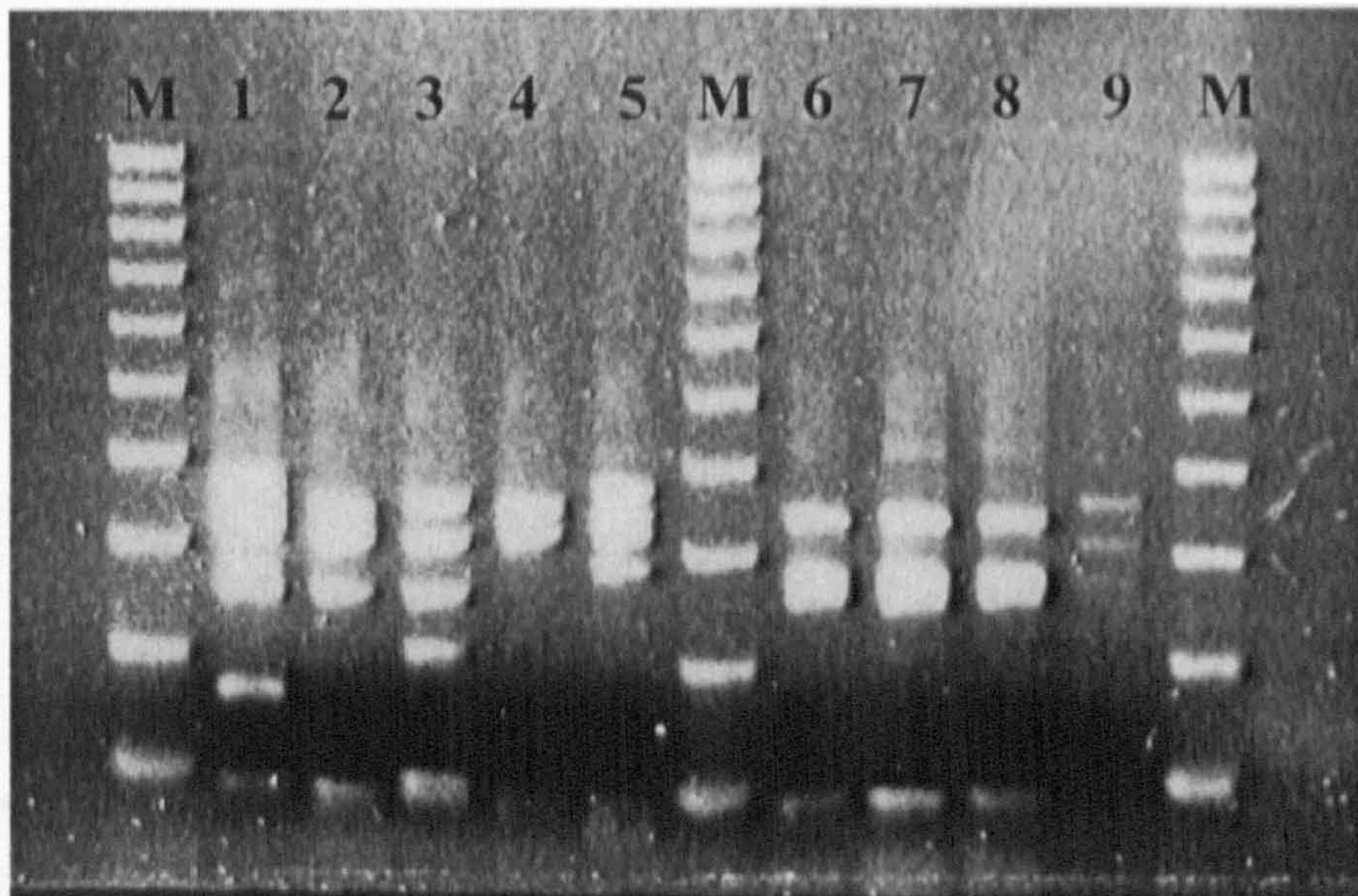


Figure 5.3.1 a) RFLP profiles generated from *Mnl*I digestion of *Lactobacillus* reference strains. Lanes: (1) *L. fermentum* ATCC 14931, (2) *L. salivarius* ATCC 11741, (3) *L. delbrueckii* NCTC 12712, (4) *L. odontolyticus* NCTC 1406, (5) *L. vaginalis* NCTC 12197, (6) *L. casei* var *rhamnosus* NCTC 6375, (7) *L. casei* var *rhamnosus* NCTC 10302, (8) *L. casei* subsp. *casei* ATCC 393, (9) *L. acidophilus* NCTC 1723, (M) 100-bp DNA ladder.

Figure 5.3.1 b) Restriction profiles generated from *Hin*fI, *Cfo*I and *Rsa*I digestion of *Lactobacillus* reference strains

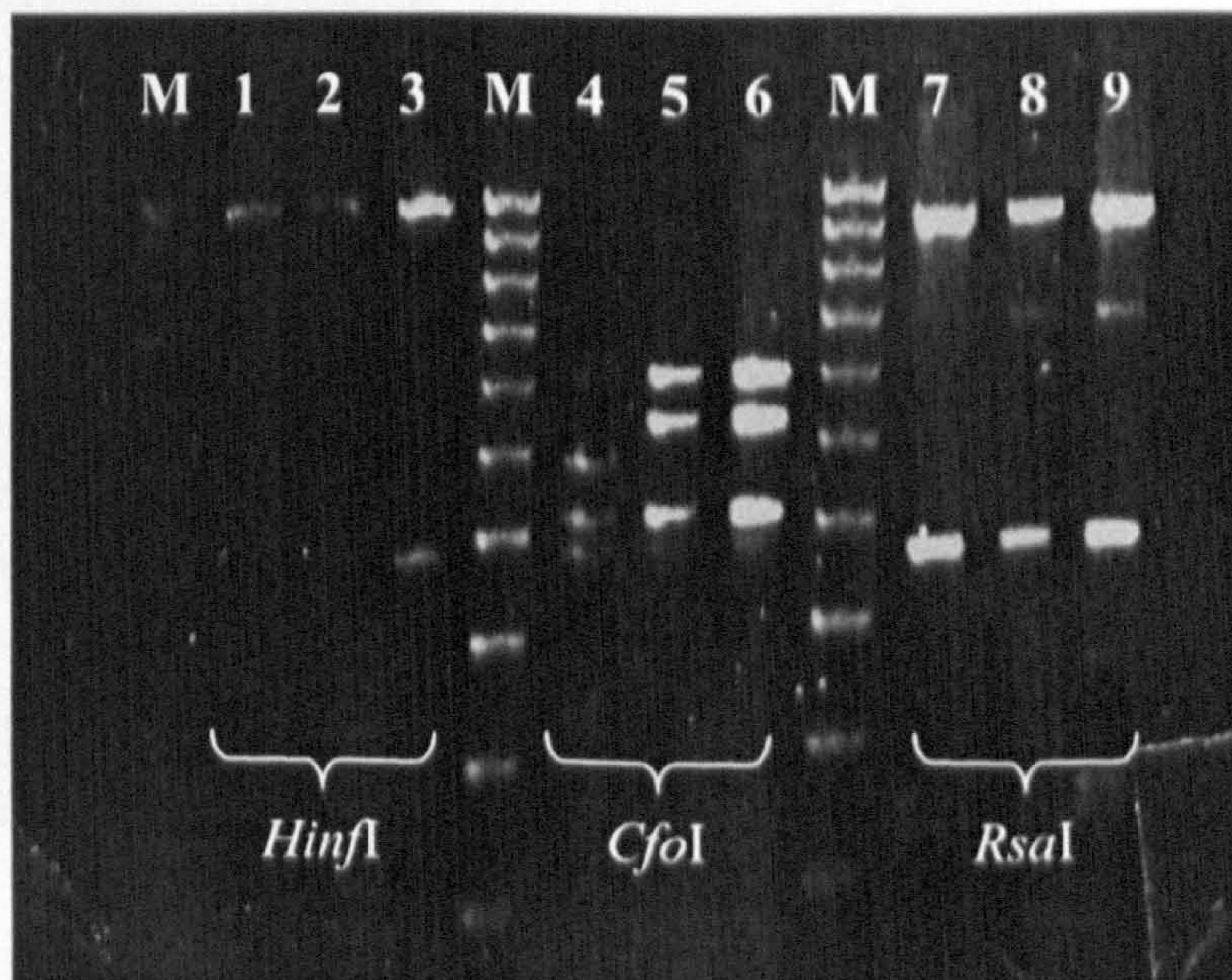


Figure 5.3.1.b) RFLP profiles generated from *Hinf*I, *Cfo*I and *Rsa*I digestion of *Lactobacillus* reference strains. Lanes (1, 4, 7) *L. casei* subsp. *casei* ATCC 393, (2, 5, 8) *L. casei* var *rhamnosus* NCTC 6375, (3, 6, 9) *L. casei* var *rhamsnosus* 10302, (M) 100-bp DNA ladder.

Figure 5.3.2 a) Typical restriction profiles generated with *Mnl*I on clinical isolates

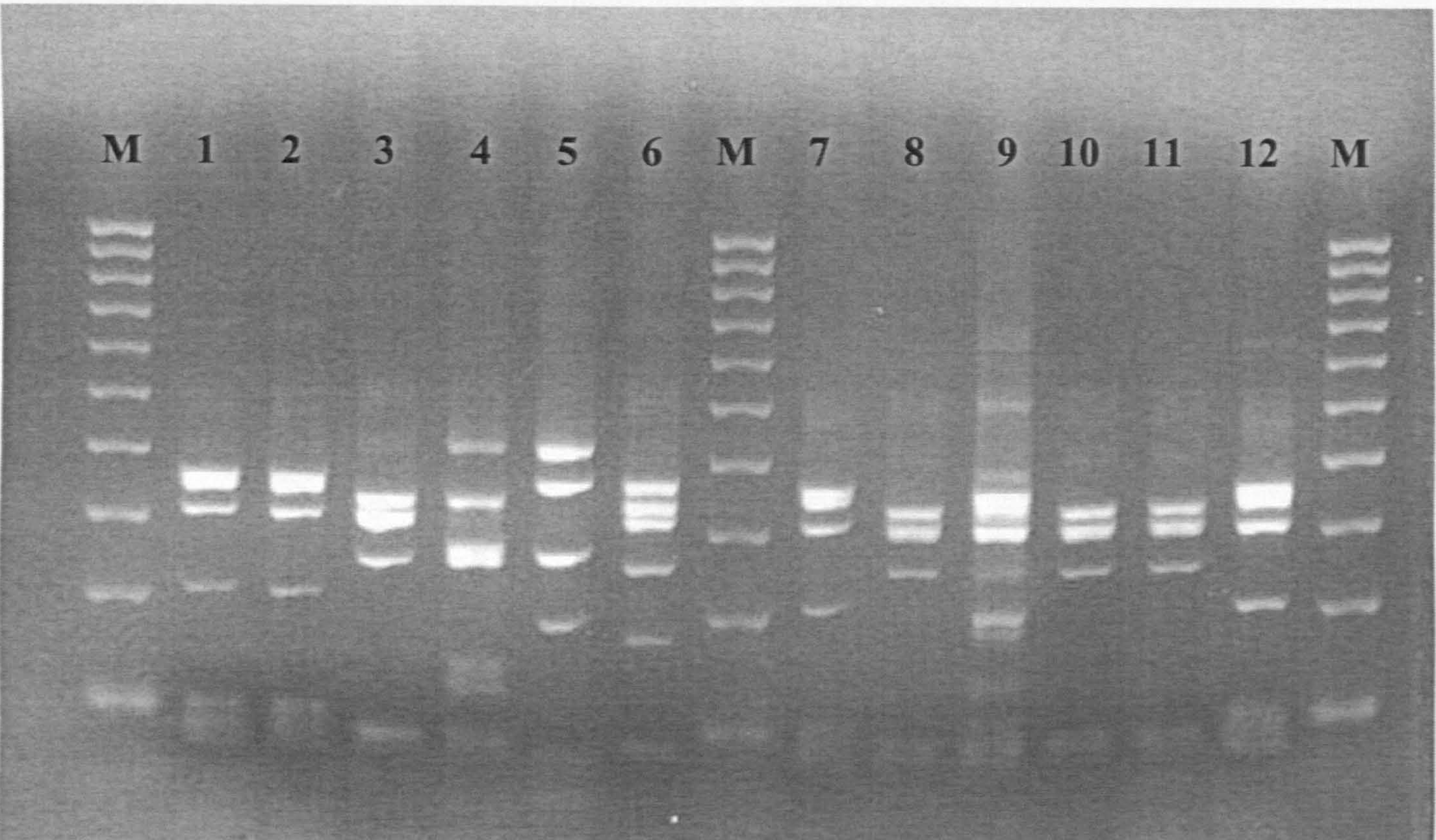


Figure 5.3.2 a) A typical RFLP profile of 12 clinical isolates digested with *Mnl*I. Lanes: (1, 2, 7, 9, 12), *L. gasseri*; (3, 8, 10, 11), *L. salivarius*; (4), *L. casei*-group; (5), *L. delbrueckii*; (6), *L. fermentum;* (M), 100 bp marker.

Figure 5.3.2 b) Restriction profiles of *L. casei* clinical isolates digested with *Cfo*I

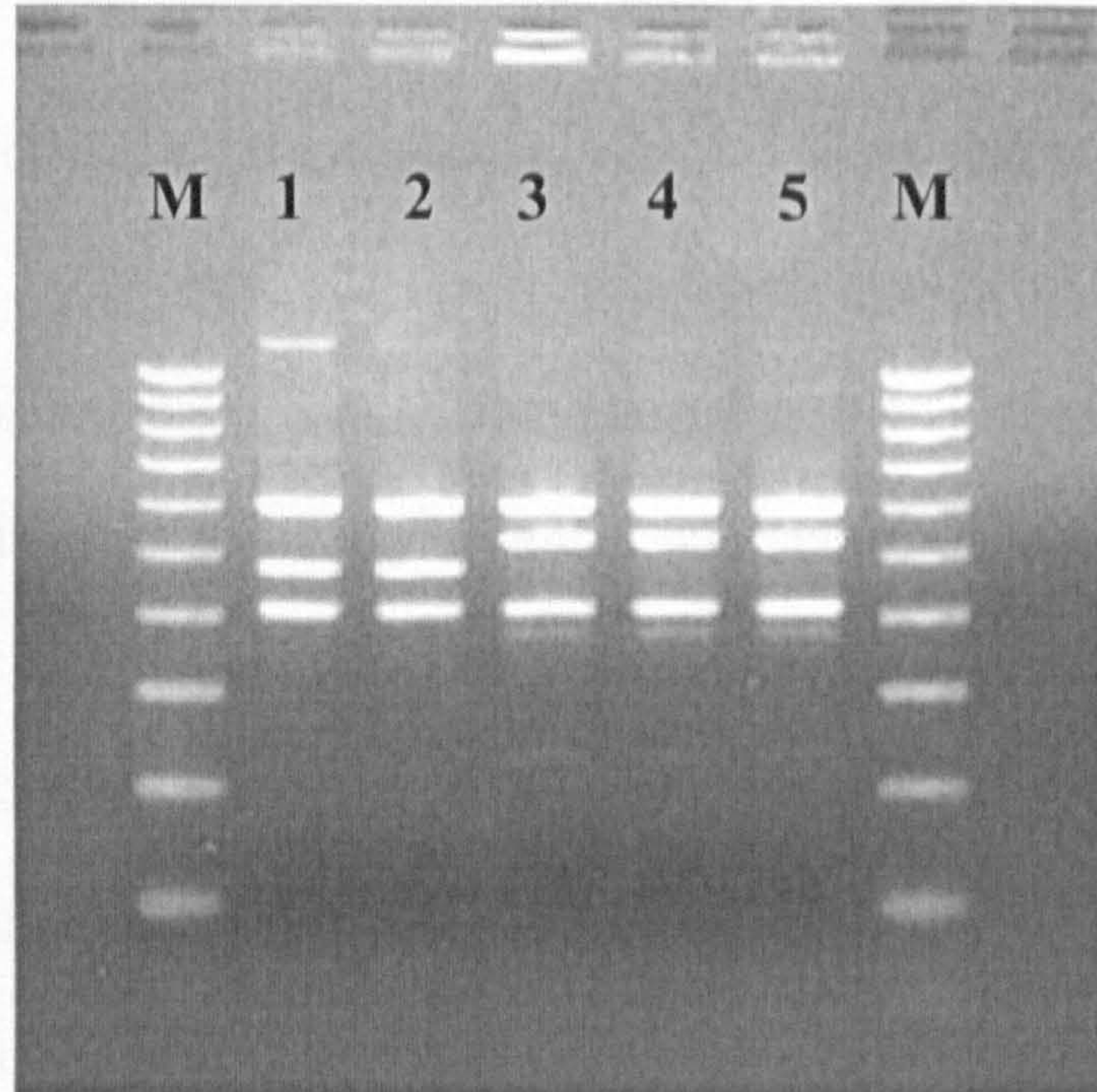


Figure 5.3.2 b) Clinical isolates digested with *Cfo*I after identification as *L. casei* species with *Mnl*I. Lanes: (1, 2), atypical *L. casei/paracasei*; (3, 4, 5), *L. casei* var *rhamnosus*; (M), 100-bp DNA ladder.

respectively. The number of isolates identified within each species was: *L. fermentum* (49); *L. salivarius* (17); *L. odontolyticus* (5); *L. casei* var *rhamnosus* (19); *L. acidophilus* (0); *L. vaginalis* (0). Table 5.3.2 a) displays the groups that each of the clinical isolates fall into and Table 5.3.2 b) displays the groups obtained for Subject CP only.

A further 122 isolates could be grouped, but not with any of the reference strains and are therefore unidentified. The most frequently isolated species [UNKNOWN1; 53/216 (24.5%)] profiled as belonging to the *L. casei*-group with *Mnl*I digestion however *Cfo*I digestion of these isolates produced a profile that was atypical to the reference strains analysed [Figure 5.3.2. a), Lanes 1, 2]. The next most common strains [UNKNOWN2; 49/216 (22.7%)] displayed a restriction profile with restriction enzyme *Mnl*I that was different from the reference strains [Figure 5.3.2. b) Lanes 1, 2, 7, 9, 12]. *Cfo*I digestion of the 57 *L. casei*-group isolates from subject CP revealed that 12 had an atypical profile to UNKNOWN 1, having one extra band at 380 bp [UNKNOWN3; Figure 5.3.3. b)]. Isolates R223 1, 2B, 3A and 6A from Subject JF (Table 5.3.2. a) all displayed a pattern that was difficult to interpret due to diffuse bands following several attempts at DNA extraction and RFLP analysis with *Mnl*I [UNKNOWN 4; Figure 5.3.2. c) Lanes 1-4]. Another four isolates were identified in the analysis of strains from Subject CP that appeared to have one strong band at 300 bp and a lighter band at 180 bp [UNKNOWN 5; Figure 5.3.2. c) Lanes 7 and 8]. Lane 5 in Figure 5.3.2 c) represents an isolate of UNKNOWN1 and Lane 6 represents an isolate of *L. fermentum*. Isolates representing each of these unidentified strains were processed for further analysis by DNA sequencing.

Table 5.3.2 a) ***Lactobacillus*** **spp. identification among clinical isolates**

Patient ID No.	Subject	Interval	*L. ferm*	*L. sal*	*L. casei* var *rhamnosus*	*L. odont*	*L. acido*	*L. delb*	*L. vag*	UNKNOWN 1	UNKNOWN 2	UNKNOWN 4
R208	SB	Exp1Wk6			3					2		
R215	JD	Exp1Wk6	3							1		
R217	AQ	Exp1Wk6		2	1			1		1		
R218	HM	Exp1Wk6		4							5	
R223	JF	Exp1Wk6			3					2		4
R229	IR	Exp1Wk6			1					4	2	
R230	BO	Exp1Wk6			1					1		
R232	BM	Baseline	4			4					5	
R240	JD	Exp2Wk1	4							1		
R244	JC	Exp1Wk1	2								3	
R249	JM	Exp1Wk1		2				1			2	
R250	EM	Exp1Wk1	1	3							3	
R252	JL	Exp1Wk1	1					1			4	
R256	JF	Exp2Wk1			4						5	
R259	AQ	Exp2Wk1		6				1			4	
R263	BM	Exp1Wk1	4		5						5	
R277	SB	Exp2Wk1	3		1							
			23	17	19	4	0	4	0	12	38	4
												121

Table 5.3.2 a) Grouping of clinical isolates into *Lactobacillus* species. Interval corresponds to the fluoridated milk project experimental stage in which the subject was participating.

Table 5.3.2 b) *Lactobacillus* spp. identification among clinical isolates from Subject CP

Patient ID No.	Interval	*L. ferm.*	*L. odont.*	UNKNOWN 1	UNKNOWN 2	UNKNOWN 3	UNKNOWN 5
R227	Baseline			4	2		
R275	Exp1Wk1			4			
R320	Exp1Wk6	6		2		2	
R361	Exp2Wk1	1		4		1	
R405	Exp2Wk6	2		5	2	1	
R452	Exp3Wk1				2	1	
R478	Exp3Wk6	2		5		2	
R005	Exp4Wk1	6		1			
R021	Exp4Wk6	1	1	11		2	2
R035	Exp5Wk1	4		2		1	
R042	Exp5Wk6	4		6		2	2
		26	1	44	6	12	4
							93

Table 5.3.2 b) Grouping of clinical isolates from subject CP into *Lactobacillus* species. Interval corresponds to the fluoridated milk project experimental stage in which the subject was participating.

Figure 5.3.2 c) RFLP profiles generated from *Mnl*I digestion of four clinical isolates from subject CP

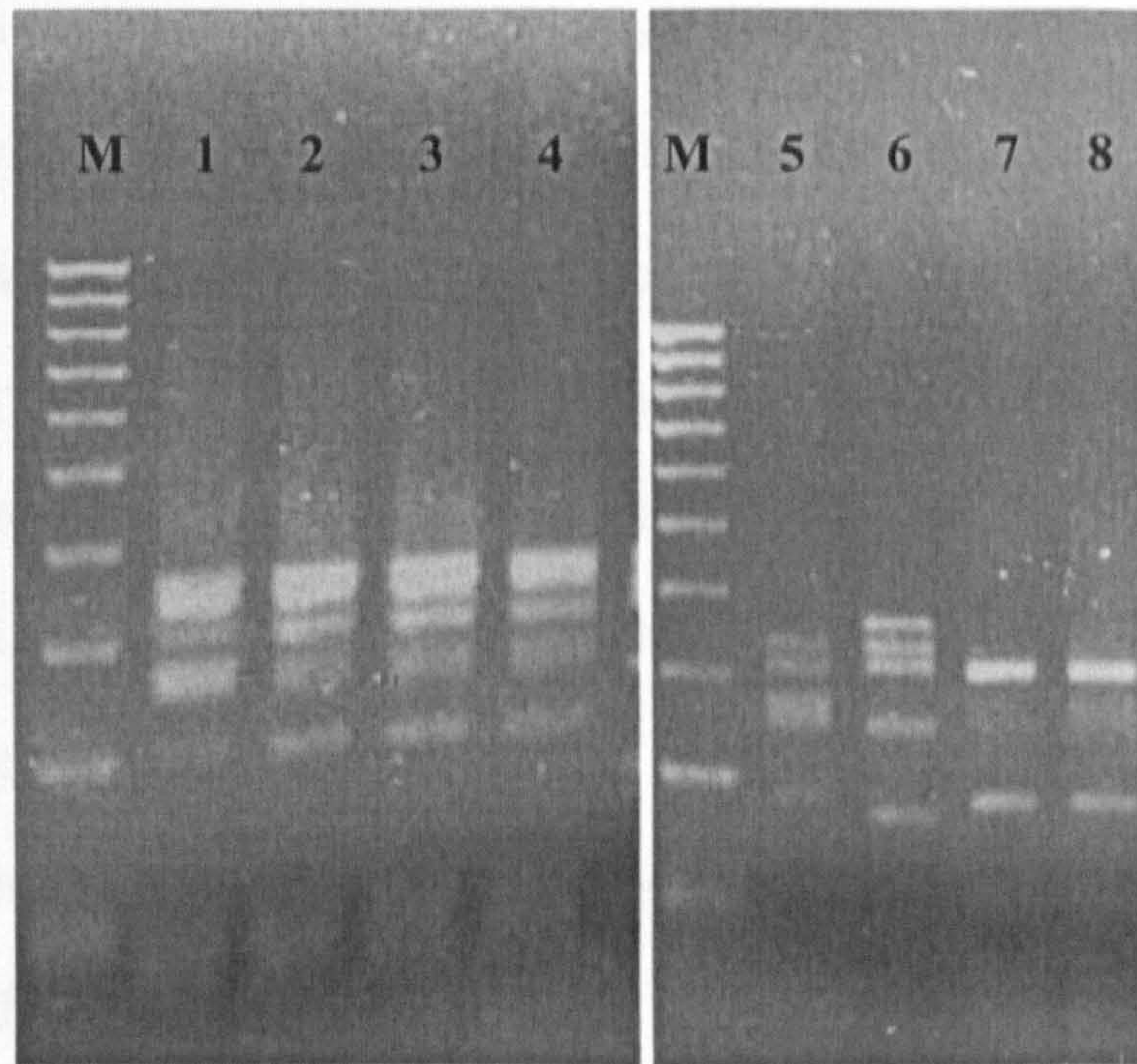


Figure 5.3.2 c) RFLP profiles generated from *Mnl*I digestion of four clinical isolates from subject CP. Lanes: (1), R223 1; (2), R223 2B; (3), R223 3A; (4), R223 6A; (5), R042 1A; (6), R042 1B; (7), R042 1C; (8) R021 1A; (M), 100-bp DNA marker.

5.3.3 DNA Sequencing

A total of five from the 122 unidentified isolates were processed for DNA sequencing, representing strains which did not correspond to any of the reference strains. Sequences generated from clones of each of these isolates were compared against the EMBL/GenBank sequence databases using the BLAST algorithm. Clones that shared 99% identity with a known sequence were positively identified as that species. To obtain a true percentage identity with clones displaying a lower score (94-97%), the ambiguous bases in the database sequence that the base-calling programme was unable to identify definitively were removed from the percentage identity scoring. This was to give a true percentage similar score between the query sequence and the sequence of the matching species in the database. The isolate representing UNKNOWN1 displayed 99% homology with *L. casei* subspecies *casei* and *L. paracasei* subspecies *paracasei*. UNKNOWN3 isolate also shared 99% homology with these two species following the removal of ambiguous bases. The representative strain of the R223 isolates (UNKNOWN4) sequenced to the same species as above, although with only 97% homology. The 16S sequences for both *L. casei* and *L. paracasei* were aligned against each other with the intention of classifying the clinical isolates. Since this alignment resulted in 97% identity between these two strains, they are likely to be different strains of the same species. The clinical isolates cannot be distinguished as definitely being *L. casei* or *L. paracasei*, therefore they are designated *L. casei/paracasei*.

DNA sequencing analysis revealed that UNKNOWN2 shared 94% homology with *L. gasseri* ATCC 33323. When the ambiguous bases were removed, the homology

increased to 98.8%. The final isolate from CP, UNKNOWN5, was identified as a member of the *Lysobacter* spp. (*Lyso. enzymogenes*) sharing 99% homology. *Lysobacter*, a Gram-negative rod, is known as a common soil bacterium (Folman *et al.*, 2003), so the identification of this isolate from this subject is highly unlikely as all these isolates were Gram tested for a positive reaction. This is most probably a contaminant.

5.3.4 Effect of Fluoridated Milk on Clinical Lactobacillus Isolates

To determine whether fluoridated milk had an effect on the prevalence of certain species of *Lactobacillus*, multiple isolates from one patient participating in a fluoridated milk study were analysed at each point of collection. The main areas of interest lie in the possibility of an effect of site on plaque bacteria and the effect of milk intake with or without fluoride. *L. casei/paracasei* and *L. fermentum* were the most frequently isolated species from this particular individual. Table 5.3.3 shows the total counts of each *Lactobacillus* strain that was isolated from Subject CP. Figures 5.3.3 a) and b) show examples of the RFLP profiles that were generated with *Mnl*I and *Cfo*I digestion of Subject CP isolates, respectively. This subject had participated in five experiments, each of six weeks duration, with at least three weeks wash-out in between.

The first measurement is a baseline count to determine extrinsic levels of bacteria before the start of the experiments. Carriage of *Lactobacillus* species is relatively low at the first two sampling time points (Baseline, Week 1 Exp. 1). After exposure to milk three times daily for six weeks *L. fermentum* is introduced into the oral cavity (Week 6, Exp. 1), with counts relatively high for the buccal and lingual sites (1, 2 and 3). The next

Table 5.3.3 Total counts of *Lactobacillus* species isolated from one subject

Interval	Site	*L. ferm.*	*L. casei/paracasei*	L.gasseri	*L. odonto.*	Lysobacter
Baseline	1		1830000			
	2		3580000	1150000		
	3			52000		
	4					
	5		1060			
	6					
Week1	1		5120000			
Exp. 1	2					
Milk	3		31600			
(x3/day)	4		1440			
	5		6800			
	6					
Week 6	1	1390000	56000			
Exp. 1	2	4070000				
Milk	3	112000	88800			
(x3/day)	4	1280				
	5	24000	2800			
	6	68000	600			
Week 1	1		6300000			
Exp. 2	2		1650000			
No	3		76000			
Beverage	4		220			
	5	40	140			
	6		460			
Week 6	1		9470000			
Exp. 2	2		2530000	192000		
No	3		1020000	8000		
Beverage	4	4600	40000			
	5	100	20			
	6		192000			
Week 1	1		70000	2000		
Exp. 3	2		298000			
Milk + F	3		150000			
(x1/day)	4		520			
	5		4600			
	6			200		
Week 6	1	1200000	464000			
Exp. 3	2		1888000			
Milk + F	3		176000			
(x1/day)	4	32400	34400			
	5		80			
	6		20			

Table 5.3.3 (cont'd) Total counts of *Lactobacillus* species isolated from one subject

Interval	Site	*L. ferm.*	*L. casei/paracasei*	*L. gasseri*	*L. odonto.*	Lysobacter
Week 1	1	240000				
Exp. 4	2	1390000				
Milk + F	3	680	100			
(x3/day)	4	40				
	5	2000				
	6	140				
Week 6	1		10300000			144000
Exp. 4	2		1630000		448000	
Milk + F	3		11580400			29600
(x3/day)	4	75200	50800			
	5		60			
	6		2000			
Week 1	1	140	200			
Exp. 5	2	848000	126000			
Milk	3	220	60			
(x1/day)	4	60				
	5					
	6					
Week 6	1	11400000	9870000			1070000
Exp. 5	2		6660000			5330000
Milk	3		624000			
(x1/day)	4	80	660			
	5	190	1090000			
	6	280				

Table 5.3.3 Total counts of *Lactobacillus* species isolated from Subject CP participating in a fluoridated milk study. Sites: (1 & 3), buccal; (2), lingual; (4, 5 & 6), palatal.

Figure 5.3.3 a) Restriction profiles of clinical isolates from Subject CP

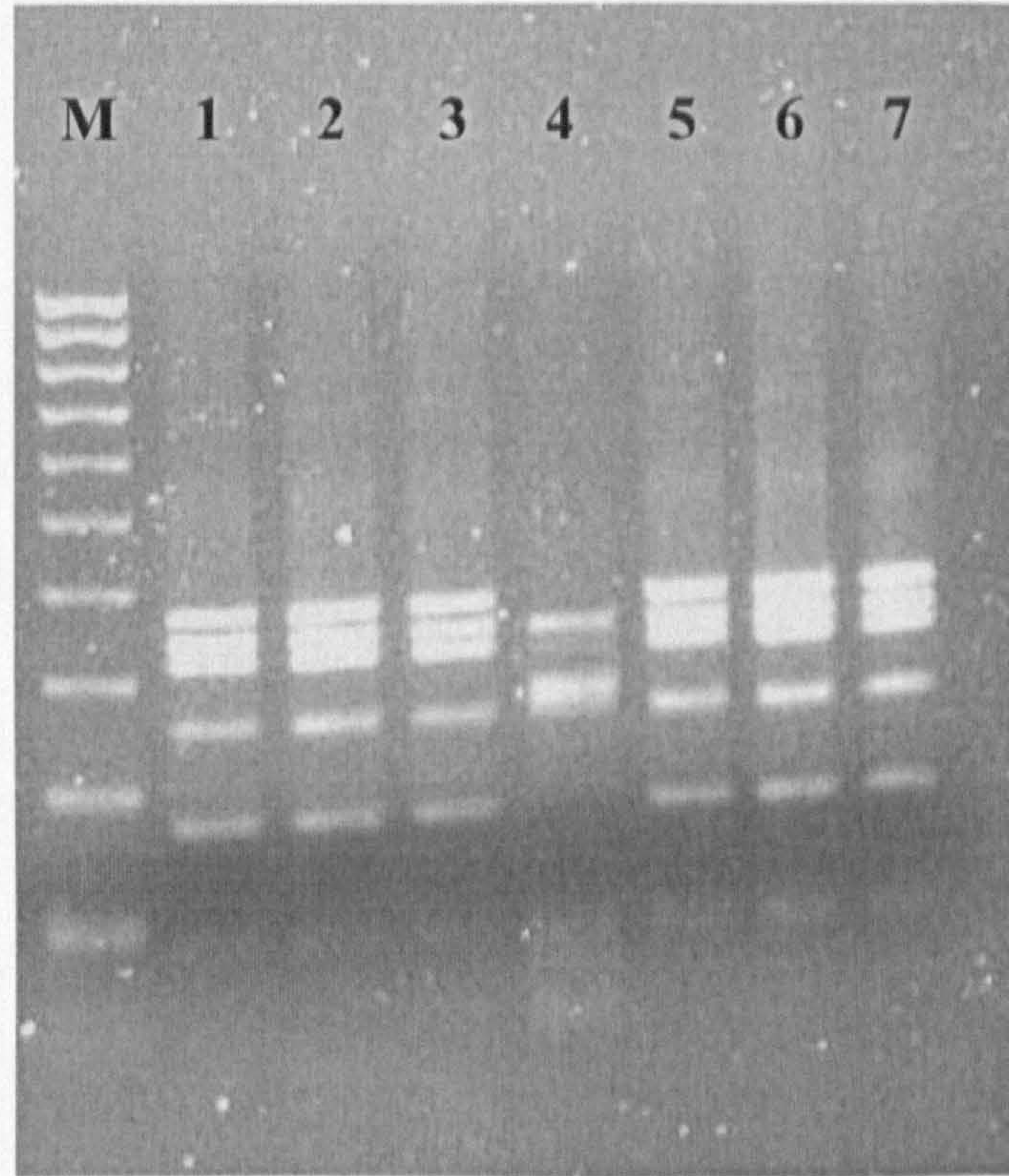


Figure 5.3.3 a) *Mnl*I digestion of clinical isolates from Subject CP. Lanes (1, 2, 3, 5, 6, 7), *L. fermentum*; (4), *L. casei*-group; (M), 100-bp DNA ladder.

Figure 5.3.3 b) Digestion of *L. casei*-group clinical isolates from CP with *Cfo*I

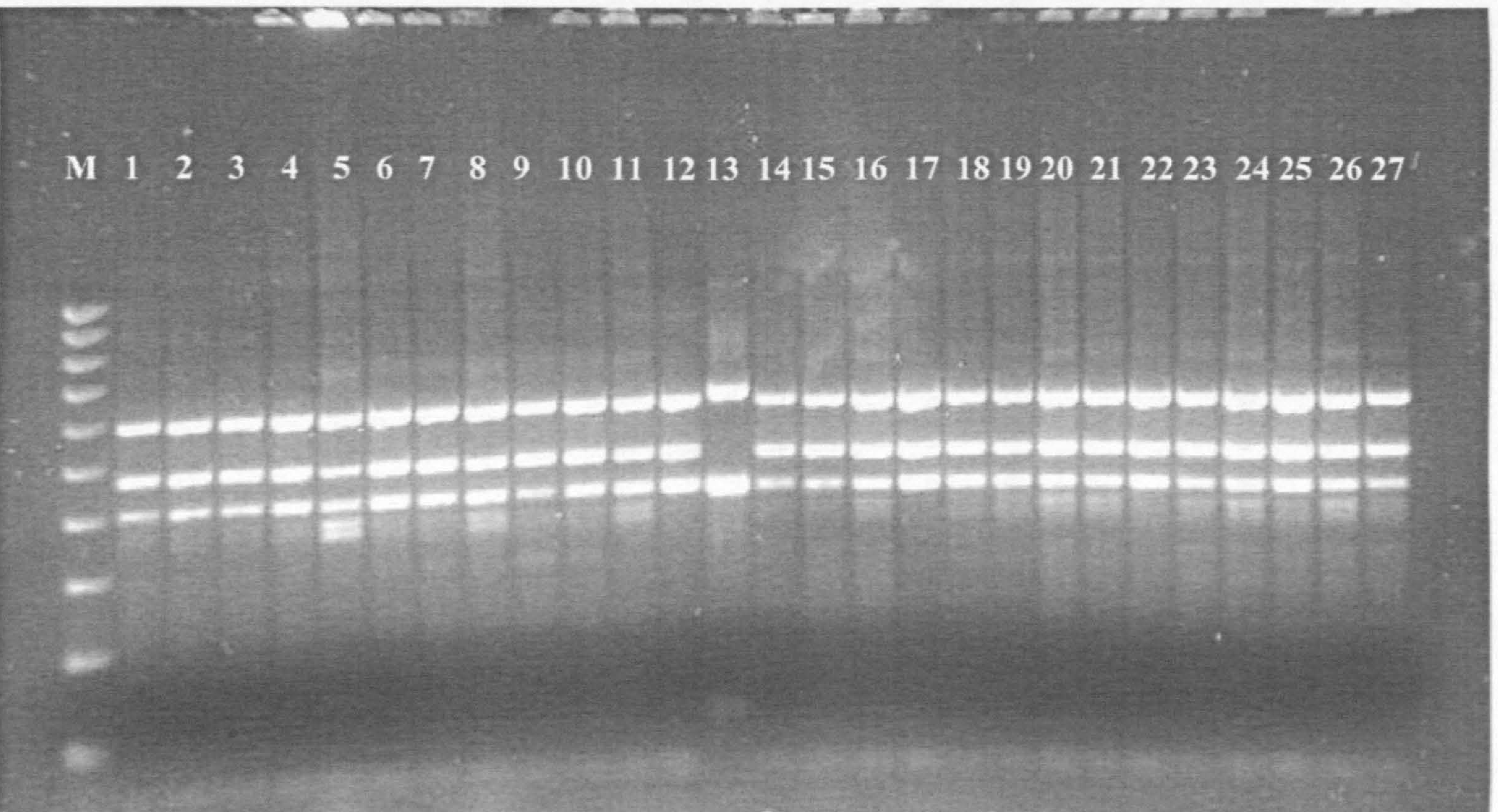


Figure 5.3.3 b) Digestion with *Cfo*I of *L. casei*-group isolates identified from Subject CP. Lanes (1-12, 14-27), atypical *L. casei/paracasei*; (13), *L. fermentum*; (M), 100-bp DNA ladder.

experiment (Exp. 2) is the control with no experimental beverage being consumed. The levels of *L. fermentum* decreased after the wash-out period (Week 1 Exp. 2) and did not increase dramatically over the six-week duration (Week 6 Exp. 2). Levels of *L. casei/paracasei* and *L. gasseri* remained relatively high with an increase apparent on the palatal sites (4, 5 and 6). The third experiment is the consumption of fluoridated milk once daily and *L. fermentum* is present in high numbers at the left buccal site (1) and in lower numbers on the right palatal site (4). *L. casei/paracasei* numbers remained high, although there was a slight decrease at two of the palatal sites. When comparing the consumption of fluoridated milk three times a day (Exp. 4), with milk alone three times a day (Exp 1), there was a noticeable decrease in the numbers of *L. fermentum* present in the oral cavity, although levels of *L. casei/paracasei* remained high. In the final experiment (Exp. 5), milk alone consumed once daily did not significantly alter the counts, given that there will be a natural increase in numbers due to plaque accumulation over the experimental period, although the counts are very high for *L. fermentum.*

5.4 Discussion

Although lactobacilli are regarded as one of the main aetiological agents implicated in dental caries (Loesche and Syed, 1973; van Houte *et al.*, 1981), there have been limited attempts to classify these bacteria and observe their behaviour over a period of time. The description in this study of a PCR-RFLP protocol for the discrimination of *Lactobacillus* species and the identification of clinical *Lactobacillus* isolates has given rise to the possibility of studying the carriage of lactobacilli by one individual over a long period of time.

The ability of RFLP analysis to distinguish between eight reference strains of *Lactobacillus* of oral origin was successfully demonstrated in this study. The profiles generated from these strains were then used to group isolates obtained from thirteen subjects. The clinical isolates had all been identified as *Lactobacillus* species by morphological and Gram characteristics. However, no attempt at speciating using biochemical methods was carried out due to cost and time limitations. The preparation of lysates of these clinical isolates was straightforward and was suitable for amplification with PCR. All the PCR products were subjected to digestion with the restriction enzyme *Mnl*I and further digestion with *Cfo*I was able to discriminate between *L. casei* strains. A large number of isolates grouped into five separate profiles that did not match any of the reference strains. A representative strain from each group was then selected and processed for DNA sequencing.

DNA sequencing of a number of the clinical isolates that were unable to be identified by RFLP analysis was successful. The identification of isolates representing *L.*

casei/paracasei was expected due to previous workers associating these organisms with active caries (Boyar and Bowden, 1985; Botha *et al.*, 1998). However, the identities of these strains could not be discriminated adequately due to the 97% homology between *L. casei* subsp. *casei* and *L. paracasei* subsp. *paracasei.* The identification of *L. gasseri* from the majority of these clinical isolates is quite surprising, as the literature does not suggest that this species is associated with the oral cavity. It appears to represent the major homofermentative *Lactobacillus* species that occupies the human gastrointestinal tract. It belongs to the *L. acidophilus* group, and within this group the species are not easily differentiated by classical methods. Most of the descriptions in the literature are related to *L. acidophilus. L. gasseri* is also utilised in the food industry, with particular reference to probiotics (Klein *et al.*, 1998). This suggests that it could be possible that this organism was present in these clinical isolates because the subjects were participating in a fluoridated milk study and therefore it was introduced via this vehicle. However, the representative isolate that was selected for DNA sequencing could have been an anomaly and therefore a larger number of isolates representing this group should be analysed before any definite association of *L. gasseri* with dental plaque is made.

The strains most commonly associated with active caries have been reported as being *L. acidophilus, L. paracasei, L. fermentum, L. plantarum, L. casei* and *L. rhamnosus.* The reference strains used in this study encompassed most of these species except for *L. paracasei* and *L. plantarum.* From the total of 216 isolates analysed by RFLP and DNA sequencing, strains representing *L. acidophilus* and *L. plantarum* were not identified. As mentioned above, *L. gasseri* is a member of the *L. acidophilus* group and therefore it is possible that this isolate has been misrepresented in this study. DNA sequencing had

been expected to result in the grouping of some of the isolates to *L. plantarum*, although this was not the case. This could be due to the fact that these subjects did not have any active caries and therefore *L. plantarum* was simply not present. The frequent isolation of *L. fermentum* and *L. casei/paracasei* is not unexpected and had the study been evaluating the progression of lesion formation on the enamel slab, there may have been an association with carriage of high numbers of these organisms with a more advanced lesion. None of the clinical isolates matched to reference strain *L. casei* subsp *casei* ATCC 393. This type strain did not originate from the oral cavity and Dicks *et al.* (1996) proposed its reclassification to *L. zeae*, however there is still some disagreement over this matter (Vásquez *et al.*, 2001).

Analysis of *Lactobacillus* strains isolated from one patient over a period of time, whilst exposed to milk and fluoride, was undertaken. The results of this are fairly inconclusive but some observations can be made. The emergence of *L. fermentum* after daily intake of milk over six-weeks suggests that milk was the main vehicle of delivery for this organism. The large reduction in numbers during the experimental protocol with no milk beverage consumption seems to support this theory. *L. fermentum* remained within the oral cavity after this time, and a decrease in numbers during consumption of fluoridated milk, three times daily, suggests that fluoride has exerted some effect on this organism. The inconsistency of isolation of some of the strains from this patient could be due to the lower levels of adhesion that lactobacilli have been shown to demonstrate (Harty and Knox, 1991). On most occasions within each experimental protocol, there is an increase in *Lactobacillus* numbers at Week 6. This is most likely to be due to the natural accumulation of plaque over time. *L. casei* strains seem to persist in the oral

cavity continuously and the data suggest that they are affected by the addition of fluoride in the diet. One point to be aware of is that Subject D was also rinsing with an additional fluoridated dentifrice and therefore was exposed to slightly higher levels of fluoride than some of the other subjects in this study. However, variations in fluoride intake generally exist within populations, with many people residing in areas with higher natural fluoride levels in water supplies. There is also the unknown intake of fluoride from other beverages and foods, which could have an additional effect.

There is the suggestion that mutans streptococci can be lost and then reappear at the same tooth site during a period of time (Redmo-Emanuelsson *et al.*, 2003). Due to the close association of lactobacilli with mutans streptococci, it seems reasonable to suggest that the same phenomenon could exist for *Lactobacillus* spp. The identification of *Lactobacillus* species from one subject over a prolonged period of time allows some preliminary observations to be made. There was not an apparent loss and reappearance of any particular species of *Lactobacillus*. The infrequency of isolation of individual strains makes any conclusions difficult regarding this question. This subject was also consuming controlled amounts of fluoride in their diet which would have affected the overall counts to some degree. Therefore, more research into this possible behavioural characteristic is required to help increase knowledge about *Lactobacillus* species within the oral cavity.

Characterisation of strains of *Lactobacillus* species with genotypic studies has been carried out over recent years. RAPD analysis (Tynkkynen *et al.*, 1999), RFLP analysis (Giraffa *et al.*, 2000), PFGE (Ferrero *et al.*, 1996), ribotyping (Ferrero *et al.*, 1996;

Tynkkynen *et al.*, 1999) and species-specific PCR (Tilsala-Timisjärvi and Alatossava, 1997; Alander *et al*, 1999) have all been reported. *Lactobacillus* species are of great importance in the food industry as they can improve food quality and maintain the microbiological balance of several habitats (Alander *et al.*, 1999). They are also of interest within the field of probiotics with *L. rhamnosus* GG and *L. acidophilus* being among the most widely used strains. Species-specific PCR for identification of these organisms is useful for identifying particular strains of interest within food preparations but has limited application when studying overall populations of *Lactobacillus* species. However, it does allow further analysis of particular species. Ferrero and colleagues (1996) used specific oligonucleotide primers to identify *L. casei* strains from which they generated genomic DNA fingerprints, separated by PFGE, and then carried out Southern blotting and hybridisation. Their results suggest that although fingerprinting did not give satisfactory evidence of polymorphisms among the strains, PFGE was useful for strain typing. This observation was supported by work carried out by Tynkkynen *et al.* (1999). However, this group of workers stated that PFGE is a laborious and expensive method, with only a limited number of samples able to be analysed.

The RFLP analysis carried out in this study has been shown to be a useful tool in distinguishing between *Lactobacillus* species from both reference strains and clinical isolates. In order to examine certain species within this group of organisms, it would be more pertinent to apply species-specific PCR to individual species and then carry out strain typing with either PFGE or ribotyping. However, for the purpose of examining the carriage of species across unrelated individuals and also from one individual over a prolonged period of time, RFLP has been proven to be successful.

CHAPTER 6: A NOVEL SPECIES-SPECIFIC PCR ASSAY FOR IDENTIFYING *LACTOBACILLUS FERMENTUM*.

6.1 Introduction

The members of the genus *Lactobacillus* are Gram-positive organisms that belong to the general category of lactic acid bacteria. They inhabit a wide variety of habitats, including vegetation and the gastrointestinal tracts of animals, and are used in the manufacture of fermented foods (Kandler and Weiss, 1986). They are also involved in the progression of caries lesions and have consistently been shown to be associated with caries in humans (Loesche and Syed, 1973; van Houte, 1980; Botha *et al.*, 1998). *Lactobacillus fermentum* is the major heterofermentative *Lactobacillus* species of the gut in man (Fons *et al.*, 1997), but it is also widely employed in industrial fermentation and as starter cultures in the dairy industry. Work carried out by Fitzgerald and colleagues (1981) demonstrated that strains of *L. fermentum* were cariogenic in conventional hamsters receiving high sucrose diets. They have since been isolated frequently from active caries lesions (Botha *et al.*, 1998). However, the majority of identification work on this organism has been carried out with regard to their involvement in the probiotics field.

The identification of *Lactobacillus* isolates by phenotypic methods is difficult because in several cases it requires determination of bacterial characteristics beyond those of popular fermentation tests (Kandler and Weiss, 1986). Identification of *L. fermentum* involves the isolation of a Gram-positive, non-spore-forming, catalase-negative, non-

motile organism. Commercial kits, such as the API 50 CHL system from bioMérieux, are also available to identify *L. fermentum* by enzymatic and sugar fermentation profiles. Since traditional methods can be time-consuming and give rise to ambiguous results, more rapid and accurate identification methods are required. Nucleotide base sequences of *Lactobacillus* 16S ribosomal RNA (rRNA) genes provide an accurate basis for phylogenetic analysis and identification (Vandamme *et al.*, 1996). The sequence obtained from an isolate can be compared to those of *Lactobacillus* species held in sequence databases. Although most of the species-specific sequences are contained in the first half of the 16S rRNA gene (V1-V3 region), identification is usually more accurate if the whole gene is sequenced (Tannock *et al.*, 1999). Universal probes or primers that will anneal to the genes that code for rRNA of all eubacteria can be designed from conserved regions of the 16S rRNA genes and species-specific primers or oligonucleotide probes can be designed from highly variable regions of 16S rRNA genes (Drake *et al.*, 1996).

Previously there have been several reports of species-specific probes for different lactic acid bacteria derived from 16S and 23S rRNA sequences (Hensiek *et al.*, 1992; Hertel *et al.*, 1993; Ehrmann *et al.*, 1994). There are no published works on the development of species-specific PCR primers for the detection of oral strains of *L. fermentum* from clinical specimens. The objectives of this study were to identify species-specific PCR primers that allow the detection of *L. fermentum* in clinical samples. The assay was applied to a variety of clinical specimens to investigate the incidence of *L. fermentum* within dentoalveolar abcesses and dental plaque.

6.2 Materials and Methods

6.2.1 Genomic DNA Extraction from Lactobacillus Reference Strains

Reference strains of the *Lactobacillus* spp were obtained from the NCTC (National Centre for Type Cultures, London, UK) and ATCC (American Type Culture Collection, Rockville, MD, USA). These cultures were reconstituted on Fastidious Anaerobe Agar (Life Technologies) supplemented with defibrinated horse blood (7.5% v/v) at 37°C for 2 days in an anaerobic chamber under an atmosphere of 85% N_2, 10% CO_2 and 5% H_2. Reference strains were harvested from the plates and genomic DNA was extracted using the Puregene DNA isolation kit (Novara Flowgen) as described in Chapter 2.

6.2.2 Preparation of Genomic DNA from Pus Samples

DNA was extracted from archival pus samples that had been obtained from patients with acute dento-alveolar abscesses. Fifty microlitres of each pus sample was diluted 10 to 100-fold in PCR diluent (10 mM Tris-HCl, pH 8.0, 10 mM NaCl, 1 mM EDTA). Thirty microlitres of SDS 10% and 3 µl of proteinase K (10 mg/ml) were added to a total volume of 300 µl of each diluted pus sample and incubated at 55°C for 3 hours. Each lysed sample was extracted twice with an equal volume of phenol:chloroform (1:1) and once with an equal volume of chloroform. DNA precipitation was performed by the addition of 0.1 volume of 3 M sodium acetate, pH 5.3, and 2 volumes of ethanol 100%, followed by mixing and incubation at -70°C for 30 minutes. The precipitated DNA was

collected by centrifugation and the pellet resuspended in 100 μl sterile, molecular-biology-grade water.

6.2.3 Preparation of Plaque Lysates

One hundred microlitre aliquots each of archival and fresh supragingival plaque samples were lysed with achromopeptidase (20 U/μl) in 10 mM Tris/HCl, 1 mM EDTA, pH7.0. These suspensions were incubated at 56°C for 30 minutes then boiled for 10 minutes. Each plaque lysate was stored at -80°C until required.

6.2.4 Design of Species-Specific Oligonucleotide Primers

Primers for use in the *L. fermentum* PCR assay were selected by the alignment of 16S rRNA gene sequences of *Lactobacillus* species and several other oral bacteria. Two primers that showed sequence specificity for *L. fermentum* at their 3' ends, compared with the corresponding regions of the other 16S rRNA genes analysed, were selected. The primer sequences were: 5′-AAT ACC GCA TTA CAA CTT TG-3′ (LF-1A; base position 203-223) and 5′-GGT TAA ATA CCG TCA ACG TA-3′ (LF-2; base position 539-519), which produce an expected amplification product of 337 base pairs. To confirm the specificity of the *L. fermentum* PCR assay, genomic DNA from each of the following *Lactobacillus* species was used as a template in a standard PCR: *L. fermentum* ATCC 14931, *L. acidophilus* NCTC 1723, *L. delbrueckii* NCTC 12712, *L. salivarius* ATCC 11741, *L. odontolyticus* NCTC 1406, *L. vaginalis* NCTC 12197, *L. casei* var *rhamnosus* (NCTC 6375, NCTC 10302) and *L. casei* subsp. *casei* ATCC 393. DNA

extracted from each of the following oral species was also used as a template in the PCR assay: *Helicobacter pylori* ATCC 43504, *Escherichia coli* NCTC 10418, *Prevotella intermedia* ATCC 25611, *Peptostreptococcus anaerobius* NCTC 11460, *Prevotella nigrescens* ATCC 25261, *Bacteroides forsythus* ATCC 43037, *Streptococcus mitis* NCTC 12261, *Candida albicans* NCPF 3093 and *Streptococcus intermedius* NCTC 11324.

6.2.5 PCR

All PCR reactions were performed in a total volume of 50 µl, comprising 5 µl of bacterial DNA template and 45 µl of reaction mixture containing 1x PCR buffer (10mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100), 1.0 unit of *Taq* DNA polymerase (Promega), 0.2 mM of each deoxyribonucleoside triphosphate and each primer at a concentration of either 0.2 µM or 0.8 µM. For increased sensitivity and specificity "hot start" PCR was used, whereby the primers are separated from other reaction components by a layer of wax (DyNAwax; Flowgen), therefore preventing the reaction from starting until the wax has melted upon commencement of thermal cycling. PCR was carried out in an OmniGene thermal cycler (Hybaid Ltd, Teddington, England). After an initial denaturation step at 94°C for 5 minutes, 35 cycles were carried out of denaturation at 94°C for 1 minute, annealing at 50°C or 55°C for 1 minute and extension at 72°C for 1 minute, followed by a final extension step at 72°C for 10 minutes. Strict anti-contamination procedures were employed when performing PCR. Negative and positive controls were included with each batch of samples that were analysed. A standard PCR reaction mixture containing 10 ng of *L. fermentum* genomic

DNA instead of sample was used as a positive control. The negative control contained water instead of sample. Five microlitres of each PCR product with 1 μl loading dye and 4 μl molecular-biology-grade sterile water was electrophoresed on a 2% agarose gel as previously described.

6.3 Results

6.3.1 Specificity and Optimisation of Primers

The specificity of the primer pair LF-1A/LF-2 was confirmed when a PCR product was obtained only with *L. fermentum* ATCC 14931 (Figure 6.3.1, Lane 1) and not with any of the other oral species that were used as templates in the standard PCR reaction.

Optimisation of this primer pair revealed that optimal yield of PCR product was obtained at a temperature of 50°C with both concentrations (0.2 μM and 0.8 μM) of primers (Figure 6.3.2, Lanes 2 and 16). However, weak, non-specific PCR products were generated with *L. casei* subsp. *casei* ATCC 393 and *L. odontolyticus* NCTC 1406 when the primers were used at a concentration of 0.8 μM (Figure 6.3.2, Lanes 10, 24 and 26). Consequently, the optimal PCR conditions employed throughout this study were a primer concentration of 0.2 μM and an annealing temperature of 50°C.

6.3.2 PCR Analysis of Clinical Specimens

The *L. fermentum* PCR assay was used to determine the prevalence of this species in archival supragingival plaque and pus aspirates obtained from subjects with acute dento-alveolar abscesses. *L. fermentum* DNA was not detected in any of the 36 plaque samples and 19 pus samples analysed [Figure 6.3.3 a) and b)]. In order to rule out the possibility of interference from inhibitors within these samples, a random selection of 5 plaque and 3 pus samples were spiked with *L. fermentum* ATCC 14931 (10 ng). This experiment

Figure 6.3.1 Specificity of the *L. fermentum* PCR assay

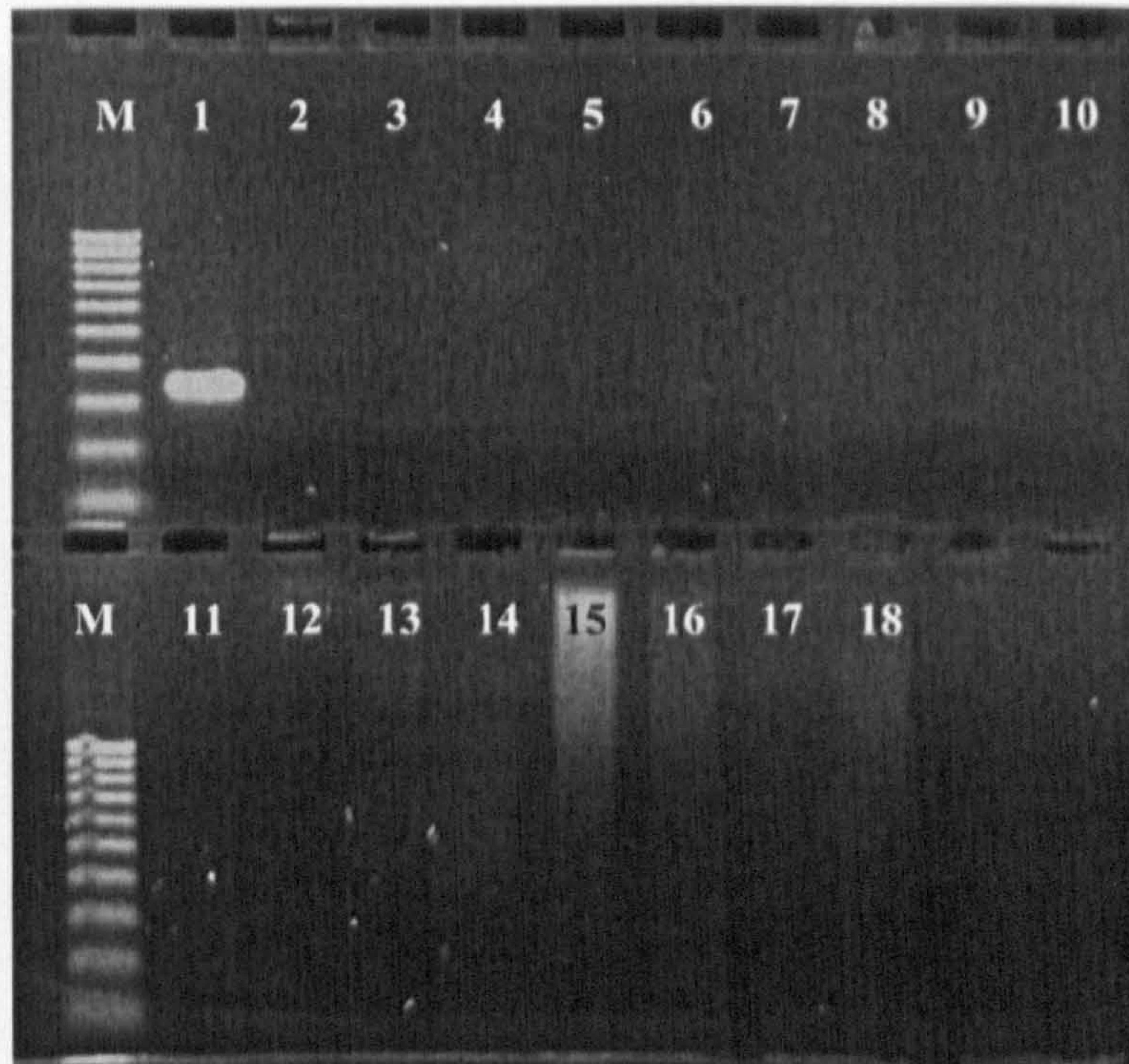


Figure 6.3.1 Specificity of *L. fermentum* primers LF-1A/LF-2. Lanes: (1), *L. fermentum* ATCC 14931; (2), *L. salivarius* ATCC 11741; (3), *L. delbrueckii* NCTC 12712; (4), *L. acidophilus* NCTC 1723; (5), *L. vaginalis* NCTC 12197; (6), *L. odontolyticus* NCTC 1406; (7), *L. casei* subsp *casei* ATCC 393; (8, 9), *L. casei* var *rhamnosus* NCTC 6375/10302; (10), *H. pylori* ATCC 43504; (11), *E. coli* NCTC 10418; (12), *P. intermedia* ATCC 25611; (13), *Peptostrep. anaerobius* ATCC 11460; (14), *P. nigrescins* ATCC 25261; (15), *B. forsythus* ATCC 43037; (16), *S. mitis* NCTC 12261; (17), *C. albicans* NCPF 3903; (18), *S. intermedius* NCTC 11324; (M), 100-bp DNA ladder.

Figure 6.3.2 Optimisation of the *L. fermentum* PCR assay

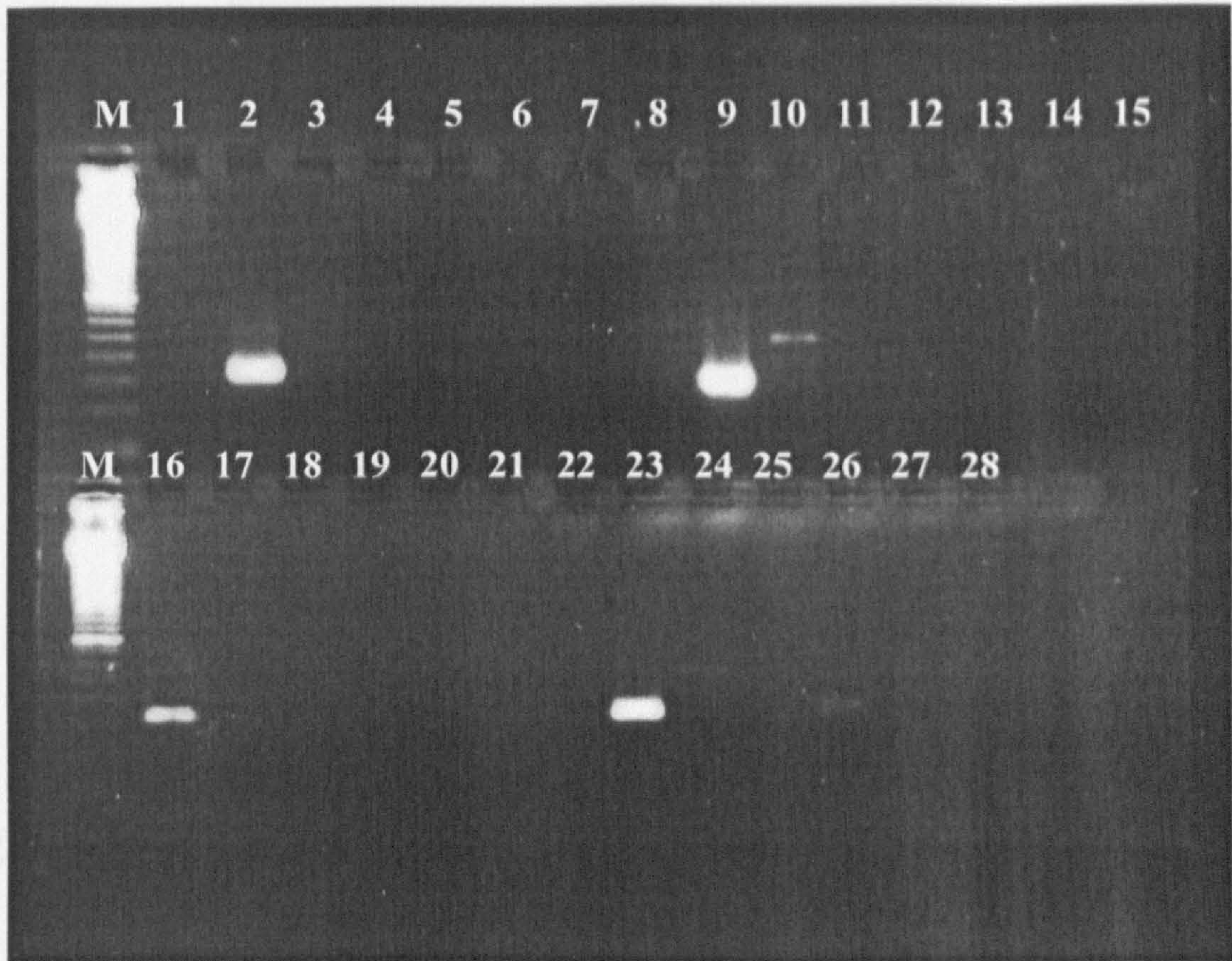


Figure 6.3.2 Optimisation of LF-1A/LF-2 primers. Lanes: (1-7), 50°C annealing temperature/0.2 μM primers; (8-14), 50°C annealing temperature/0.8 μM primers; (15-21), 55°C annealing temperature/0.2 μM primers; (22-28), 55°C annealing temperature/0.8 μM primers; (1, 8, 15, 22), *L. salivarius* ATCC 11741; (2, 9, 16, 23), *L. fermentum* ATCC 14931; (3, 10, 17, 24), *L. casei* subsp. *casei* ATCC 393; (4, 11, 18, 25), *L. acidophilus* NCTC 1723; (5, 12, 19, 26), *L. odontolyticus* NCTC 1406; (6, 13, 20, 27), *L. vaginalis* NCTC12197; (7, 14, 21, 28), *L. casei* var *rhamnosus* NCTC 10302; (M), 100-bp DNA ladder.

Figure 6.3.3 a) *L. fermentum*-specific PCR analysis of supragingival plaque samples

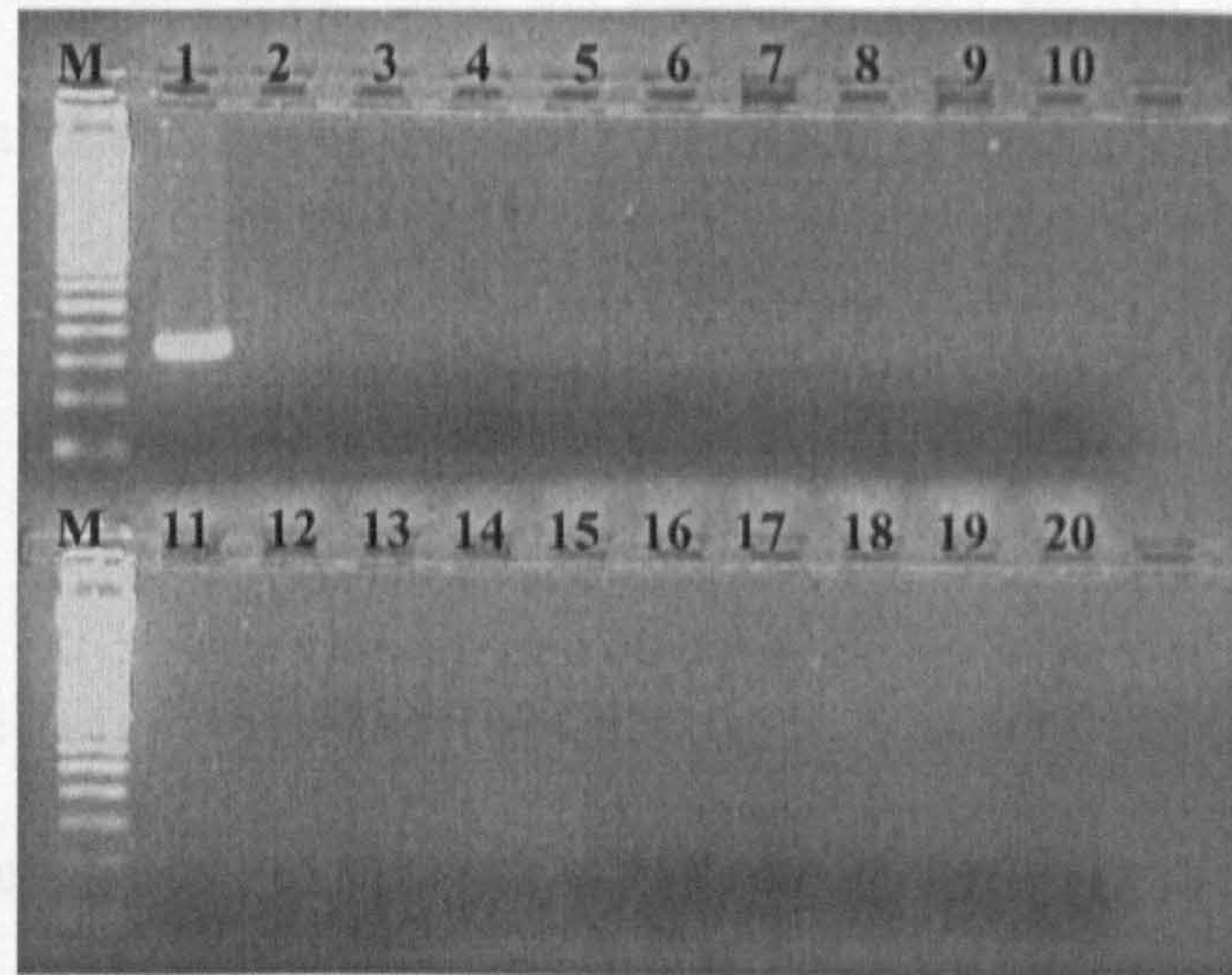


Figure 6.3.3 a) *L. fermentum*-specific PCR analysis of archival supragingival plaque samples. Lanes: (1), *L. fermentum* (positive control); (2-19), plaque samples; (20), negative control; (M), 100-bp DNA marker.

Figure 6.3.3 b) *L. fermentum*-specific PCR analysis of dento-alveolar pus samples

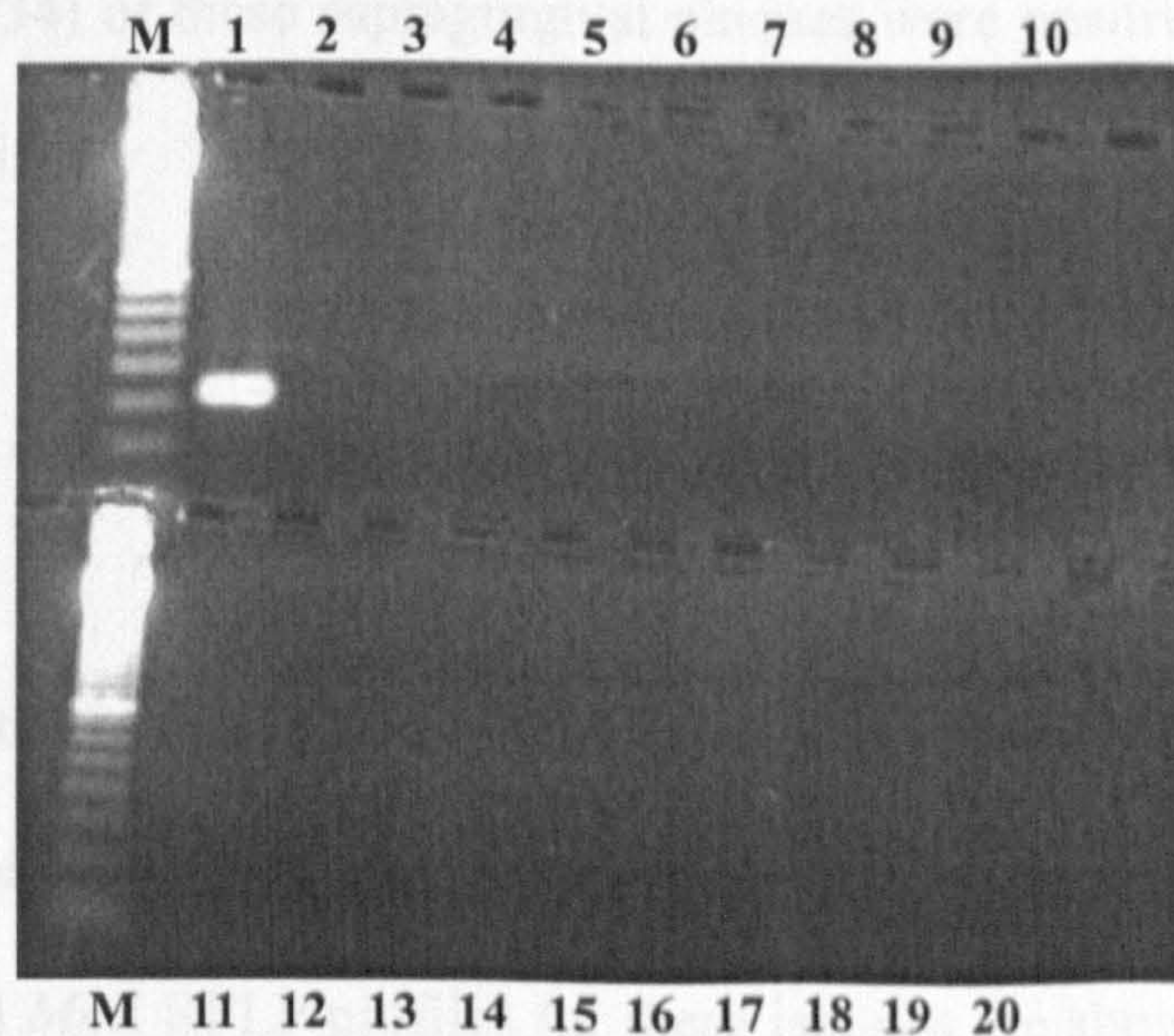


Figure 6.3.3 b) *L. fermentum*-specific PCR analysis of archival dento-alveolar pus samples. Lanes: (1), *L. fermentum* (positive control); (2-19), pus samples; (M), 100-bp DNA marker.

demonstrated that there were no inhibitors influencing the PCR assay [Figure 6.3.3 c)], since *L. fermentum* was detected when the samples were spiked and not in unspiked samples. The original observations are therefore valid.

6.3.3 Direct PCR on RFLP Positive/RFLP Negative Plaque Samples

Supragingival plaque samples recently collected as part of a fluoridated milk study were analysed for the presence of *L. fermentum*. Previous RFLP analysis (*see* Chapter 5) had identified 15 plaques samples from which *L. fermentum* had been isolated and its identity verified by RFLP analysis. Direct species-specific PCR analysis of these 15 plaque samples revealed three which contained *L. fermentum* [Figure 6.3.4 a) Lanes 8, 13 and 16]. Further analysis of 19 plaque samples originally negative for *L. fermentum* through culture techniques revealed a single positive sample [Figure 6.3.4 b) Lane 4]. Therefore, 11.8% (4/34) of these supragingival plaques were positive for *L. fermentum* by direct PCR analysis.

6.3.4 Direct PCR on Pure Isolates Identified as L. fermentum/Uncertain

In Chapter 5 of this thesis, RFLP analysis revealed four isolates that had profiles very similar to *L. fermentum* but which could not be classified as any other oral *Lactobacillus* species. The atypical *Mnl*I RFLP profiles for these isolates are shown in Figure 6.3.5a. In an attempt to clarify this classification, direct species-specific PCR was carried out on these four isolates and nineteen other isolates that had profiles corresponding to *L. fermentum*. A positive result was obtained for all nineteen samples that had been

Figure 6.3.3 c) *L. fermentum*-specific PCR analysis of plaque and pus samples spiked with *L. fermentum*

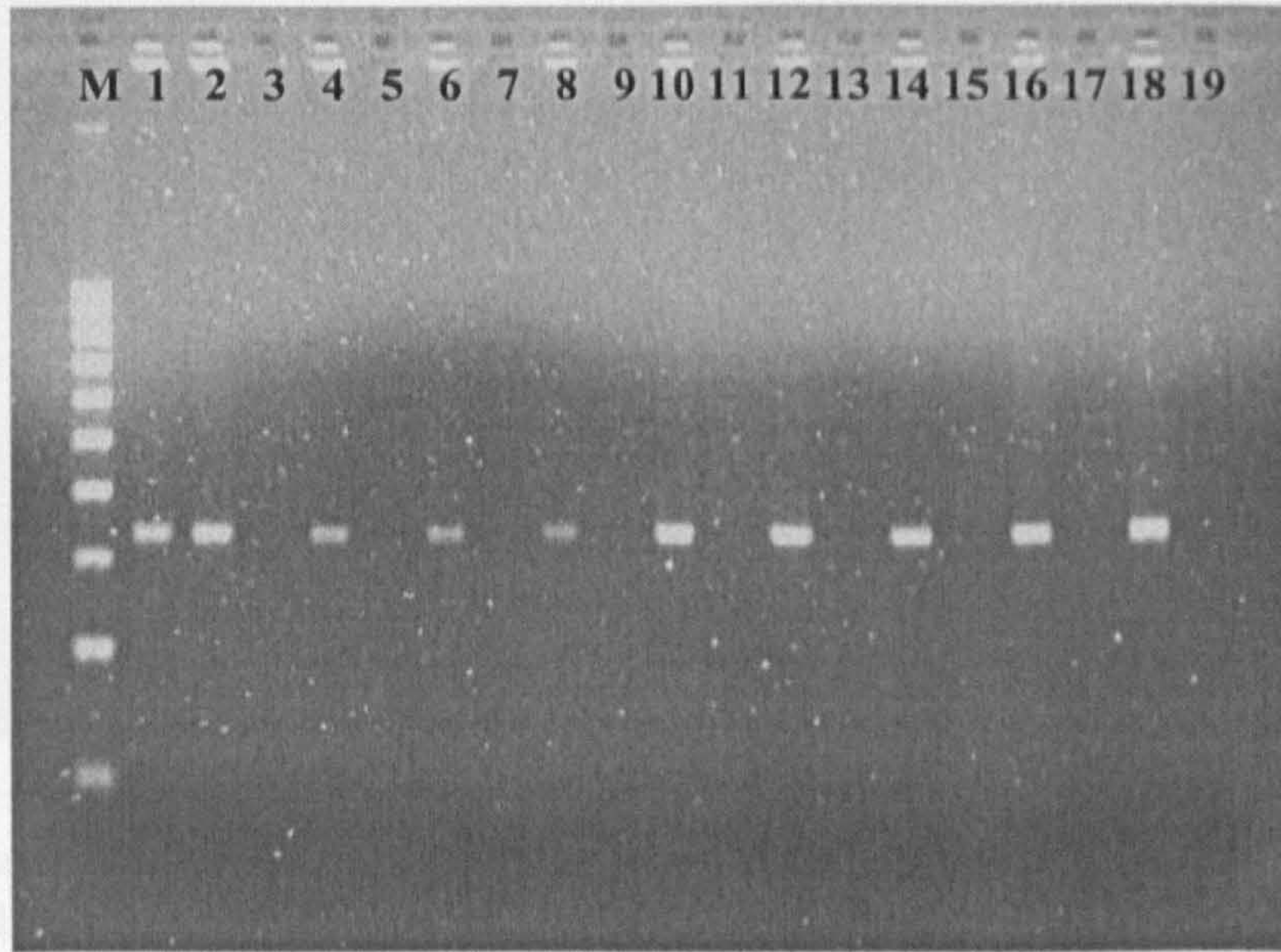


Figure 6.3.3 c) *L. fermentum*-specific PCR carried out on a selection of archival plaque and pus samples spiked with 10 ng of *L. fermentum*. Lanes: (1, 2), *L. fermentum* ATCC 14931; (3), plaque 1915; (4), plaque 1915 + *L. f*; (5), plaque 1937; (6), plaque 1937 + *L. f*; (7), plaque 1942; (8), plaque 1942 + *L. f*; (9), plaque 222; (10), plaque 222 + *L. f*; (11), plaque 248; (12), plaque 248 + *L. f*; (13), pus 96/227; (14), pus 96/227 + *L. f*; (15), pus 96/630; (16), pus 96/630 + *L. f*; (17), pus 96/867; (18), pus 96/867 + *L. f*; (19), negative control.

Figure 6.3.4 a) *L. fermentum*-specific PCR analysis of culture-positive plaque samples

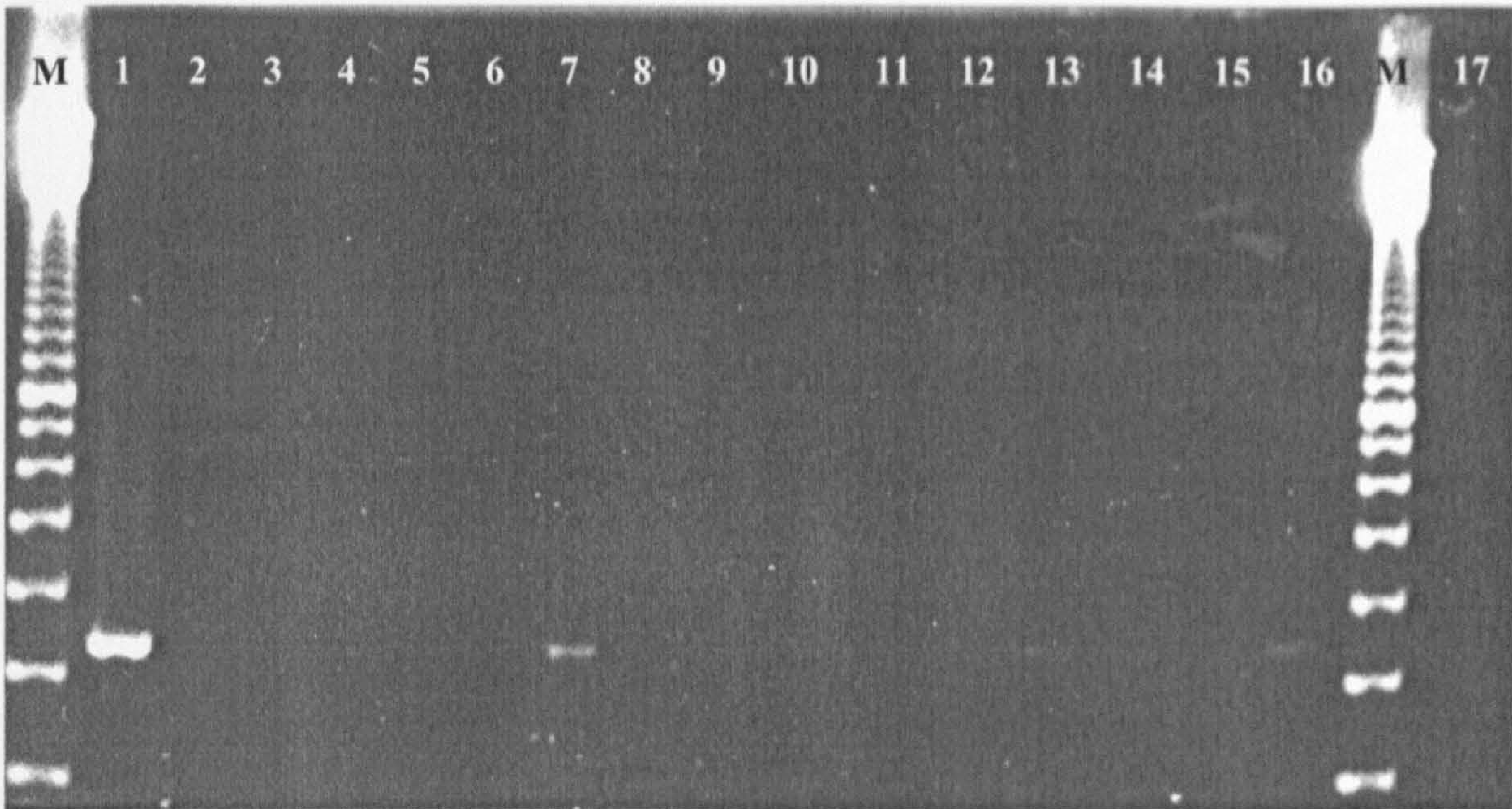


Figure 6.3.4 a) Detection of *L. fermentum* in 15 culture-positive plaque samples. Lanes: (1), *L. fermentum* ATCC 14931 (positive control); (2-16), clinical samples; (17), negative control; (M), 100-bp DNA ladder.

Figure 6.3.4 b) *L. fermentum*-specific PCR analysis of culture-negative plaque samples

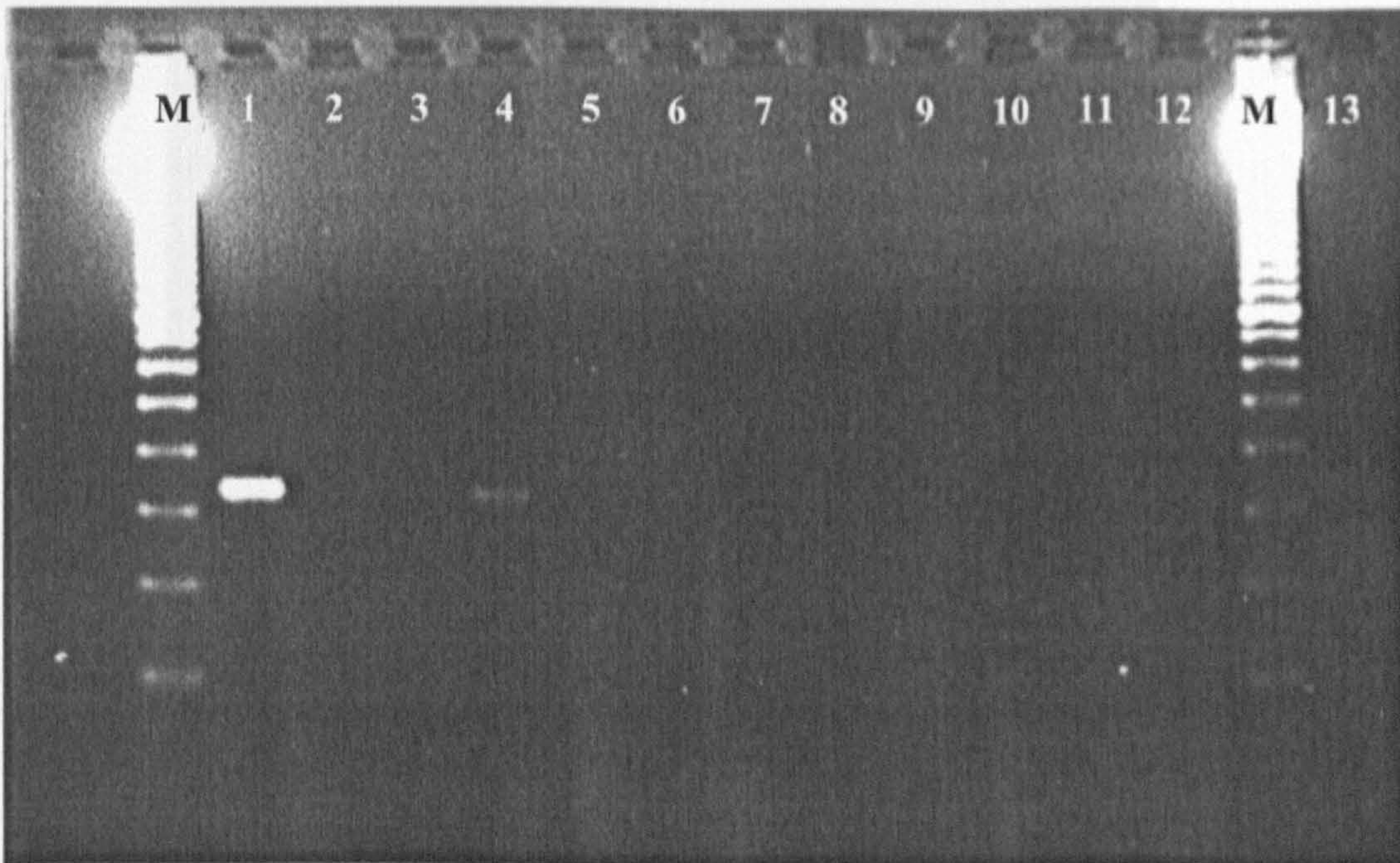


Figure 6.3.4 b) Detection of *L. fermentum* in 19 culture-negative plaque samples. Lanes: (1), *L. fermentum* ATCC 14931 (positive control); (2-12), clinical samples; (13), negative control; (M),100-bp DNA ladder.

Figure 6.3.5 a) RFLP profiles of unknown clinical isolates

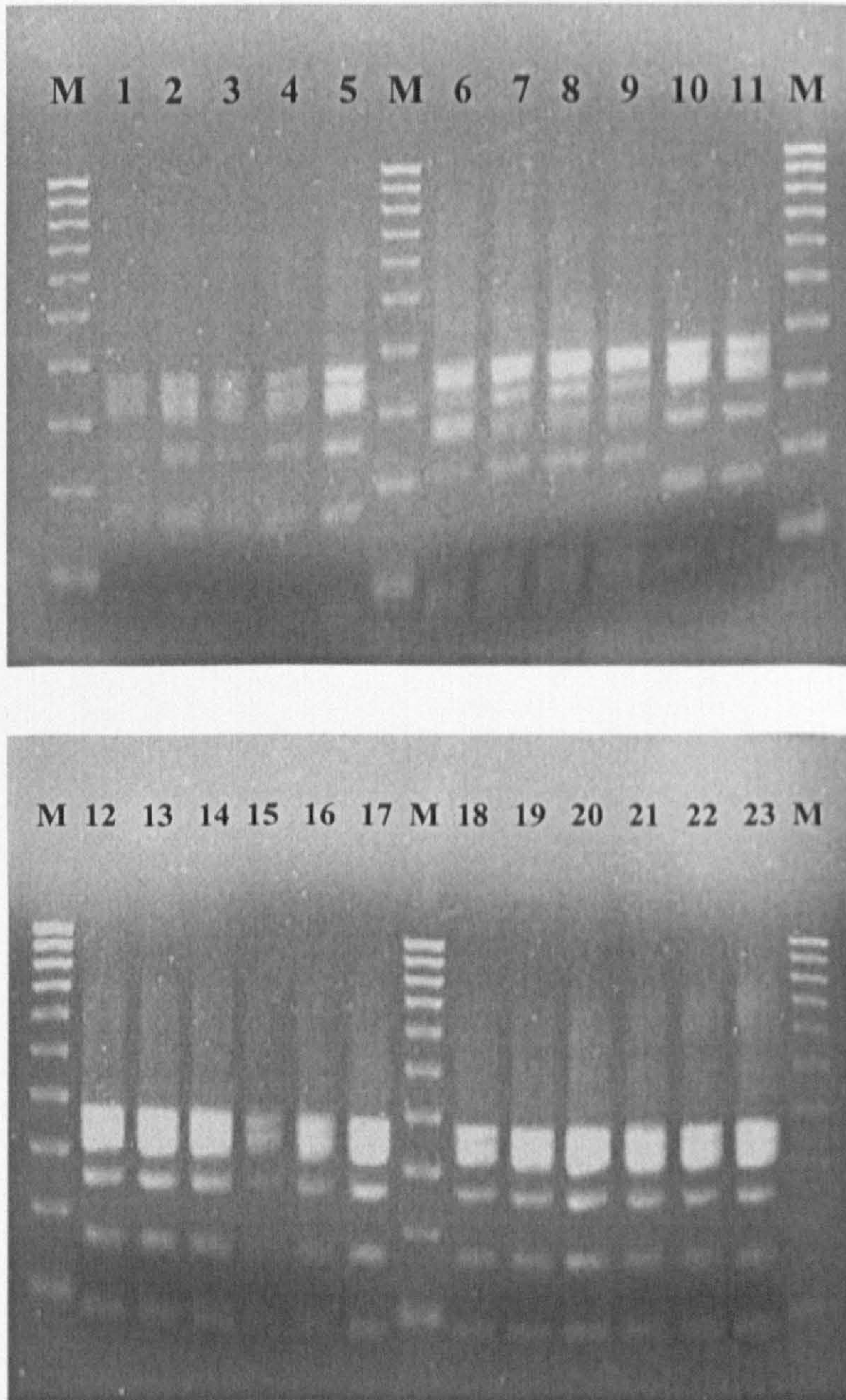


Figure 6.3.5 a) RFLP patterns generated by digestion of DNA from clinical isolates with *Mnl*I. Lanes: (1), *L. fermentum* ATCC 14931 (positive control); (2-5, 10-23), *L. fermentum* clinical isolates; (6-9), unknown clinical isolates; (M), 100-bp DNA marker.

confirmed as *L. fermentum*, but no PCR product was generated for the four unknown isolates [Figure 6.3.5 b)]. This confirmed that the unknown clinical isolates were not *L. fermentum*.

Figure 6.3.5 b) *L. fermentum*-specific PCR analysis of unknown clinical isolates

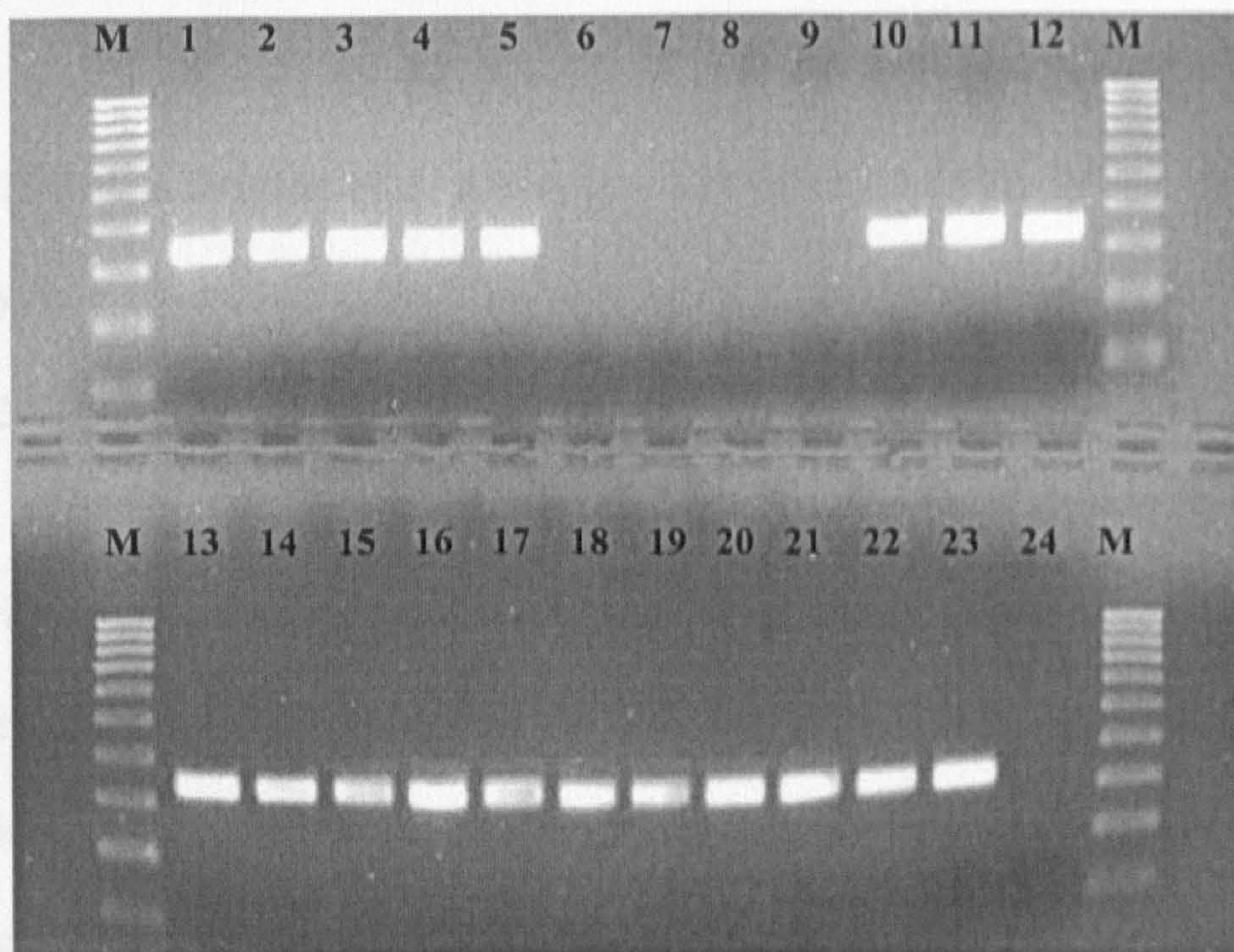


Figure 6.3.5 b) Analysis of unknown clinical isolates with the *L. fermentum*-specific PCR assay. Lanes: (1), *L. fermentum* (positive control); (2-5, 10-23), *L. fermentum* clinical isolates; (6-9), unknown clinical isolates; (24), negative control; (M), 100-bp DNA marker.

6.4 Discussion

The aims of this study were to develop a novel PCR assay that was specific for *L. fermentum* and apply this to the direct detection of *L. fermentum* DNA in clinical samples. The identification of *L. fermentum* and other members of this genus is based on carbohydrate fermentation profiles, morphology and Gram stain reaction. The API 50 CHL system, which utilises the characteristics of bacterial sugar fermentation and enzymatic activities, has been used for the identification of *L. fermentum*. However, these conventional methods often lead to ambiguous results and even misidentifications. Song and colleagues (2000) compared the results of API 50 CHL tests with DNA-DNA hybridisation and showed that only 30.2% of the *Lactobacillus* strains tested were correctly identified by the API 50 CHL tests.

Another problem with characterising oral members of the *Lactobacillus* genus is that strains found in the saliva are phenotypically close (Richard *et al.*, 2001). Discrimination between *L. rhamnosus* and *L. plantarum* has been recorded as difficult due to the small variation in phenotypic characters. Genotypic methods are becoming the most commonly used taxonomic tools for the characterisation of lactobacilli, although very few have been developed to enable more accurate identification of oral lactobacilli. As *L. fermentum* has been frequently identified from active caries lesions, this study set out to develop a rapid and reliable technique for the identification and detection of this strain in various oral clinical specimens.

A PCR assay that is specific for the detection of *L. fermentum* was developed and employed in this study. This assay was shown to be specific for *L. fermentum* as no other bacterial species, including others within the closely related *Lactobacillus* genus, were detected with the PCR primers designed. When clinical samples were analysed with the PCR assay, there was no detection of *L. fermentum* DNA in the archival supragingival plaque samples and pus aspirates from dento-alveolar abscesses. Therefore, *L. fermentum* does not appear to be an aetiological agent in dento-alveolar abscesses. RFLP analysis of supragingival plaque samples obtained from a recent fluoridated milk study suggested that *L. fermentum* was present in a number of these samples. The PCR assay was applied 34 of these specimens, of which 4 (11.8%) were positive for *L. fermentum* DNA. The low detection rate of *L. fermentum* in plaque samples which were known to harbour this organism, is unexpected and difficult to explain. Within the sampling environment there is an uneven distribution of bacteria. Vortex-mixing is carried out in an attempt to alleviate this problem, but an even distribution may not always be attained. One of the major advantages of molecular techniques, such as PCR, over conventional culture techniques is the ability to analyse smaller volumes of sample, but this may also be a drawback. When the plaque samples were processed for culture, a large volume of sample was required. This means that there is likely to be a greater chance of detecting the organism in the sample. Once all the microbiological sampling had been carried out, the samples were then stored for later molecular analysis. The volume of plaque remaining varied depending on earlier sampling techniques, which could have ultimately resulted in a sample that was deficient in many of the bacterial populations we would have expected. It may also be of interest

to investigate whether the species-specific PCR carried out on ten-fold serial dilutions of the plaque samples would increase the detection of *L. fermentum*.

Other molecular techniques have been applied in an attempt to identify *L. fermentum*, mainly from food products. Vogel *et al* (1994) utilised whole-cell protein patterns and 16S rRNA patterns to demonstrate the different phylogenic locations of *L. fermentum* and *L. plantarum*. Ribotyping (Rodtong and Tannock, 1993; McCartney *et al.*, 1996; Zhong *et al.*, 1998), pulsed field gel electrophoresis (McCartney *et al.*, 1996) and random amplified polymorphic DNA analysis (Veyrat, 1999; Nigatu, 2000) have all been applied to the identification of *L. fermentum*. Species-specific oligonucleotide probes have been applied to the successful identification of other important *Lactobacillus* species (Nakagawa *et al.*, 1994; Drake *et al.*, 1996; Tilsala-Timisjärvi and Alatossava, 1997; Yasui *et al.*, 1997; Lucchini *et al.*, 1998; Alander *et al.*, 1999; Chagnaud *et al.*, 2001). However, there are no studies that report the use of specific oligonucleotide primers with direct PCR for identifying *L. fermentum* and their application to clinical samples.

In conclusion, this study reports the use of a novel, species-specific PCR assay that can detect *L. fermentum* in clinical samples and that can be used to confirm the identification of isolates obtained from microbiological culture of clinical samples. The application of this assay could be useful in studies investigating the incidence of *L. fermentum* within the oral cavity, especially within saliva, and its influence on the caries process.

CHAPTER 7: GENERAL DISCUSSION

The micro-organisms implicated in the aetiology of dental caries have been extensively studied, with *Lactobacillus* species used as indicator organisms of caries since the early 1940's (Arnold and McClure, 1941; Becks *et al.*, 1944). However, since then the proposed importance of *Streptococcus mutans* in the caries process has relegated *Lactobacillus* species to a less prominent position. Much of the research into preventing dental caries has examined ways of inhibiting the action of *S. mutans* and rendering the organism less cariogenic. For example, studies have involved the inhibition of enolase, which is the main enzyme involved in the glycolytic process, or the inhibition of glucan production by targeting glucosyltransferases (Marsh and Martin, 1999). At one point, vaccination was thought to be the way forward, but unfortunately the initial vaccine prototypes cross-reacted with heart tissue. Since then, the antigens have been defined and purified, but side effects are still possible and therefore governing bodies are not keen to promote immunisation for a disease that is not life-threatening (Bowden, 1996).

Fluoride is one of the most promising agents in the fight against caries. Many studies have shown that delivering this compound through vehicles such as toothpastes, supplements, mouthwashes, water, salt and milk have all been able to reduce caries incidence and promote better oral health. However, dental caries still persists and brings misery to many young children and their parents with a great deal of unnecessary pain and suffering, not to mention the spiralling costs of dental care (Becker *et al.*, 2002). A solution to this problem is obviously required but there are many complex social and scientific barriers to be overcome, for example opposition to fluoridation of public water

supplies and general public mistrust of scientific findings. Studies are required to identify the best means possible of delivering fluoride to those most at risk from dental caries and also to determine the optimum concentrations, frequency of exposure and also the implications of fluoride intake on the plaque microflora, with particular reference to *S. mutans* and *Lactobacillus* spp.

The fluoridated milk study described in Chapter 3 attempts to answer some of the questions relating to each of the above points. This study was originally planned to form the bulk of this thesis, but unforeseen methodological problems encountered from the outset in the clinical component has resulted in greater emphasis being placed on molecular identification of the most important cariogenic micro-organisms, *S. mutans* and *Lactobacillus* spp. This chapter will attempt to address the problems that were encountered in the fluoridated milk study and will also discuss the various molecular identification techniques utilised in the ensuing chapters.

7.1 Methodological Problems in Chapter 3

As discussed in Chapter 3, it has been recognised that the overall nature of the fluoridated milk study was a limiting factor. This study involved investigation of the effects of fluoridated milk on both the demineralisation and remineralisation of artificially-created enamel caries lesions. The effect of the fluoride on plaque bacteria was also under investigation. The investigation into the influence of tooth site meant that it was impossible to study locations of interest using models such as partial dentures or intra-oral devices, therefore complete denture wearers were deemed the most

appropriate study group from all aspects. Thus, whilst the eventual target population for fluoridated milk is young children, the only population that this type of experiment could be carried out on was at the opposite end of the spectrum, i.e. mature adults/elderly. All considerations such as independent or dependent living, reduced salivary flow due to medications, and diet changes have indicated that the diagnostic implications of salivary bacterial counts could be different from those established for children and young adults (Loesche *et al.*, 1995). With this in mind, it was important to have strict selection criteria to ensure that external influences were kept to a minimum. Adequate salivary flow was important as low salivary flow rate leads to an increase in plaque and micro-organisms in saliva. In low salivary flow rates, the clearance time becomes prolonged and buffering capacity decreases leading to an increase in aciduric bacteria, which do not mimic the conditions within a younger persons mouth (Almståhl and Wikström, 1999). Good general health was also a necessary requirement, with certain medications viewed as exclusion criteria, due to their ability to reduce salivary flow rates. Unfortunately the implications of this type of study were particularly evident in the microbiological screening of the subjects. Although *S. mutans* and *Lactobacillus* spp. have been identified from denture plaque (Theilade *et al.*, 1983), their carriage is variable, especially between individuals. This became evident when screening potential participants, many of whom were not colonised with *S. mutans*. As a result, the original requirement for participants to be carrying *S. mutans* as well as lactobacilli was adapted to include the carriage of lactobacilli alone. Loesche *et al.* (1995) reported that *S. mutans* levels were higher in subjects that had partial dentures or one complete denture but also some natural dentition present, therefore, it had been hoped that the

incorporation of enamel slabs into the dentures may increase the chance of isolating *S. mutans*. Unfortunately, this did not occur and so the data on *S. mutans* were limited.

The decision to investigate the effect of site also proved to be a limiting factor, for two main reasons. The first was due to the large number of samples generated for the microbiological analysis. Every subject had plaque sampled from each of the six slabs, and per sample, seven primary plates (2 fastidious anaerobe agar, 2 Mitis-salivarius bacitracin and 3 Rogosa) were inoculated. After incubation, a maximum of three of these plates were required for enumeration of the bacteria. From the selective agar plates, a representative of each morphologically different isolate was subcultured for further identification by Gram stain and sugar fermentation tests. Each isolate was subsequently stored using the Protect system. This volume of work generated from one subject meant that the study number for this section of the project had to be limited to under half the original number (14/30). It also meant that there could only be a maximum of nine subjects reviewed in one week, to allow time for follow-up processing of the samples. Poor retention of the slabs was the second major limiting factor of site and as mentioned in Chapter 3, attempts were made to improve retention. Various cements were tested *in situ* and eventually embedding the slabs with acrylic was the most, although not entirely, successful approach. The resulting missing data contributed to the limitations on the statistical analysis.

The results that were obtained from this study were not entirely as predicted. The first hypothesis concerning the effect of different treatment protocols, was that subjects consuming fluoridated milk with an additional fluoridated dentifrice would have lower

bacterial counts and proportions of cariogenic micro-organisms than those not consuming fluoride. The effect of treatment was observed within the group of subjects participating in the treatment only protocols. No beverage consumption and milk with fluoride consumed three times a day had the lowest lactobacillus counts. However, no effect of treatment was seen in the treatment and dentifrice group. This may have been due to the fluoride-containing dentifrice applications masking other treatment effects. This observation may suggest that in those regularly brushing their teeth with a 1100 ppm F dentifrice twice daily, the effect on plaque counts of consuming fluoridated milk up to three times a day would be minimal. However, further study with larger subject numbers would be required to confirm this hypothesis. It is also possible that with a larger study number, the treatment effects that were observed within the treatment only group would have been more prominent, and therefore statistically significant. However, as has been discussed, a larger study number was impossible to achieve within this time-scale and so modifications would have to be made to the overall study design. Ideally, there should be more than one person working on the microbiological analysis, since the volume of plates is overwhelming and species identification can be very labour intensive. Storage of the isolates following identification was also time-consuming and for the purpose of this study alone, it is not really necessary. As a result of the retention problem of the palatal slabs, it may be more pertinent to focus on the buccal and labial slabs for the microbiological aspect of the study, to reduce interference caused by missing data and zero counts within the statistical analysis.

The second hypothesis was with respect to site, where it was anticipated that the site of the enamel slab would exert an effect on the counts. It was anticipated that plaque

collected from sites with low salivary flow, would have higher counts and proportions of acidogenic and aciduric bacteria, than sites with more rapid clearance of sugars and metabolic end products due to higher salivary flow rates. The six oral locations analysed were situated on the upper denture, two at buccal sites (Sites 1 & 3) with good salivary flow rates, and one labial (Site 2) and three palatal sites (Sites 4, 5 & 6) with poor salivary flow rates. Slabs at the three palatal sites were frequently dislodged and so the proportions of aciduric bacteria in the total plaque bacteria were eventually analysed at the buccal and labial sites only. The results were able to confirm the hypothesis to a certain extent. Analysis revealed that the buccal sites had similar proportions of aciduric bacteria but that the labial site had greater proportions than the left buccal site (Site 3). There was also an observed subject effect on the data, which is not entirely surprising and Monsenego (2000) also recorded intra-individual variations in bacterial counts. This researcher was investigating the presence of micro-organisms on the fitting complete denture surface and as a consequence of these variations they too had to adapt their analysis to account for this effect.

A complicating factor was identified when statistical analysis was carried out on the proportions of lactobacilli in the total counts. For a number of specimens, the lactobacillus counts were greater than the total counts, which caused the proportions to become negative in value. The lactobacilli should have grown well on the fastidious anaerobe agar but there seem to have been occasions where there could have been interference from an inhibiting factor. However, the growth of lactobacilli on the selective Rogosa medium is typically luxuriant and the numerical inconsistency may result from the comparison of counts across different growth media.

Overall, this study has attempted to address the main questions that were detailed at the start of this investigation. The study design was ambitious and unfortunately a large number of methodological problems that arose during its prosecution. Adjustments were made to overcome some of these difficulties but the validity of some of the data is questionable. The objectives set out regarding the effects of fluoridated milk were not fully achieved in this study and many of the questions remain unanswered. A more comprehensive insight into the effects of fluoridated milk with regard to plaque composition would have been desirable. Nevertheless, some useful data have been generated, which may help in the interpretation of results from the enamel demineralisation and remineralisation study which ran in parallel.

7.2 Molecular Microbiological Considerations

Despite the limitations imposed by the fluoridated milk study on the longitudinal microbiological analysis of populations of *S. mutans* and *Lactobacillus* spp., further analysis of selected clinical isolates of these two organisms using various molecular identification techniques was possible and forms the majority of the work presented in this thesis.

Chapter 4 describes the use of ribotyping to identify strain variation among isolates of *S. mutans* within participating subjects in the fluoridated milk study. Genetic differences amongst *S. mutans* isolates are already well documented and various methods have been established to study genetic heterogeneity, such as restriction endonuclease analysis (Kulkarni *et al.*, 1989) and restriction fragment polymorphism analysis (Colby *et al.*,

1995). The problem with both these techniques when analysing *S. mutans* is the complicated band patterns that are achieved, rendering the profiles difficult to interpret and variations almost impossible to visualise. However, Saarela *et al.* (1996) described the use of arbitrarily primed polymerase chain reaction (AP-PCR) to discriminate between *S. mutans* and *S. sobrinus* species. The technique was successful and reproducible but its discriminative power was slightly poorer than the ribotyping technique that they had described three years earlier (Saarela *et al.*, 1993). Their conclusion was that AP-PCR would be more suitable for epidemiological studies investigating large numbers of mutans streptococcal isolates. Further studies by Li and Caufield (1998) have applied AP-PCR to mutans streptococci in an attempt to carry out genotyping of bacterial strains from mother and child pairs and reported results comparable to those of chromosomal DNA fingerprinting.

When developing the protocol for investigating the *S. mutans* isolates collected from the fluoridated milk study, it was the discriminative power of ribotyping that made it the technique of choice. The ribotyping technique that Saarela *et al.* (1993) described utilised an rRNA probe to hybridise to the chromosomal digests and reduce the number of bands that are detected. These investigators had already determined that restriction enzyme *Hin*dIII gave the best visualisation of strain diversity and they recorded that plasmid pKK3535, which consists of the cloning vector pBR322 and the *rrnB* rRNA operon of *E. coli*, was able to hybridise to the restriction fragments. This earlier work permitted the development of the assay for use in this study.

An interesting observation from this work was the identification of particular gender-specific variations amongst genotypes of the seven subjects analysed. This finding may be worth investigating with a larger study number to see if it is a generalisable observation or was only apparent due to the small study number and the different oral environment provided by that of denture-wearers. As stated earlier, *S. mutans* carriage varies in denture wearers and epidemiological studies have not been carried out on isolates from this source. The majority of work carried out in a similar area involves the intrafamilial relationships of *S. mutans* genotypes, with the bulk of strains isolated from children identifying closely to that of the mother and not of the father (Davey and Rodgers, 1984; Li and Caufield, 1995; Kozai *et al.*, 1999). The present, small-scale study suggests that there could be sharing of genotypes within unrelated female subjects. More extensive work in this field may be useful to determine whether genotype carriage is conserved within females since the reasons behind this phenomenon could provide greater insight into how strains of *S. mutans* are acquired.

Another interesting aspect of this particular investigation was the suggestion that *S. mutans* genotypes can be 'lost' then reappear later at the same site. This was observed in a number of subjects over the course of the study. The growth of mutans streptococci on the teeth is localised as they may occur on one or several tooth surfaces, but not in others, within the same oral cavity (Lindquist and Emilson, 1990). Transmission of the organism from one tooth surface to another does not readily occur. Stability of colonisation by mutans streptococci in the mouths of children has been shown in numerous studies, although some genotypes altered in prevalence (Caufield and Walker, 1989; Kulkarni, *et al.*, 1989; Alaluusua *et al.*, 1994; Redmo-Emanuelsson and

Thornquist, 2000). Research has not yet clarified how many mutans streptococcal genotypes may colonise a distinct tooth site and if a particular genotype persists on a site over a period of time (Redmo-Emanuelsson *et al.*, 2003). The data presented in Chapter 4 suggests that *S. mutans* does have the ability to disappear and then reappear at the same site over an extended period of time. Longitudinal investigations into the conditions which lead to the loss of *S. mutans* could possibly be advantageous in the fight against caries.

Microbiological identification of clinical isolates of *Lactobacillus* species was only carried out to genus level in Chapter 3, due to the cost of identifying to species level with commercial enzymatic and biochemical kits as well as the documented inaccuracy of these identification methods. Botha *et al.* (1998) carried out a series of biochemical tests and fermentation patterns to identify homo- and heterofermentative lactobacilli within a caries group and a control group of subjects. A total of 153 confirmed lactobacilli were isolated and 15 characteristics were analysed for each isolate. Although the authors were confident with this technique, it is obviously a time-consuming exercise, especially when analysing a large number of isolates. There is also the issue of reader interpretation when examining colour changes and one person may produce a different profile from another researcher for the same isolate. Nigatu (2000) published results on the evaluation between the discriminative power of API 50 CH fermentation profiles and randomly amplified DNA (RAPD) analysis. The results revealed a disagreement between the RAPD and API 50 CH data, which displayed the reduced accuracy of the phenotypic procedure to clearly identify *Lactobacillus* isolates.

Restriction endonuclease analysis (REA) was one of the first molecular identification techniques described for the typing of bacterial species. This technique involves the digestion of chromosomal DNA to generate banding profiles for analysis. Unfortunately this generates large numbers of bands within the profiles which makes strain discrimination complicated and unreliable. Subsequently more refined methods have been developed for strain typing and identification which result in profiles with fewer bands. RAPD involves the random priming of usually one short oligonucleotide primer (8-10 base pairs in length) which results in a smaller more manageable number of bands than REA. RAPD analysis is becoming a rapid and relatively cheap genetic tool for the identification of lactic acid bacteria (LAB) found in the food industry (Dykes and Von Holy, 1994; Du Plessis and Dicks, 1995). However, there are concerns over the reproducibility of RAPD profiles, for example between different laboratories with different PCR machines. Restriction fragment length polymorphism (RFLP) analysis involves digestion by restriction enzymes but this technique is generally carried out on a specific region of the genome, usually a gene such as the 16S rRNA gene. This technique detects base changes in a sequence that results in the gain or loss of a restriction enzyme recognition site. RFLP analysis has been applied to identify a wide range of bacteria, including oral species such as *Actinobacillus actinomycetemcomitans, Haemophilus aphrophilus, Haemophilus paraphrophilus* and *Peptostreptococcus* spp. (Riggio and Lennon, 1997; 2003).

Approximately sixteen hundred isolates were identified and stored during the course of the fluoridated milk study. Only a small proportion (12.5%) of these was analysed further with the PCR-RFLP protocol described in Chapter 5. The ability to discriminate

between *Lactobacillus* species was demonstrated with reference strains initially, but subsequent application to clinical isolates also allowed species determination. The use of restriction enzymes *MnlI* and *CfoI* produced a small enough number of bands to allow variation to be observed, but not too many to cause confusion when analysing the profiles.

The PCR-RFLP assay proved to be a useful and reliable tool for species determination within dental plaque and the use of DNA sequencing to identify isolates that did not match any of the reference strains was also advantageous. Sequencing is more time-consuming and costly than other identification methods, but is the most discriminatory and consequently more accurate method for identification and typing of bacterial strains. The use of PCR-RFLP was easier and less time-consuming than sequencing and proved to be a reliable identification method since the reference strains were available for cross-referencing. The research carried out by Botha *et al.* (1998) identified *L. paracasei* and *L. rhamnosus* as the predominant lactobacilli in carious lesions and their control subjects. The PCR-RFLP protocol and DNA sequencing also described *L. paracasei* as one of the most frequently isolated species from dental plaque, although discrimination between *L. casei* and *L. paracasei* could not be made. Ward and Timmins (1999) described specific primers for the discrimination of the three strains in the *L. casei* group, which could be applied here to identify these strains accurately.

The present study suggests that *L. gasseri* was also a common isolate. *L. gasseri* belongs to the *L. acidophilus* group and reports on the carriage of this organism in the oral cavity seem to vary. Boyar and Bowden (1985) frequently isolated *L. acidophilus*

from carious dentine but Botha *et al.* (1998) isolated it in small numbers. Further investigations of the isolates identified as *L. gasseri* from this study could be made by PCR-RFLP analysis of the reference strain for *L. gasseri* and possibly further sequencing of a larger number of representative isolates. Lucchini *et al.* (1998) describe a primer pair for detecting *L. gasseri* in faecal samples, therefore this protocol could be applied to the conformation of the identity of the *L. gasseri* oral isolates. The overall conclusion from this study is that PCR-RFLP could prove to be useful for determining the prevalence and frequency of the most cariogenic *Lactobacillus* species within the oral cavity and longitudinal effects of external factors such as fluoride or probiotic-containing foods could be monitored.

The use of PCR-RFLP is applicable to study the prevalence and identification of *Lactobacillus* spp. across a large number of species. However, identification of particular species can be achieved by designing species-specific probes and primers based on 16S and 23S rRNA gene sequences (Hensiek *et al.*, 1992; Hertel *et al.*, 1993; Ehrmann *et al.*, 1994). *L. fermentum* has previously been described as one of the most predominant heterofermentative *Lactobacillus* spp. in active caries lesions (Botha *et al.*, 1998). Chapter 6 describes a rapid and reliable method for the identification of this organism. As mentioned earlier, Ward and Timmins (1999) designed primers which, when used in conjunction with primers to the conserved regions of the 16S rRNA gene, gave a specific amplification fragment from each of the species in the *L. casei* group. This assay was applied to 63 isolates identified from cheese as belonging to the *L. casei* group by sugar fermentation profiles. The 63 isolates grouped to either *L. paracasei* or *L. rhamnosus*. Many other studies have documented the use of one species-specific

primer and one general bacterial primer in the identification of many *Lactobacillus* spp. from dairy products to human intestinal flora (Drake *et al.*, 1996; Daud Khaled *et al.*, 1997; Berthier and Ehrlich, 1998; Song *et al.*, 2000; Chagnaud *et al.*, 2001). In order to identify *L. fermentum* from clinical isolates, two PCR primers specific for *L. fermentum* were designed from the comparison of 16S rRNA sequences of *Lactobacillus* spp. and other oral micro-organisms, and analysis showed that they were able to identify samples containing this particular species. Further analysis of plaque samples that were originally culture-negative for *L. fermentum* revealed a small number of positive plaque samples. This was an expected finding, as PCR is more specific and sensitive than conventional culture methods. However, an unexpected observation was that the species-specific PCR assay was unable to identify *L. fermentum* in a number of plaque samples that had already proved positive through culture and PCR-RFLP analysis. A tentative theory suggesting that sampling anomalies may be a factor has been given in Chapter 6. Overall, the species-specific PCR assay was rapid and reliable for the identification of *L. fermentum* isolates identified from conventional culture techniques of dental plaque samples. This assay could be used in the identification of this organism when isolated by culture from a variety of specimens, including food products where they are important organisms in starter cultures.

The studies presented in this thesis highlight the logistical and technical difficulties that can be encountered when attempting to carry out a clinical trial and also the advantages of employing molecular techniques for identifying and subtyping two of the most cariogenic micro-organisms. Utilisation of such molecular techniques in future clinical studies may eventually lead to a greater understanding of dental caries and elucidate some of the underlying mechanisms of this common oral disease.

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