The Effect of Fixed Orthodontic Brackets on the Bacterial Composition of Dental Plaque in Adolescents

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Dental Science (Orthodontics)

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Abstract

BACKGROUND: Demineralisation around orthodontic brackets is a considerable risk of orthodontic treatment. It can occur very early during treatment and involve a large number of teeth with aesthetic and dental health consequences. Early detection of demineralisation is very important in terms of treatment and prevention.

The presence of bacteria is one of the requirements for demineralisation to take place and it is unclear whether the type of bacteria in the plaque or the quantity of certain types of bacteria are significant influences on demineralisation.

AIM: To identify any general changes in the bacterial composition of dental plaque in adolescents undergoing fixed orthodontic appliance therapy. A secondary aim is to determine the incidence of white spot lesion development and if this was related to the identified red fluorescent plaque.

STUDY DESIGN: Prospective longitudinal cohort study.

METHODS: Fourteen 11 to 23 year old consecutive patients attending the Orthodontic Department of Liverpool University Dental Hospital were recruited for this study. Toothcare^{TM} and QLF were used to identify red fluorescent plaque and enamel demineralisation on the labial surfaces of the anterior teeth before and after the placement of fixed orthodontic appliances. The bacterial composition of the red fluorescent supragingival plaque was determined by DNA extraction, polymerase chain reaction amplification of the 16r rRNA gene and denaturing gradient gel electrophoresis.

RESULTS: The incidence of white spot lesions was recorded as 4.2% of the total surfaces of the teeth included as detected with QLF, the development of white spot lesions was not associated with the presence of red fluorescent plaque. There were differences in the bacterial composition of red fluorescent supragingival plaque in terms of *P. gingivalis, S. mutans* and *S. gordonii* between different participants, although changes in the plaque composition between visits for the same participant was not significantly associated with the development of white spot lesions in adolescents.

CONCLUSIONS: With the limitations of this study, the placement of fixed orthodontic brackets in adolescents does not significantly change the bacterial composition of red fluorescent supragingival plaque around the brackets and that the development of white spot lesions cannot be correlated to red fluorescent plaque alone.

Chapter 1

Introduction

The development of enamel demineralisation known as white spot lesions adjacent to orthodontic brackets remains a significant clinical problem in orthodontics (Årtun and Brobakken 1986; Gorelick et al., 1982; Mizrahi 1983; Øgaard et al., 1988). The overall prevalence of white spot lesions amongst orthodontic patients has been reported to be anywhere between 2 and 96 % (Årtun and Brobakken 1986; Gorelick et al., 1982; Mizrahi 1982; Mizrahi 1983; Øgaard et al., 1988; Zachrisson and Zachrisson 1971). This large variation can be the result of the different methods used to assess and score the presence of decalcification, type of orthodontic appliance used (bands or bonds), whether idiopathic enamel opacities were included or excluded, and if any fluoride regime was used during treatment. The formation of these lesions has been related to the prolonged retention of bacterial plaque, which is due to inadequate oral hygiene. Enamel demineralisation around the brackets (white spot lesions), can be seen as early as four weeks following placement of fixed orthodontic appliances (O'Reilly and Featherstone 1987; Øgaard et al., 1988). Fixed orthodontic appliances create new plaque retentive areas and present an oral hygiene challenge for the patient. This leads to an increase in the volume and number of bacteria within dental plaque (Huser et al., 1990; Rosenbloom and Tinanoff 1991).

1.1 Dental caries

According to the World Health Organisation dental caries is one of the most prevalent chronic diseases in the world and is the primary cause of oral pain and tooth loss. An estimated five billion people worldwide had experienced dental caries, and its treatment is estimated to account for 5-10% of health costs in industrialised countries (Petersen 2003).

Dental caries is the localised destruction of susceptible dental hard tissues by acidic by-products from bacterial fermentation of dietary carbohydrates. It is a multifactorial disease that starts with microbiological shifts within the complex biofilm and is affected by salivary flow and composition, exposure to fluoride, consumption of dietary sugars, and by preventive behaviours such as teeth cleaning (Selwitz et al. 2007). It was described as a chronic disease that progresses slowly in most individuals and in the absence of treatment can progress from initial loss of mineral at the ultra structural level to total destruction of tooth structure. Caries lesion development is not continuous, but a highly dynamic series of alternating periods of progression and arrest/regression (Takahashi and Nyvad 2008).

1.2 Pathogenesis

Dental caries lesions result from a shift in the ecology and metabolic activity of the biofilm, whereby an imbalance in the equilibrium between tooth mineral and biofilm fluid has developed (Selwitz et al., 2007). Dental caries develops where microbial deposits are allowed to form biofilms that are not frequently removed or

disturbed by mechanical wear (mastication, attrition, abrasion from brushing, flossing or toothpicks).

The plaque biofilm is a prerequisite for caries lesions to occur. It is characterised by continued microbial activity resulting in continued metabolic events in the form of minute pH fluctuations. Dental caries lesions are the product of innumerable metabolic events in biofilms, which have covered the tooth surface. When this outcome results in a cumulative loss of mineral from the tooth of such a magnitude, the porosity in the enamel gives rise to a decrease in enamel translucency. This early stage in enamel lesion formation will manifest itself as a white-spot lesion. The shape of the lesion reflects where the biofilm has been allowed to grow and remain for prolonged periods.

1.3 The role of dental biofilm in the aetiology of dental caries

Dental plaque is a general term for the complex but organised cooperative community of microorganisms that develops on the tooth surface, embedded in a matrix of polymers of bacterial and salivary origin. It forms via an ordered sequence of events, resulting in a structurally and functionally organised species rich microbial community (Marsh 2004).

For many years, there were two main schools of thoughts on the role of plaque bacteria in the aetiology of dental caries. Firstly, the specific plaque hypothesis proposed that, out of the population of organisms comprising the resident plaque

microflora, only a single or very small number of species were actively involved in the disease (Marsh 1994).

Many studies have suggested that *Streptococcus mutans* are the major group of bacteria associated with human dental caries. That is mainly due to their frequent isolation from cavitated carious lesions, their highly acidogenic and aciduric nature, their ability to produce water insoluble glucans and their capability of inducing caries formation in animals when fed a sucrose rich diet (Takahashi and Nyvad 2008). It was believed that dental caries is a specific treatable infection and that mutans streptococci and lactobacilli are responsible for the majority of human dental decay (Loesche 1986). *Streptococcus mutans* colonise the fissures of the teeth soon after they erupt and that inevitably causes decay unless other bacteria occupy the fissure depths, and then there is a possibility that decay will not take place or at least be greatly reduced.

A systematic review by Tanzer examined the evidence concerning microbial causes and associations with dental caries in humans. The included studies comprised of 25 interventional randomised clinical trials and 79 prospective and retrospective longitudinal and case control studies. The majority of these studies support a strong positive statistical association of mutans streptococci with carious lesions. The overall conclusion of the review suggested a central role of mutans streptococci in the initiation of caries on smooth surfaces and crown fissures of teeth (Tanzer et al., 2001).

In contrast, the non-specific plaque hypothesis considered that disease is the outcome of the overall activity of the total plaque microflora, so not only the bacteria that produce acid is the causative agent but also species that produce alkali or

consume lactate are involved in the carious process. Thus, a heterogeneous mixture of microorganisms could play a role in disease (Thomas and Nakaishi 2006).

Some recent studies indicate that the relationship between mutans streptococci and caries is not absolute. Whereas high proportions of mutans streptococci may persist on the teeth surfaces without leading to lesion development, caries can develop in the absence of these species. A cross-sectional study showed that 10% of subjects with rampant caries in the permanent dentition did not have detectable levels of *S. mutans* (Aas et al., 2005) and suggested that health and disease related bacterial species other than *S. mutans* are likely to play an important role in caries progression.

In addition to the specific and non-specific plaque hypotheses, an alternative hypothesis has been proposed (Marsh 1994; Takahashi and Nyvad 2008). In brief, it proposes that the organisms associated with disease may also be present at sound sites, but at levels too low to be clinically relevant. Disease is then a result of a shift in the balance of the resident microflora driven by a change in local environmental conditions.

A dynamic model was proposed in an attempt to explain the changes in the ecology of dental plaque that lead to the development of a carious lesion (Marsh and Martin 2009). According to that model, acidogenic or aciduric bacterial may potentially be found naturally in dental biofilm, but at neutral pH, these organisms are weakly competitive and may be present only as a small proportion of the total plaque community. In such situation the acid production by such bacteria is clinically insignificant or may be counteracted by other bacteria, and the processes of demineralisation and remineralisation are in equilibrium. If the frequency of

fermentable carbohydrates intake increases and/or salivary flow is impaired, then the biofilm spends more time below the critical pH for enamel demineralisation (approximately pH 5.5). The effect of this on the microbial ecology would be twofold. Firstly, conditions of low pH favour the proliferation of aciduric and acidogenic bacteria especially mutans streptococci and lactobacilli, but not exclusively so (Van et al., 1994), and tips the balance towards demineralisation. Secondly, greater numbers of aciduric bacteria such as mutans streptococci and lactobacilli in plaque would result in more acid being produced at even faster rates, thereby enhancing demineralisation further.

Bacteria other than mutans streptococci and lactobacilli could also make acid under similar conditions, but at a slower rate, or could initiate lesions in the absence of other aciduric species in a more susceptible host. If highly aciduric species were not present initially, then the repeated conditions of low pH coupled with the inhibition of competing organisms might increase the likelihood of colonisation of mutans streptococci or lactobacilli.

In this hypothesis, caries is a consequence of changes in the natural balance of the relevant plaque microflora brought about by an alteration in local environmental conditions (e.g. repeated conditions of high sugar and low plaque pH) (Marsh and Martin 2009). The hypothesis also acknowledges the dynamic relationship that exists between the microflora and the host, so that the impact of alterations in key host factors (diet and saliva flow) on plaque composition is also taken into account.

1.4 Development of dental plaque biofilm

Dental plaque is a general term for the complex but organised cooperative community of microorganisms that develops on the tooth surface, embedded in a matrix of polymers of bacterial and salivary origin (Overman 2000). Dental plaque forms via an ordered sequence of events, resulting in a structurally and functionally organised species rich microbial community. According to Marsh, the development of plaque biofilm can be subdivided into several stages (Marsh 2004).

a. Acquired pellicle formation: the formation of plaque begins with the rapid non selective deposition of several salivary proteins onto all exposed intraoral surfaces, that takes place as soon as a tooth surface is cleaned (Nassar et al., 1995). The thickness of the pellicle ranges from 50-150nm after 24 hours and is influenced by the shear forces acting at the site of formation (Hannig and Balz 1999).

b. Transport of microorganisms and reversible attachment: microorganisms are generally transported passively to the tooth surface by the flow of saliva. As the cell approaches the pellicle-coated surface, long range but relatively weak physiochemical forces are generated. The DLVO (Derjaguin-Landau-Verwey-Overbeek) theory explains the long-range interaction forces between microorganisms and pellicle layer (Liang et al., 2007). This theory states that the total interactive energy of two smooth particles is determined solely by the sum of the van der Waals attractive energy and the repulsive electrostatic energy.

c. Pioneer microbial colonisers and irreversible attachment: within a short time, initial weak physiochemical interactions may become irreversible due to adhesins on the microbial cell surface becoming involved in specific, short-range interactions with complementary receptors in the acquired pellicle. The initial colonisers constitute of a highly selected part of the oral microflora. They are organisms able to withstand the high oxygen concentrations and to resist the various removal mechanisms of the oral cavity such as swallowing, chewing and salivary and crevicular fluid outflow (Sbordone and Bortolaia 2003). Within minutes coccal bacterial appear, and they are mainly *S. sanguinis, S. oralis and S. mitis*. Once attached, these pioneer populations start to divide and form microcolonies. These early colonisers become embedded in bacterial extracellular slimes and polysaccharides together with additional layers of adsorbed salivary proteins and glycoproteins. The irreversible attachment of cells to the tooth involves specific short range stereochemical interactions between components on the microbial cell surface (adhesins) and complementary receptors in the acquired pellicle.

d. Coaggregation/coadhesion and microbial succession: over time, the plaque microflora becomes more diverse. The biofilm shifts away from the initial preponderance of streptococci to a biofilm with increasing proportions of *Actinomyces* and other Gram positive bacilli. Some organisms that were unable to colonise the pellicle-coated tooth surface are able to attach to already-adherent pioneer species by further adhesion-receptor interactions (coaggregation/co-adhesion) (Palmer et al., 2003).

e. Mature biofilm formation: the microbial diversity of plaque will increase over time due to successive waves of microbial succession and subsequent growth. The growth rate of individual bacteria within plaque slows as the biofilm matures. Confluent growth on the tooth surface produces a biofilm with 3-dimensional structure. Some of the adherent bacteria synthesise extracellular polymers, which will make a major contribution to the plaque matrix (Rosan and Lamont, 2000).

Microorganisns in the mature biofilm can adhere firmly to tooth surfaces unless subjected to shear forces such the ones resulting from brushing or chewing. Some bacteria can actively detach themselves from within the biofilm so as to be able to colonize elsewhere. *Streptococcus mutans* can synthesise an enzyme that can cleave proteins from its own surface and thereby detach itself from a mono-species biofilm.

1.5 Importance of plaque identification

Plaque detection is important not only to the patient but also to the clinicians and researchers. It is crucial for the patients to enable them to effectively clean their mouth. The clinicians need it to assess plaque levels and to decide on patients' suitability for dental procedures such as orthodontic treatment. From a researcher's point of view plaque levels are important to test oral hygiene products or periodontal therapies. However, detecting the mere presence or absence of dental plaque is not a useful tool for assessing the risk of dental caries or periodontal disease. Because dental plaque is composed of hundreds of different bacterial species, where not all of

which are pathogenic, it is more important to detect the virulent bacterial species that are linked to oral diseases.

1.6 Plaque quantification techniques

1.6.1 Disclosing

If plaque is present in large amounts it can be clearly seen without any aid. But in small quantities, and because it is generally colourless, plaque is usually stained prior to assessment. Common disclosing agents used include erythrosine (E 127 red). In certain products this erythrosine is usually combined with a blue dye (E 133). Disclosing is a very effective method for patients' oral hygiene improvement. But for the researcher, it lacks precision, objectivity, sensitivity, specificity and reliability that are required for clinical trials (Pretty et al., 2005).

1.6.2 Computer based plaque analysis

This technique utilises the analysis of photographs of tooth surfaces following disclosing. This is followed by analysis and measurement of plaque covered areas. It has been shown by a number of studies that computer based plaque analysis is more reliable, more precise, more objective and more sensitive than classic plaque indices (Pretty et al., 2005). The negative aspect of this technique is that it only measures area of plaque coverage and not depth.

1.6.3 Fluorescein disclosing and digital plaque image analysis (DPIA)

This technique relies on the use of fluorescein as a disclosing agent (Lang et al., 1972) and subsequent digital measurement of the fluorescent component. Under UV light, fluorescein disclosed plaque would appear yellow green in comparison to the dark oral tissues. Image analysis is then carried out for each tissue. On the computer, plaque appears red, dental hard tissues are blue and soft tissues are green making the separation of various oral tissues clear and unambiguous.

The technique permits smaller amounts of plaque to be measured and smaller changes between treatments to be detected due to its increased sensitivity (Smith et al., 2001). But the patient will be at risk of demineralisation, because in order to achieve good fluorescence, the pH of the system has to be low. In addition, the system is expensive and the plaque quantification is limited to the facial surfaces of the anterior teeth (Sagel et al., 2000).

1.6.4 Plaque quantification using 3D co-ordinate data

This method is based on the digitising of standard replicas of models with a co-ordinate measuring machine. A mathematical model of the surface is constructed and visualised from captured three-dimensional co-ordinates with the aid of supporting software. Changes in the depth of plaque can be detected by superimposition of models (Jovanovski and Lynch 2000).

For purposes of superimposition, the plaque located around the mid-line of the coronal half has to be removed. This coud have a negative impact on caries studies. The other down fall of this system is its reliance on direct impressions of the teeth because the laser scanner cannot be used within the mouth.

1.6.5 Plaque detection with quantitative light-induced fluorescence (QLF)

This technique relies on the ability of plaque bacteria to produce red fluorescing components. The phenomenon of red fluorescence in carious dental hard tissues and in dental plaque and calculus was first reported in the 1920's (Lennon et al., 2006). When plaque is present in large amounts on tooth surfaces, it fluoresces deep red or bright orange (Pretty et al., 2005; van der Veen and de Josselin de Jong 2000). Red auto fluorescence is presumed to originate from bacterial products that are chromophores of porphyrins (Konig et al., 1998), but the exact nature of the fluorescing chromophores is still not known.

Studies on the relation between red auto fluorescence of plaque and red fluorescence of isolated bacteria from that plaque using QLF found that red-fluorescing plaque comprised only about 62% of the total plaque that was found around the gingival margin corresponding to initial plaque formation (van der Veen et al., 2006).

Lennon and her group (Lennon et al., 2006) investigated a selection of cultivable bacteria associated with caries and periodontal disease for fluorophore production by visual assessment. It was found that *F.nucleatum* and the streptococci fluoresced

green, lactobacilli fluoresced orange-red, the actinomycetes fluoresced red and *P. intermedia* fluoresced bright red.

A different study (Coulthwaite et al., 2006) looked at the microbiological origins of green, orange and red fluorescence in dental plaque to evaluate the relationship between plaque age and fluorescence. The study utilised the QLF technique to identify fluorescence from denture plaque rather than plaque on natural teeth. Based on culture methods; it was reported that red fluorescent plaque contained both red and green fluorescent organisms. Moreover, obligate anaerobic bacteria were the source of red fluorescence; *C. albicans* were the source of orange fluorescence, cariogenic streptococci were the source of green fluorescence and lactobacilli had no typical fluorescence (Theilade et al., 1983; Verran and Maryan 1997; Verran and Whitehead 2005).

1.7 Methods of isolation and identification of dental plaque bacteria

Traditionally, bacterial identification was dependent on culture-based techniques (Mohania et al., 2008; Pratten et al., 2003). However, cultivation does not provide a full picture of the complex and diverse plaque bacterial community because a large number of oral bacteria are not amenable to culture-methods available. Despite the limitation of this approach, nearly 500 bacterial strains have been recovered from the subgingival crevice (Kroes et al., 1999). Recent studies with molecular techniques have shown that the bacterial diversity is underestimated when using cultivation based techniques, and that only 40-50% of the bacteria in the human oral cavity have been cultured (Kumar et al., 2005a; Paster et al., 2001).

The rapid development of molecular biological techniques during the past 15 years have lead to an increased use of culture-independent methods to supplement or replace culture based detection, identification and typing of bacteria. Molecular techniques involve the study of cellular macromolecules such as DNA, RNA and proteins.

1.7.1 PCR based methods (conventional, multiplex, real time)

Polymerase chain reaction (PCR) is a technique used to amplify a single or few copies of a piece of DNA across several orders of magnitude generating millions or more copies of a particular DNA sequence. The method relies on thermal cycling, that is cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA.

This is the most commonly used technique in molecular-based oral bacterial epidemiology, mainly due to its simplicity, sensitivity and cost-effectiveness (Rogers et al., 1991). A large number of oral bacterial studies employ specific oligonucleoside primers targeting the 16s rRNA gene in the sample. 16s rRNA gene sequencing enables the identification of rare bacteria with unusual phenotypic profiles, slow growing bacteria, novel bacteria, diagnosis of culture-negative infections alongside routine bacterial identification (Woo et al., 2008).

1.7.2 Hybridisation-based techniques

These techniques are based on the spontaneous pairing of two strands of nucleic acid double helix. DNA probes, usually oligonucleotides, have been used to probe bacterial DNA from the oral cavity. Oligonucleotide probes are short single stranded DNA sequences of 15 to 30 bases complementary to their target sequence. Soon after their development, they were used for identifying and detecting oral microorganisms (Dix et al., 1990; Moncla et al., 1990; Tsai et al., 2003).

In most studies using the 16s rRNA gene cloning and sequencing technique to investigate oral bacterial community diversity, plaque samples were collected form distinct oral sites. These include subgingival dental surfaces (Kumar et al., 2003; Kumar et al., 2005b; Kumar et al., 2006; Muyzer et al., 1993; Zijnge et al., 2006), supra gingival dental surfaces (Becker and Marcus 1971; Li et al., 2007), tongue surfaces (Kazor et al., 2003; Riggio et al., 2008) and from root canals (Siqueira et al., 2007; Siqueira and Rocas 2005). It should be noted that sampling from distinct oral anatomical sites reflects the microbial community related to that distinct site and cannot be generalized to represent the whole of the oral microbiota.

1.7.3 Denaturing gradient gel electrophoresis (DGGE)

Among molecular biology methods, PCR amplification of conserved regions of the 16s rRNA gene, followed by cloning and sequencing of PCR products has been widely used. As an alternative to the cloning approach, which can be time consuming and expensive laborious procedure, genetic finger printing techniques such as DGGE can be used as an alternative. These techniques can provide a profile representing the genetic diversity of a bacterial community for a given environment.

DGGE has been one of the most commonly used techniques to study bacterial communities from diverse ecosystems (Muyzer et al., 1993). It is an electrophoretic method that can detect differences between DNA fragments of the same size but with different sequences. Since PCR products from a given reaction are of similar basepair size, conventional separation by agarose gel electrophoresis results only in a single DNA band that is largely non-descriptive. During DGGE, the PCR products encounter increasingly higher concentrations of chemical denaturants (formamide and urea) as they migrate through the polyacrylamide gel and PCR products will begin to denature at a threshold denaturant concentration. When DNA denatures, it opens in a domain which is anchored by a higher melting domain or domains that have not melted. At this point the migration rate is slowed down. When the fragments completely denature, the migration becomes a function of size. This technique is capable of identifying a single base change in a segment of DNA (Figure 1.1).

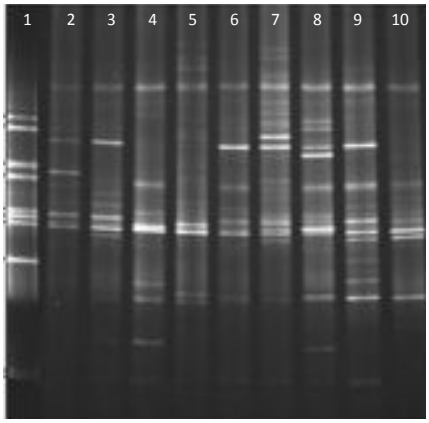

Figure 1.1: PCR-DGGE profiles of PCR-amplified bacterial 16s rDNA segments. **Lane** 1: reference marker created from 16s rDNA of a number of bacterial species, **lanes** 2-10 samples collected from the stimulated saliva of caries-free subjects where each band corresponds to a different bacteria, adapted from Li et al., 2005

1.8 DGGE and dental plaque

Traditional studies of oral microorganisms relied heavily upon cultivation methods. More recently a number of studies used the PCR-DGGE method to determine the microbial diversity in the oral environment. The microbial diversity in saliva and dental plaque for caries risk assessment and caries ecology were studied using DGGE and compared to the migration pattern of 25 known bacterial strains (Li et al., 2005). The 20 subjects were defined as caries free or caries active. The study found that the total cultivable bacterial counts and the total number of detectable bands on the DGGE was significantly higher in the caries-free group. However, there was no significant difference in the *S.mutans*, a prominent caries associated bacterium, levels between the two groups. The study suggested that a portion of the oral microbiota of the caries-active individuals may be absent, suppressed or replaced.

In another study that characterised the microflora of dental carious lesions by a combined cultural and molecular analysis (Munson et al., 2004). Five subjects whose carious dentine was removed and collected from the teeth were studied. The cultural analysis involved incubation of the samples on blood agar, Rogosa agar, and fastidious agar. The molecular analysis involved 16s rRNA gene isolation and sequencing. The results confirm the diversity of the bacterial community found in dental caries with 44 identified taxa out of the total of 95 taxa identified, 31 of which were not described previously.

Li and co-workers reported another study assessing the feasibility of using PCR-based DGGE methods to monitor changes in the microbial composition of dental

plaque in a clinical single-blind study. Plaque samples from the interproximal area of molar teeth from twelve individuals with a mean age of 41.6 years were collected. A total of three samples were collected from each individual; baseline, 24 hours after prophylaxis and four days after tooth brushing. Samples from each individual were pooled and subjected to PCR-DGGE analysis. The results showed that the mean number of detected PCR amplicons was significantly different between the three groups, with samples collected from sites after brushing showing less amplicons, confirming the effect of fluoride on microbial growth (Li et al., 2006).

The PCR-DGGE approach was employed to identify as much of the endodontic bacterial community in the root canals of teeth with evidence of chronic apical periodontal lesions (Machado de Oliveira et al., 2007). Samples collected from eight single-rooted teeth were subjected to PCR-DGGE analysis and bands (total of 12) were removed from the gel and sequenced. The results of the sequencing analysis revealed the presence of both cultivable and uncultivable species. Six bands were closest to *Fusobacterium* species. Two bands were 97-99% matching to *Bacteroides* species. Two bands had high similarities to *Prevotella*. Three bands were identified as *Escheirchia coli*. Representatives of the genera *Dialister, Synergistes, Eubacterium* and *Peptostrepococcus* were also identified.

Another study aimed to characterise the diversity in the complex dental plaque of children with severe early-childhood caries in comparison to caries-free controls (Li et al., 2007). Twelve children with caries and eight caries-free children with a mean age of 4.7 years were included in this study and pooled plaque samples were collected from the proximal surfaces of molars and canines. Following PCR-DGGE analysis, distinct amplicons from the DGGE gels were excised, cloned and

sequenced. A total of 92 distinct amplicons were detected from the overall DGGE profiles. On average, the total number of detectable bands was significantly higher in the caries-free group. The diversity of bacteria in the caries-free group was greater than in the caries-group. The overall conclusion was that the microbial diversity and complexity of the microbiota in dental plaque are significantly less in severe early-childhood caries than in caries-free children.

In a clinical study, aimed to detect all bacterial species associated with caries in primary and permanent teeth and to determine the bacterial profiles associated with different disease states (Aas et al., 2008). The subjects included in this study were 15 who had caries in the primary teeth and a group of 14 age matched controls, and 36 who had caries in the permanent teeth and a group of 25 age matched controls. The collected plaque samples were pooled for the controls but not for the caries subjects. Total DNA was isolated, 16s rRNA gene isolated and PCR amplified, cloned and sequenced to determine species identities. The results showed that the bacterial profile of intact enamel of healthy subjects differed significantly from the bacterial profiles of intact enamel from diseased subjects. There was a greater diversity associated with caries in permanent teeth suggesting that the presence of *S.mutans* does not necessarily indicate caries activity. *Actinomyces* and species other than *S.mutans* streptococci were predominant in white-spot lesions, while other acid producers were found at their highest levels later in disease. The overall conclusion was that bacterial profiles change with disease states and differ between primary and secondary dentitions.

1.8.1 Limitations of the PCR-DGGE method

PCR-DGGE is a molecular method that has been shown to be reliable, reproducible, rapid and inexpensive (Muyzer 1999) but as with any other method it has its limitations. These limitations can be related to the PCR or the DGGE stages of analysis. It is limited to its resolution and sensitivity. Bias during DNA extraction and amplification of the 16s rRNA gene is another factor as it is based on the assumption that DNA is equally extracted from all bacterial species (Siqueira, Jr. and Rocas 2005; Suzuki and Giovannoni 1996). It relies on the quality and reproducibility of bacterial sample processing and DNA extraction. Also, only microorganisms that are present in relatively high concentrations are represented on the gel (Muyzer and Smalla 1998), therefore not all of the microbial population within a given habitat appear on the DGGE profile. Another limitation is the fact that single species with multiple rRNA gene copies can display a DGGE profile with multiple bands (Machado de Oliveira et al., 2007).

1.9 Effect of orthodontic appliances on the plaque microflora

A number of different studies examined the effects of orthodontic appliances on the microbial profile of dental plaque. Corbett et al. (Corbett et al., 1981) looked at the effect of orthodontic bands and archwires on the prevalence of *S.mutans* in plaque. Pooled plaque samples were collected from 18 patients and 18 controls then plated and analysed for the presence of *S.mutans* colonies. The result showed that banded patients had greater buccolingual *S.mutans* plaque population, patients with a history of caries had significantly higher levels of *S.mutans*, and caries-free banded

patients had significantly more *S.mutans* infected sample sites than did non-banded caries free subjects.

A longitudinal clinical study to investigate the influence of orthodontic brackets on the relative number of *S.mutans* in dental plaque where a number of plaque samples were collected before and during fixed orthodontic treatment over a period of one month. The percentage of *S.mutans* continued to increase from the last pre-bracket sample to the last bracket sample (Mattingly et al., 1983).

Another study found that the levels of *S.mutans* and lactobacilli increase after the insertion of fixed orthodontic appliances and the use of chlorhexidine significantly reduces the numbers of lactobacilli. A difference in caries incidence on the buccal surfaces of bonded teeth in the treated group compared to the untreated controls was found, although it was not statistically significant (Lundstrom and Krasse 1987a; Lundstrom and Krasse 1987b).

The effect of orthodontic bands on the gingival tissues and the microbial composition of dental plaque was investigated on ten subjects undergoing orthodontic treatment using dark-field microscopy. The results showed a significant increase in the percentage of spirochetes, motile rods, filaments and fusiform and a decrease in cocci after banding accompanied by signs of gingivitis (Huser et al., 1990).

The effect of two different methods of ligation (ligature wires and elastomeric rings) on the development of plaque around orthodontic appliances was studied using a split mouth clinical study. Saliva and plaque samples were collected from 12 patients aged between 12 and 14 years and analysed for *S.mutans* and lactobacilli. The results showed that the number of *S.mutans* and lactobacilli increases

significantly in saliva after the insertion of fixed appliances. In plaque a greater number of microorganisms were found on the lateral incisor, which was attached to the arch-wire with an elastomeric ring compared to the incisor ligated with stainless steel wire (Forsberg et al., 1991).

A cross sectional study aimed to evaluate the levels of *S.mutans* in the saliva of patients before, during and after orthodontic treatment. Saliva samples were collected from 75 subjects at different stages of orthodontic treatment. The results showed that subjects in active orthodontic treatment had significantly higher total number and percentage of *S.mutans* compared to the subjects in retention, at post retention and the untreated age matched controls. The overall conclusion was that orthodontic treatment does not result in any long-term elevation of *S.mutans* levels (Rosenbloom & Tinanoff 1991).

The effect of certain orthodontic bonding composites and a glass ionomer cement on the adhesion of a strain of Streptococcus mutans was examined. Four different composites and one glass ionomer cement were tested for microbial growth. *In vitro* analysis showed that materials with rough surfaces attached more bacteria than ones with smooth surfaces. A stronger correlation was found between surface free energy of the material and the amount of *S.mutans* growth suggesting that surface free energy has a greater influence than surface roughness on bacterial colonisation (Blunden et al., 1994).

A link between different materials used to bond orthodontic appliances and the amount of plaque accumulation or its bacterial composition was attempted. Whole saliva from 10 healthy individuals provided microcosm plaque that was grown on discs of a range of bonding materials. Their results indicated that the accumulation

of *in vitro* supragingival plaque is not affected by the type of bonding material used, but biofilms formed over freshly made bonding materials known to release fluoride did not contain *S.mutans* (Badawi et al., 2003).

The prevalence of *Candida* and Enterobacteriaceae in a group of 50 adolescents during fixed orthodontic appliance therapy over only a 3 month follow-up period was measured. The results showed a significant increase in plaque index after the insertion of fixed orthodontic appliance and a significant increase in candidal number mainly *C.albicans* detected by the imprint technique (Hagg et al., 2004).

The short-term effect of the placement of orthodontic brackets on the subgingival micribiota and periodontal parameters was studied. The study lasted for three months only and included two groups of subjects: 30 patients who were about to receive orthodontic treatment and 30 untreated controls. Microbiological analysis of subgingival samples showed that scores for bleeding on probing, plaque index and gingival plaque index increased after bracket placement. The number of periodontopathic species was elevated following bracket placement (Naranjo et al., 2006).

The effect of fixed orthodontic appliances on the periodontal tissues and the microbiological composition of subgingival dental plaque in adolescents was studied, and it was found that the values of periodontal indices and the growth of pathogenic and anaerobic bacteria increases after the placement of fixed orthodontic appliances (up to 3 months) and start to decrease after 6 months of fixed appliance placement. This transitional increase does not have a destructive effect on the deep periodontal tissues (Ristic et al., 2007; 2008).

The influence of orthodontic treatment on the numbers of opportunistic bacteria and fungi in the oral cavity of 42 patients was examined. It was found that the isolation frequencies of opportunistic bacteria and fungi increase during orthodontic treatment (Kitada et al., 2009).

A scanning electron microscope technique was used to quantitatively analyse the formation of bacteria on supra- and subgingival surfaces of orthodontic bands on 10 patients. The results showed that despite the presence of supragingival biofilm, no mature subgingival biofilm was found on the orthodontic bands. A demarcation line was found on all bands corresponding to a supragingival biofilm and the absence of a mature subgingival biofilm (Demling et al., 2009).

DNA probe analysis was used to describe the distribution of various levels of putative periodontal pathogens in child and adolescent population, and to monitor changes in the levels these pathogens during and after fixed orthodontic treatment. The results showed that the count of 6 of the pathogens increased significantly after 6 months of treatment but returned to pre-treatment levels by 12 months (Thornberg et al., 2009).

The caries risk factors in children undergoing orthodontic treatment with sectional brackets were investigated. The results showed that the levels of mutans streptococcus remained unchanged during and after active orthodontic treatment while the levels of lactobacilli in the caries high risk group were significantly increased (Sanpei et al., 2010).

The evidence available to date suggests that the placement of a fixed orthodontic appliance can lead to a transient increase in the volume of plaque, gingival inflammation and hyperplasia, an increase in the number of bacteria with a

disproportionate increase in *S.mutans* that would fall back to normal levels on removal of the appliance, and that materials used have some effect on bacterial levels where bands have similar effects as bonds.

1.10 Detection of initial caries lesions (white spot lesions)

Due to the high prevalence of white spot lesion development in orthodontic patients, many clinical and laboratorial studies focussed on investigating this phenomenon using different techniques. For clinical examination, visual inspection is a non-invasive method of caries detection on accessible smooth surfaces. Figure 1.2 shows an example of white spot lesions as a result of poor oral hygiene and plaque accumulation at the gingival margin, whereas orthodontic white spot lesions are seen around the orthodontic bracket at the labial surface of the tooth.

Visual inspection is simple, quick and cost-effective, but because lesions are usually first detectable clinically at the white spot stage, they can be fairly advanced lesions by the time they are seen. Routine dental radiographs cannot detect early enamel white spot lesions. Approximately 30%-40% of mineral loss is necessary before an early enamel lesion can be detected radiographically and it can take up to 9 months or longer for demineralisation to appear on the radiograph (Lang et al., 1987).

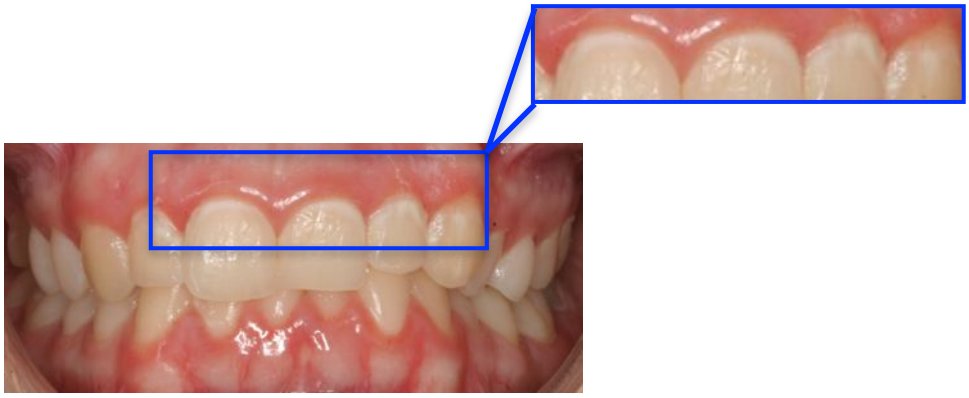

Figure 1.2: The atypical clinical appearance of white spot lesions seen at the gingival margins of the upper incisors.

Conventional diagnostic techniques lack sufficient sensitivity and specificity for early detection of lesions and cannot provide information on caries activity (Choo-Smith et al., 2008).Various optical methods have been developed to quantify enamel demineralisation and remineralisation. These include non-fluorescent methods of photographs and optical caries monitor, and fluorescent methods relying on the use of ultraviolet or laser light, which can be a particularly dangerous form of radiation especially to the eyes (Angmar-Mansson and ten Bosch 1993).

Quantitative Light Induced Fluorescence (QLF) is a valuable tool for the precise detection and quantification of initial caries lesions and for monitoring demineralisation or remineralisation on smooth surfaces (Heinrich-Weltzien et al., 2005). It exploits natural fluorescence emitted by fluorophores from dentine and enamel (Figure 1.3). In demineralised enamel, the light absorption and scattering properties are altered, resulting ultimately in less fluorescence being emitted and caught by the camera lens from a demineralised surface than from a sound enamel surface (Gmur et al., 2006).

Figure 1.3: Tooth image with QLF where the contrast between sound and demineralised enamel is clear (Image obtained from Inspektor research systems BV website, The Netherlands).

The device produces light from an arc lamp (with a peak intensity of 370 nm) that passes through a blue filter a long a liquid light guide to a hand piece that can be directed at the tooth surface. Enamel auto-fluorescence is then detected using an intra oral camera. The reflected light passes through a yellow high pass-filter of 520 nm in front of the camera. The images are then stored, processed and analysed with a customised software (Inspektor Research System BV Amsterdam, The Netherlands) (Al-Khateeb et al., 1997) .

Images obtained from digitally converted photographic slides were compared with those of QLF to measure enamel demineralisation surrounding an orthodontic bracket *in vitro* (Benson et al., 2003). It was found that both the area of demineralisation and a relative assessment of mineral loss of a white spot lesion surrounding an orthodontic bracket can be recorded and quantified reproducibly by either of the two techniques. The repeatability of using image analysis to measure

demineralisation from a photographic image is very similar to the technique of analysing a fluorescent image of the tooth using the customised software with QLF.

The prevalence and severity of white spot lesions in orthodontic patients after fixed appliance therapy were studied using QLF in comparison to visual inspection (Boersma et al., 2005). The results showed that the same distribution pattern of white spot lesions was detected with QLF and visual inspection. However, more lesions were found with QLF than with visual inspection and the difference between the percentages of caries affected surfaces in boys (40%) and girls (22%) were significant. Other factors such as age, treatment period or eating frequency did not show a significant correlation.

Longitudinal monitoring of the behaviour of white spot lesion directly after fixed appliance treatment as well as 6 weeks, 6 months and 2 years later by means of QLF in 51 subjects showed that many patients develop white spot lesions during fixed appliance treatment. A total of 351 carious surfaces were detected that did not resolve after de-bonding of the appliance. Out of those, 171 lesions remained stable, 145 lesions showed some improvement and 35 lesions got worse (Mattousch et al., 2007).

Early detection and diagnosis will allow dentists to counsel and assist patients to prevent the progression of caries and prevent the need for invasive and irreversible restorative procedures.

1.11 Prevention and treatment of white spot lesions

Enamel demineralisation prevention starts with tooth brushing instructions and diet advice to ensure that the patients have and are able to maintain an adequate level of oral hygiene throughout treatment. Other preventative methods include topical fluorides, oral irrigation systems, electric toothbrushes and sonic and ultrasonic toothbrushes. Boyd and co-workers (Boyd et al., 1989) studied the difference between a rotary electric tooth brush and commercial tooth brushes on the periodontal status during fixed orthodontic treatment. The results supported the fact that rotary electric toothbrushes can be more effective in maintaining the periodontal health of adolescents undergoing fixed appliance therapy.

Another factor that plays a role in prevention is the fluoride-releasing cementing agents. Glass ionomer cements and resin-modified glass ionomer cements have been shown to have a remineralisation effect on demineralised enamel adjacent to orthodontic bands and brackets (Basdra et al., 1996; Donly et al., 1995; Sadowsky et al., 1981). Despite these preventative measures white spot lesions still occur. In this case efforts should be focussed on remineralising these early lesions and preventing their progression.

Good standard of oral hygiene using a standard dentifrice daily is a starting point. The addition of the daily use of 0.05% sodium fluoride rinses can be an effective aid to tooth brushing (Stookey et al., 1985; Stookey et al., 1993). Professional preventative dental care includes the application of topical fluorides such as 1.23% acidulated phosphate fluoride (APS), 2% sodium fluoride and 8%

stannous fluoride. Another agent that can have a remineralisation effect is casein phosphopeptide-amorphous calcium phosphate (CPP-ACP).

When enamel remineralises fluoride, calcium and phosphate ions penetrate the surface zone and precipitate on sound enamel at the margins of the subsurface demineralised lesion (Silverstone et al., 1988). The remineralisation process does not recreate enamel prisms and the remineralised enamel has a higher calcium, phosphate and fluoride content. For that reason it appears whiter than natural enamel.

A number of clinical and laboratorial studies were conducted to investigate the effect of professional topical treatments on the remineralisation potential of white spot lesions. Buchalla and co-workers (Buchalla et al., 2002) evaluated the fluoride uptake, retention and remineralising efficacy of a single dose application of highly concentrated fluoride solution (10,000 ppm) in a double blind, placebo controlled, randomised crossover *in situ* study and concluded that a single application of this highly concentrated fluoride solution increases enamel remineralisation.

Altenburger and colleagues (Altenburger et al., 2009) aimed to test whether 1.0% amine fluoride fluid is better than a 0.5% solution in terms of fluoride retention and mineral change in initial caries enamel lesion over a period of 28 days using a double-blind, placebo-controlled, randomised cross over *in situ* study. The results suggested that the use of 1% fluoride resulted in a significantly higher remineralisation compared with the use of 0.5% fluoride.

Lee and his group (Lee et al., 2010) studied the remineralisation effect of three topical fluoride regimes on initial carious lesions. 2% sodium fluoride solution, 1.23% acidulated phosphate fluoride gel and 5% sodium fluoride varnish were used.

The results showed no significant difference between the three regimes on the remineralisation of early enamel carious lesions.

It is important to realise that fluoride is only effective as a remineralising agent in the early stages of lesion development and cannot achieve full remineralisation even with high concentrations. One difficulty with enamel remineralisation is the preferential remineralisation of the outer enamel surface which in turn can slow or prevent the complete remineralisation by restricting mineral ion diffusion into deeper regions of the lesion (Larsen and Fejerskov 1989; ten Cate et al., 1996; ten Cate and Duijsters 1983). ACP-CPP has been shown to slow the progression of caries significantly and to promote the regression of early lesions (Reynolds et al., 2003). More recent studies by Lata et al. (Lata et al., 2010) aimed to investigate the remineralisation potential of fluoride, ACP-CPP and the combination of both on early enamel lesions. The results showed that fluoride varnish is more effective than ACP-CPP cream in remineralising the early enamel lesion at the surface level and the combination of both does not provide any additive remineralisation potential. All three treatments were not effective in remineralising the lesion at the subsurface level.

The effect of ACP-CPP tooth mousse on the remineralisation of bovine enamel was evaluated using circulatory polarised images (Wu et al., 2010). The results showed that ACP-CPP can promote remineralisation and the combination of fluoride toothpaste and ACP-CPP tooth mousse improves the remineralisation effect.

The use of natural products as medicines has increased recently. The combined effect of nano-hydroxyapatite and Galla chinensis (a Chinese herbal medicine) on the remineralisation of initial enamel lesions was evaluated (Huang et al., 2010) and the results showed that there was a significant effect of the combined nano-hydroxyapatite and Galla chinensis on promoting the remineralisation of initial enamel lesions and that more mineral deposition occurred in the body of the lesion leading to a reduction of lesion depth.

Chapter 2

Aims

2.1 Aims

The aims of this study were to make use of novel diagnostic methods including Quantitative Light-Induced Fluorescence (QLF) and DNA amplification to identify any general changes in the bacterial composition of supragingival red fluorescent dental plaque in adolescents undergoing fixed orthodontic appliance therapy. A secondary aim was to determine the incidence of white spot lesions and if there was any relationship with the identified red fluorescent plaque.

2.2 Null Hypotheses

1. There is no difference in the general bacterial composition of red fluorescent supragingival dental plaque before and after the placement of fixed orthodontic appliances in adolescents undergoing fixed orthodontic appliance therapy.
2. There is no association between red fluorescent dental plaque and the development of white spot lesions in adolescents undergoing fixed orthodontic appliance therapy.

Chapter 3

Study design

3.1 Study design and participants

3.1.1 Study design

A prospective non-interventional 12-month longitudinal cohort investigation of potential changes in the general composition of supragingival red fluorescent dental plaque and the incidence of white spot lesions in adolescents undergoing orthodontic treatment using fixed brackets.

3.1.2 Participants

A sample of thirty 11 to 23 year old patients scheduled to have fixed orthodontic appliance therapy at the Orthodontic Department of Liverpool University Dental hospital was to be recruited for this study. Based on a recommendation from a statistician, the sample size was selected on the basis of the probability of identifying plaque constituents, which are common in the target population. A sample size of 30 will give a 95% probability of identifying plaque constituents, which occur in at least 10% of the target population.

Plaque samples and QLF images were obtained during the participants' routine orthodontic appointment, which is usually every six weeks.

3.1.3 Exclusion criteria

Due to the effect of antibiotics on plaque bacterial microflora, any participant who had received antibiotic therapy in a period less than eight weeks prior to their participation in the study, or at any time during the study, were excluded.

3.2 Ethical approval

Ethical approval was granted by the North West Ethics Committee, Liverpool; a representative of the National Research Ethics Committee (reference 09/H1005/63). Approval from the research and development department of the Royal Liverpool and Broadgreen University Hospitals Trust was also obtained (reference 3822). The University of Liverpool acted as a co-sponsor with the Royal Liverpool and Broadgreen Hospitals NHS Trust (reference UoL000454).

Potential participants and their parents/guardians were given verbal and written information about the study. Six weeks after the introduction to the study, patients and their parents/guardians willing to participate in the study were asked to sign the consent/assent forms.

Chapter 4

Identification and collection of red fluorescent plaque

Red fluorescence of plaque is thought to originate from porphyrins, which are by-products of bacterial metabolism (Coulthwaite et al., 2006, Pretty et al., 2005). When plaque is present in large amounts on tooth surface, it fluoresces deep red or bright orange (Pretty et al., 2005, van der Veen and de Josselin de Jong 2000). Obligate anaerobes are the source of red fluorescence and cariogenic streptococci are the source of green fluorescence (Verran and Whitehead 2005; Coulthwaite et al., 2006), section 1.6.5.

4.1 Method

4.1.1 Identification of red fluorescent plaque

Red fluorescent supragingival plaque was identified with Toothcare^{TM}, which is a hand held device based on the QLF principle, when viewed with a special red filter (Figure 4.1). The labial/buccal surfaces of maxillary and mandibular the six anterior teeth (central incisors, lateral incisors and canines) were scanned for red fluorescent plaque. Order of inspection was: the lateral incisors, followed by the central incisors and finally the canines.

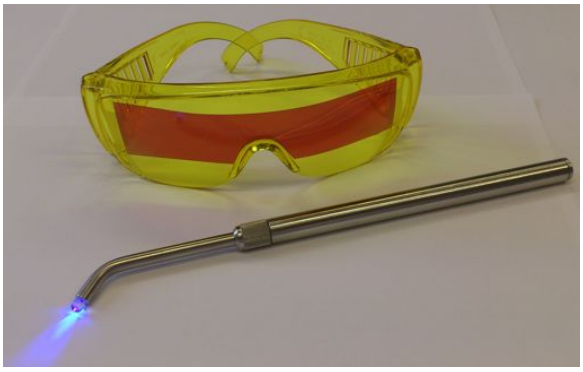

Figure 4.1: The toothcare™ device and the specially filtered goggles used to identify red fluorescent plaque.

The site from which the dental plaque was collected from was noted on a chart (Figure 4.2). When no red fluorescent plaque could be identified, none of the plaque was collected, even if gross plaque deposits were clearly seen without the use of Toothcare™.

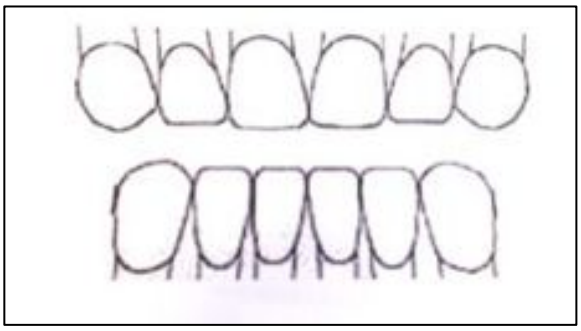

Figure 4.2: The chart used to note the position of the red fluorescent plaque once identified with Toothcare™.

4.1.2 Collection of red fluorescent plaque

Once the red fluorescent plaque was identified on a single tooth's surface, a sterile dental instrument was used to remove the plaque from the tooth surface. The collected plaque was transferred into a sterile Eppendorf® 1.5 ml microcentrifuge tube. The plaque sample was kept on ice on clinic until it was ready to be transferred and kept at -20^{o} C for further analysis.

4.2 Results

A total of 14 participants agreed to take part in this study, following attainment of consent/assent, red fluorescent plaque samples were identified and collected before the placement of the orthodontic brackets and at every follow up orthodontic appointment (approximately every six weeks) for a total period of twelve months (Table 4.1). Where no red fluorescent plaque was identified, no samples were collected. For example, for participant 17, only one red fluorescent plaque sample was identified at one visit and during the six remaining visits no red fluorescent plaque samples were identified.

Table 4.1: Number of visits and red fluorescent plaque samples collected per participant if identified with Toothcare™.

Participant	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Number of visits	7	8	9	9	5	7	6	5	9	7	8	9	8	7
Total number of red fluorescent plaque samples collected.	7	7	2	9	4	5	6	1	9	6	8	8	7	1

For the 14 participants a total of 80 plaque samples were collected with a mean of 5.71±2.76 samples per participant .The total number of visits was variable because the participants were seen during their regular orthodontic appointment. Any cancellations of appointments meant that the samples were not collected at a constant interval and the total number of samples collected varied accordingly. The mean number of visits per participant was 7.43 ± 1.40. There was only one drop out (participant 5) as that participant moved away from the area. The plaque samples collected from that participant were included in the analysis. One of the participants attended only 5 appointments during the 12 months follow up period. That was due to multiple cancellations and failed appointments. The labial surfaces of the upper right lateral incisor followed by the upper right canine were the most common sites where red fluorescent plaque was collected (Figure 4.3).

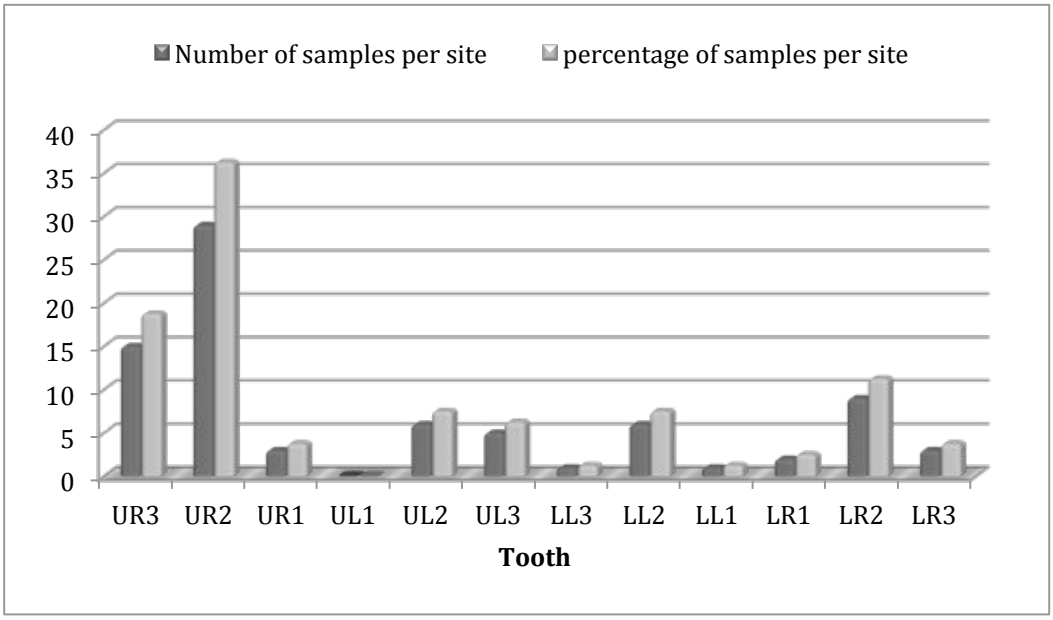

Figure 4.3: The distribution of red fluorescent plaque collected from each tooth.

16.4% of the sites where red fluorescent plaque was detected (n=80) showed signs of demineralisation identified with QLF, and 83.6% of the sites where red fluorescent plaque was detected did not show any signs of demineralisation when assessed using QLF (Figure 4.4). The overall incidence of white spot lesions was not related to the presence of red fluorescent plaque when analysed using chi square test, resulting in a p value >0.05 (x^2= 3.3) (Figure 4.5).

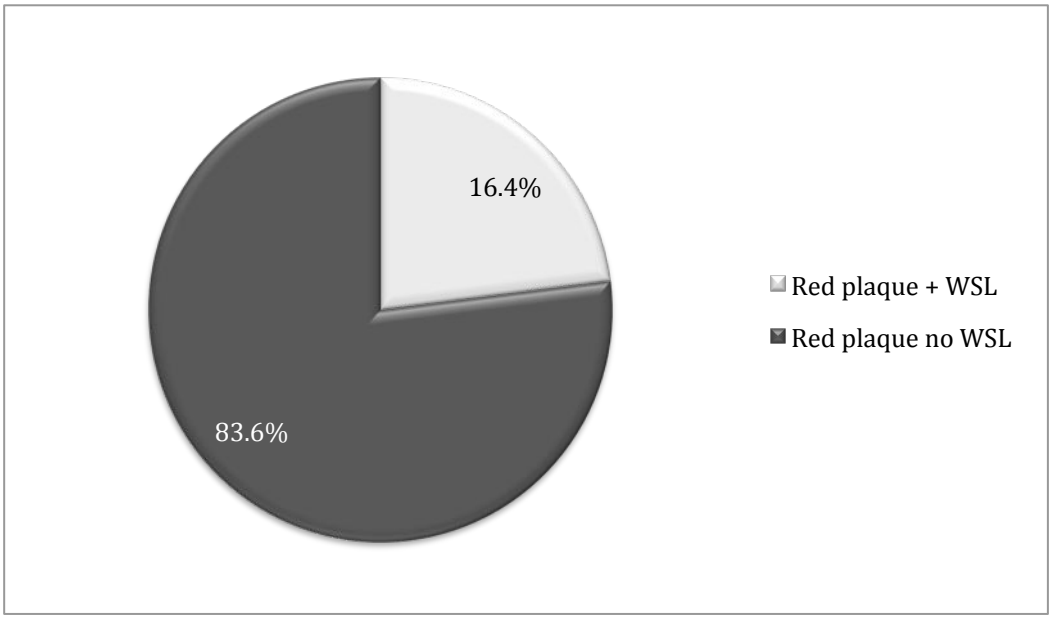

Figure 4.4: The relationship between red fluorescent plaque and white spot lesions as a percentage of the total number of tooth surfaces with red fluorescent plaque.

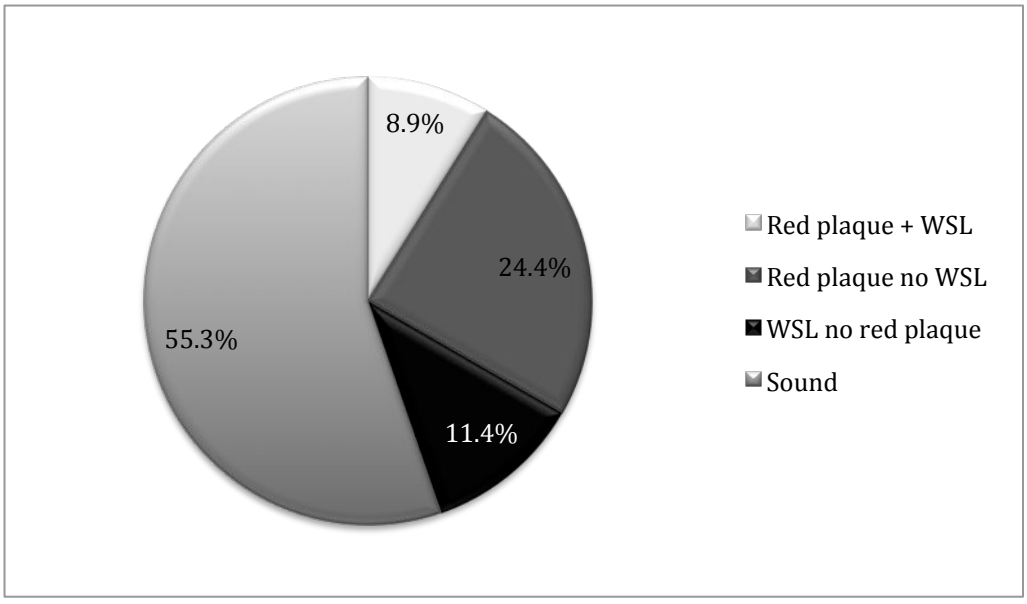

Figure 4.5: The relationship between red fluorescent plaque and white spot lesions as a percentage of the total number of tooth surface.

Chapter 5

Preliminary dental plaque analysis

PCR amplification of conserved regions of the 16s rRNA gene followed by DGGE analysis has been widely used as molecular biology methods of isolation and identification of dental plaque bacteria. Molecular techniques are used to overcome the limitations encountered when using cultivation-based techniques (section 1.7).

I. Methods

5.1 Total bacterial DNA extraction

5.2.1 Isolation of genomic DNA

To isolate the bacterial genomic DNA, MasterPure^{TM} Complete DNA and RNA Purification Kit (Epicentre Biotechnologies) was used. The following protocol that was provided with the purification kit (Epicentre Biotechnologies) was modified and then followed to lyse the cells and release the DNA from the nuclei:

1. The collected frozen plaque sample was thawed and re-suspended in 1ml of DNAse free water.
2. Five µl of Proteinase K solution (0.18 mg/ml, [50 mM Tris-Hcl (pH 7.5)], 100 mM NaCl, 0.1 mM EDTA, 10 mM $CaCl_2$, 0.1% Triton® X-100, 1 mM dithiothreotol in 50% glycerol solution), and 30µl of 10% sodium dodecyl sulphate (SDS, 0.5%) were added to the sample and mixed by inversion.
3. The sample was then incubated in a water bath at 55^{o} C for 1 hour and inverted occasionally.

4. The cells were then pelleted by centrifugation; the supernatant discarded leaving approximately 25µl of liquid.
5. The pelleted cells were then re-suspended by vortexing for 10 seconds.
6. For each sample, 3µl of Proteinase K was diluted into 300µl of Tissue and cell lysis solution. The solution was added to the sample and mixed thoroughly.
7. The sample was then incubated in a water bath at 65^{o} C for 15 minutes, mixed every 5 minutes by vortexing.
8. The sample was then cooled to 37^{o} C, by placing it in a water bath, for 10 minutes.
9. Three µl of 5µg/µl RNase A was then added to the sample and mixed thoroughly.
10. The sample was incubated at 37^{o} C for 30 minutes.
11. The sample was then left on ice for 3-5 minutes before proceeding with total DNA precipitation.

5.2.2 Total DNA precipitation

Following the isolation of the bacterial genomic DNA the following was undertaken to precipitate the DNA and remove any bacterial protein from the sample:

1. For every 300µl of lysed sample, 100µl of MPC protein precipitation reagent was added and mixed by vortexing vigorously for 10 seconds.

2. The debris (denatured protein) was pelleted by centrifugation at 4°C for 10 minutes at ≥10,000 x *g* in a micro-centrifuge. If the resultant pellet was clear, small or loose, an additional 25µl of MPC protein precipitating reagent was added to the sample, mixed vigorously and centrifuged to pellet the protein again.
3. The pellet was then discarded, and the clear supernatant transferred to a clean micro-centrifuge tube.
4. 500µl of cold isopropanol was added to the recovered supernatant. The tube was then inverted 30-40 times.
5. The DNA was pelleted by centrifugation at 4°C for 10 minutes at ≥ 10,000 x *g* in a micro-centrifuge.
6. The isopropanol was carefully discarded without disturbing the DNA pellet. The DNA pellet was then left to air dry.
7. Finally the pellet was re-suspended in 10µl of TE (10 mM Tris-HCl [pH 7.5], 1 mM EDTA) buffer and stored at 4°C (if required) for further analysis.

5.2.3 Identification of genomic DNA

Following the isolation of bacterial genomic DNA, the sample was detected on 0.8% (w/v) agarose gel to confirm the successful isolation of the DNA. Agarose (0.8 g) was dissolved in 100ml of TBE buffer with 4µl of ethidium bromide and 2µl of the sample, 8µl of DNAse free water and 2µl of loading dye were loaded. The gel was run at 80V for 30 minutes. The gel was then viewed under U.V. Light to detect the presence of DNA.

5.2 Amplification of bacterial DNA by PCR

5.2.1 PCR reaction

PCR was used to amplify the 16S rRNA gene of the suparagingival plaque samples. A combination of the forward primer 27F (5'-AGAGTTTGATCMTGGCTCAG-3',) and the reverse primer 1492R (5'-TACGGYTACCTTGTTACGACTT-3') obtained from Sigma-Aldrich®, 20µM, were used to amplify the 16S rRNA gene hyper-variable region. PCR amplification was performed using Px2 Thermal cycler (Thermo Fisher Scientific Inc). The amplification consisted of 30 cycles of denaturation at 95^{o}C for 1 minute, annealing at 54^{o}C for 1minute and extension for 1 minute at 72^{o}C. The first cycle was preceded by an initial template denaturation step of 5 minutes at 95^{o}C, and the last cycle was followed by a final extension step of 15 minutes at 72^{o}C. The PCR programme was obtained from Li et al. (Li t al., 2007).

A 50µl reaction mix volume was used containing 25µl of PCR 2X Master mix, (which contained 50 units/ml *Taq*DNA polymerase [pH 8.5], 400µM of each: dATP, dGTP, dCTP, dTTP, 3mM $MgCl_2$, Promega, UK Ltd), 2.5µl, 10µM of each primer, 2.5µl of genomic DNA and 17.5µl of nuclease free water.

5.2.2 Identification of PCR products

To confirm the successful amplification of the 16S rRNA gene region, 2.5% (w/v) agarose gel electrophoresis was done. The samples were run at 80V for 1.5 hours. 50 bp DNA Step Ladder (Promega UK Ltd) was used as a marker. The gel was then viewed under UV light to identify the PCR products stained with ethidium bromide. Ethidium bromide (2µl) was added to the agarose gel before setting.

5.3 Generation of DGGE profile

DGGE was performed with *D GENE*^{TM} Denaturing Gel Electrophoresis System, Bio-RAD, Germany, to separate the PCR products. The PCR amplicons were subjected to 7.5% (w/v) acrylamide 40% Acrylamide/Bis (37.5:1) gels in TAE buffer with a denaturing gradient ranging from 30% to 60% denaturant. The high and low density denaturing solutions were loaded into two different syringes and attached to the manual gradient delivery apparatus (Figure 5.1).

The polymerisation process was initiated by the addition of 100µl of Ammonium persulfate (APS) and 10µl of TEMED to each 25 ml of acrylamide denaturing solution. The gels took approximately two hours to fully polymerise. The electrophoresis was performed at 60^{o} C. The samples were run for 3 ½ hours or until the blue dye line was close to the bottom of the gel (Figure 5.2).

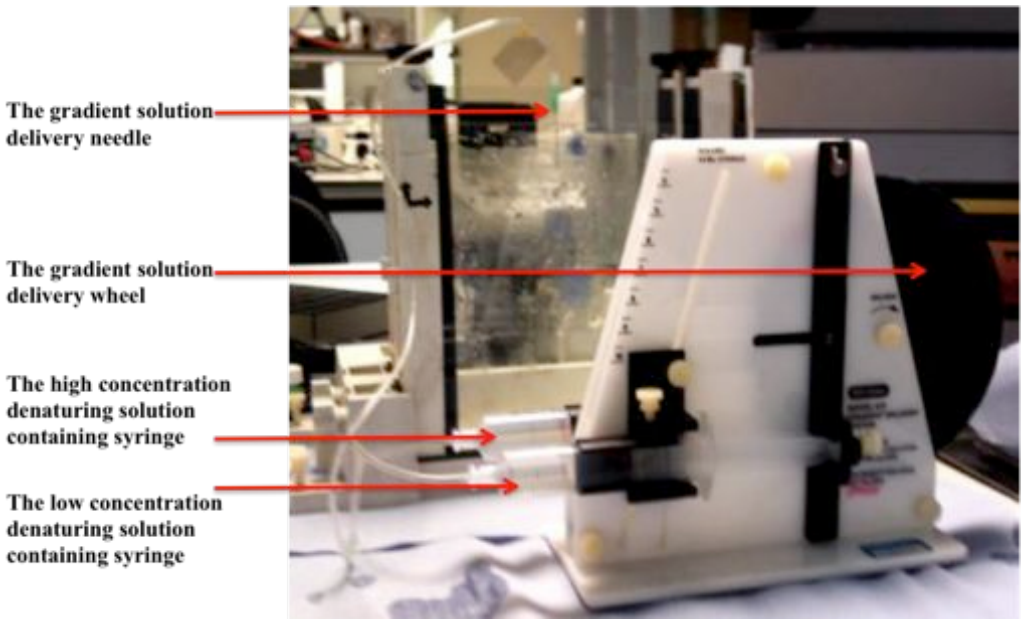

Figure 5.1: The gradient delivery apparatus

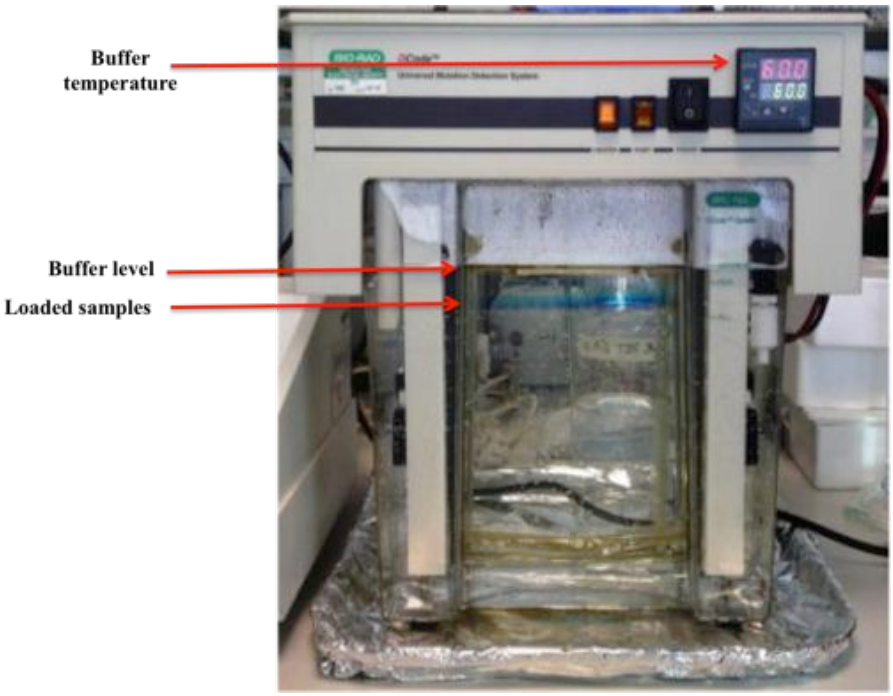

Figure 5.2: Fully assembled DGGE apparatus with the samples loaded and seen as blue horizontal lines.

When the gels were poured as a continuous gel and the comb inserted at the top of the glass plates, the wells did not form well and the top of the gel did not polymerise uniformly. A layer of 4% (w/v) acrylamide stacking gel was subsequently poured at the top of the gel to stack the samples and allow adequate well formation.

The parallel gradient was poured using Model 475 gradient delivery system (Bio-RAD, Germany). Few drops of ethanol were added to the top surface of the gel to facilitate the formation of a level top of the gel. Twenty-five wells comb was used to form the wells within the stacking gel. For stock solution preparation and protocol refer to appendix F.

5.3.1 Silver staining

The bands were then viewed following silver staining of the gel. Silver staining was used because it has been shown to be at least fivefold more sensitive than ethidium bromide staining, with a detection limit of 2.5 ng for total DNA (Gottlieb and Chavko 1987). For stock solutions and protocol refer to appendix G.

II. Results

For the 14 participants who agreed to take part in this study, a total of 80 plaque samples were collected with a mean of 5.71±2.76 plaque samples per participant. The number of visits was variable because the participants were seen during their regular orthodontic appointment. Any cancellations of appointments meant that the samples were not collected at a constant interval and the total number of samples collected varied accordingly. There was only one drop out (participant 5) as that participant moved away from the area. The plaque samples and QLF images collected from that participant will be included in the analysis. One of the participants attended only 5 appointments during the 12 months follow up period. That was due to multiple cancellations and failed appointments.

Because the samples were collected from a single site from the identified tooth, the volume of dental plaque was very small. Difficulties were encountered initially when attempted to extract the gemonic DNA from these collected samples. Later on, genomic DNA was successfully extracted from the samples, although to varying amounts depending on the volume of the plaque sample collected (Figure 5.3).

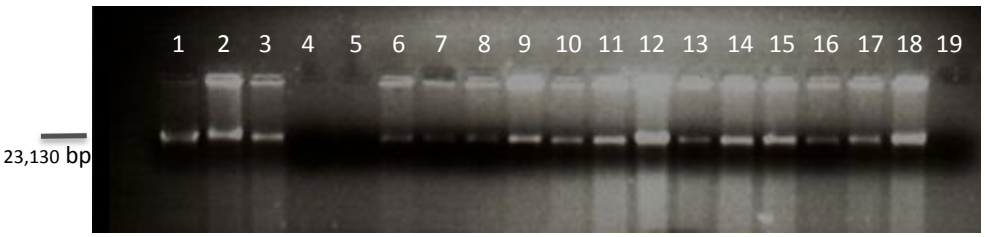

Figure 5.3: The agarose gel (0.8%) profile of genomic DNA stained with ethidium bromide. **Lane 1**: marker (lambda DNA); **lanes 2-19** plaque samples from different participants.

The amount of PCR product was also variable depending on the initial amount of collected plaque and the recovered genomic DNA. Although for some of the samples, the genomic DNA could not be detected on the agarose gel, the 16s rRNA gene was successfully amplified by the PCR reaction and clearly detected on the agarose gel (see lanes 4,5 19 Figure 5.1 and lanes 5,6,20 Figure 5.2). When compared to the 50 base pair ladder used, the size of the PCR product was estimated to be 1,500 base pairs (Figure 5.4).

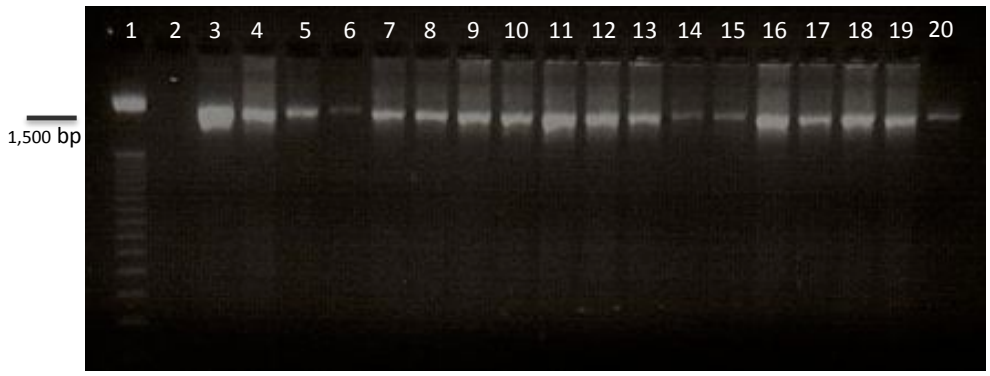

Figure 5.4: The agarose gel (2.5%) profile of 16s rRNA gene-PCR product stained with ethidium bromide. **Lane** 1: 50 bp marker; **lanes 2**: -ve control; **lanes 3-10**: Plaque samples from different participants.

When the DGGE profile was produced, most of the separation of the DNA fragments took place at the top of the gel. It was difficult to distinguish the different bands at the top of the gel. The few bands that could be distinguished further down the gel, were not only few but also faint and difficult to identify (Figure 5.5).

From the bands that could be identified, it was seen that the band separation profile of the two bacterial controls (*P. gingivalis* ATCC33277 and *S. gordonii* DL1) was distinctly different from the separation profile of the plaque samples. This suggests that these bacterial species are unlikely to be present in the supra gingival plaque of adolescents. Individual participant variation was also noted as different band profiles were identified for the samples collected from different participants.

Looking at the plaque samples collected from the same individual, the overall band pattern was the same for samples collected before and during the 12-month period following the placement of fixed appliances. Some of the bands were noted to become darker after time, for example the first identified band from the top of the

gel in lanes 4-8 in Figure 5.5. That might be explained by a change in the amount of certain bacteria, rather than the introduction of new bacterial species.

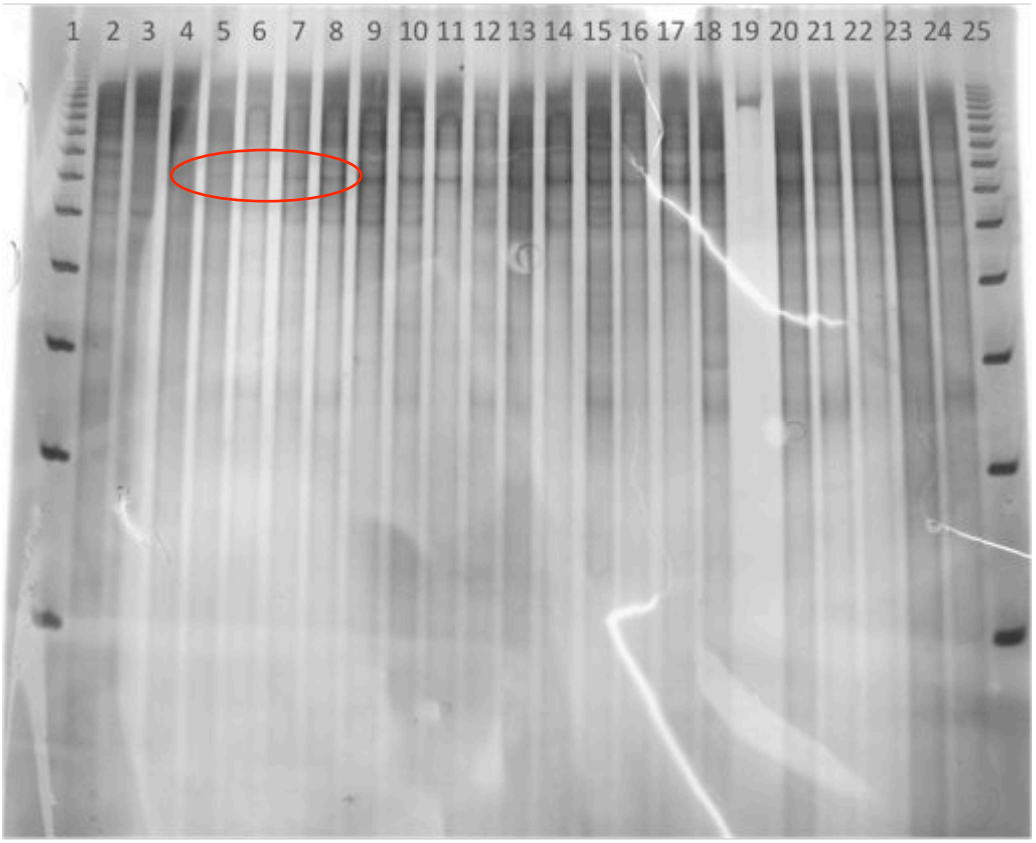

Figure 5.5: PCR-DGGE profiles from red fluorescent dental plaque samples and laboratory strains of oral bacteria stained with silver staining. **Lanes 1, 25**: 50 bp ladder, **lane 2**: *P gingivalis* (ATCC 33277), **lane 3**: *S. gordonii* (DL1), **lanes 4-8**: participant 1 plaque samples1, 3, 5, 6, 7; **lanes 9-13** participant 2 plaque samples 1,2,3,4,5; **lanes 14-16**: participant 3 plaque samples 1,2,5; **lanes 17-22**: participant 4 plaque samples 1,2,3,4,5,6; **lanes 23-24**: participant 5 plaque samples 1,2. The red circle highlights the change in darkness of the first identified band from the top of the gel for participant 1, lanes 4-8.

The overall picture suggested that following the placement of bonded orthodontic brackets; there might be changes in the number or quantity of the bacterial species already present in the plaque rather than a change in the bacterial species in the plaque community.

A definitive conclusion cannot be drawn from these results. This is due to the fact that the number of the bands appeared to be too high in the upper part of the DGGE profile, closely related and single bands could not be distinguished. Changes in the band profile might be present in that narrow area of the gel. This cannot be ruled out because the bands are difficult to distinguish in that area.

In order to overcome this issue a different denaturing concentration gradient was used so that the bands travel a longer distance into the gel and can be separated further. Since most of the band separation is taking place at the lower denaturant concentration, a denaturant solution of a gradient between 15% and 50% was used instead of 30% and 60% (section 6.2).

In another approach to overcome this issue is to use nested PCR primers following the ≈ 1,500-bp 16s rRNA gene pre-amplification with the universal 16s rRNA gene sequence primers. The second nested amplification would produce a smaller PCR products (≈ 250 bp) and more distinguishable bands on the DGGE profile. Nested GC-clamp PR primers to allow better and more accurate separation of the PCR amplification products and the possibility for a more definite conclusion to be drawn form these results were used.

The nested PCR amplicons will denature quickly as they are of smaller size DNA double strand that will dissociate and completely denature at lower denaturant concentrations and will not travel further down the denaturant gel. The dissociation and complete denaturation of the DNA double strand is prevented by incorporating a GC-rich high melting temperature domain (GC Clamp) at one end of the PCR amplicons. This is achieved by using a PCR primer with a 5'GC-rich tail. Increasing the melting temperature of the tail end allows the detection of single base differences between the different PCR amplicons (Myers et al., 1985).

When GC-clamps were first developed, they were 300 bp long then later developed to be as small as 40 bp (Rettedal et al., 2010; Sheffield et al., 1989).

Chapter 6

Validation of GC-Clamp PCR primers

6.1. Primer design

Two sets of GC- rich sequence (GC-clamp) PCR primers based on the protocol described by Muyzer (Muyzer et al., 1993) were used to amplify the V3 variable conserved region of the 16s rRNA genes. The following primers bought from Sigma Aldrich were used: primer 1 (357F-GC): 5'- CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3', primer 2 (518R): 5'- ATT ACC GCG GCT GCT GG-3'.

The PCR amplification was performed with Px2 Thermal Cycler (Thermo Fisher Scientific Inc.) as follows: 25µl of maser mix, 1µl (10 µM) of primer 1, 1µl (10 µM) of primer 2, 1µl of initial PCR product and 2µl of nuclease free water for a total volume of 50µl of the PCR reaction mix.

The PCR reaction programme consisted of: a denaturing stage at 95^{o}C for 5 minutes, 10 cycles of denaturation at 94^{o}C for 30 seconds, 56^{o}C for 30 seconds and 72^{o}C for 1 minute, 26 cycles of annealing at 92^{o}C for 30 seconds, 53^{o}C for 30 seconds and 72^{o}C for 1 minute and a final extension stage at 72^{o}C for 10 minutes. The PCR amplification products were then detected by electrophoresis in 2.5% agarose gel, stained with ethidium bromide and visualised under U.V. light (Figure 6.1). The resultant fragment size was between 200 and 250 bp, which corresponds to the 233bp size reported by Muyzer et al.(1993).

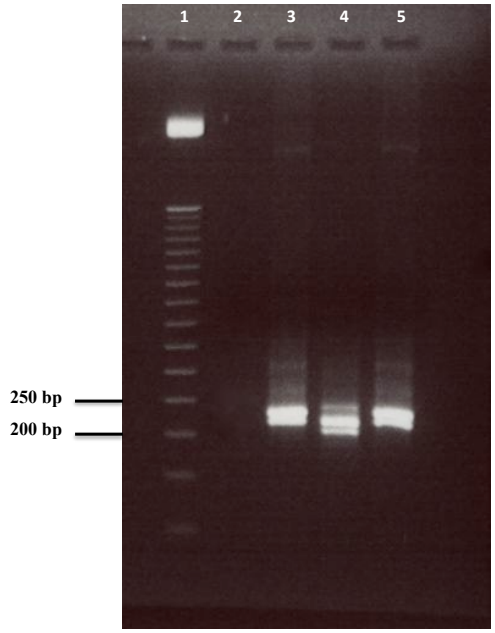

Figure 6.1: Agarose gel (2.5%) profile of 16s rRNA gene GC-Clamp PCR product stained with ethidium bromide. **Lane 1**: 50 bp marker; **lanes 2**: -ve control; **lane 3**: *P.gingivslis* (ATCC 33277), **lane 4**: *S. mutans* (NCTC 10449), **lane 5**: *S. gordonii* (DL1).

The exact size of the PCR fragment was calculated using the calibration curve constructed from the measured distance each band of the DNA marker had travelled and using the equation corresponding to the linear line of best fit. The resultant calculated size was 233.5 bp (Figure 6.2).

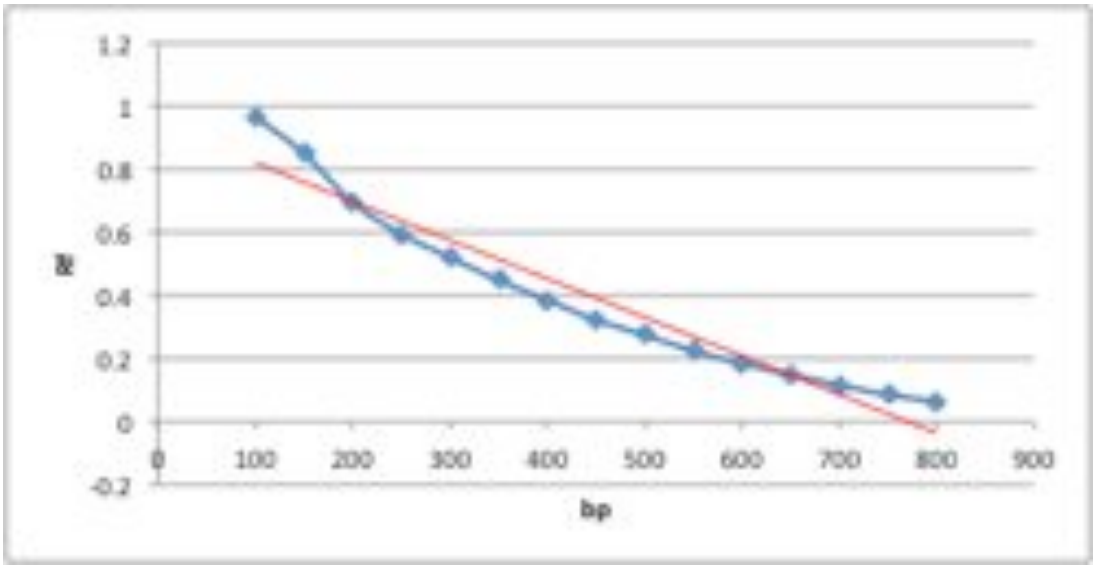

Figure 6.2: Calibration graph of bp size and the corresponding distance travelled for each band of the 50 bp DNA Marker.

6.2 DGGE conditions optimisation

DGGE analysis was performed as described earlier (refer to section 5.3), but due to the smaller size of the second PCR products, two denaturation concentration gradients were tested. The first ranged from 30%-60% and the second ranged from 25% to 45%. Both denaturation concentrations were successful in isolating the PCR fragments but the gradient range of 25%-45% appeared to differentiate more bands when compared to the higher concentration gradient (Figures 6.3, 6.4).

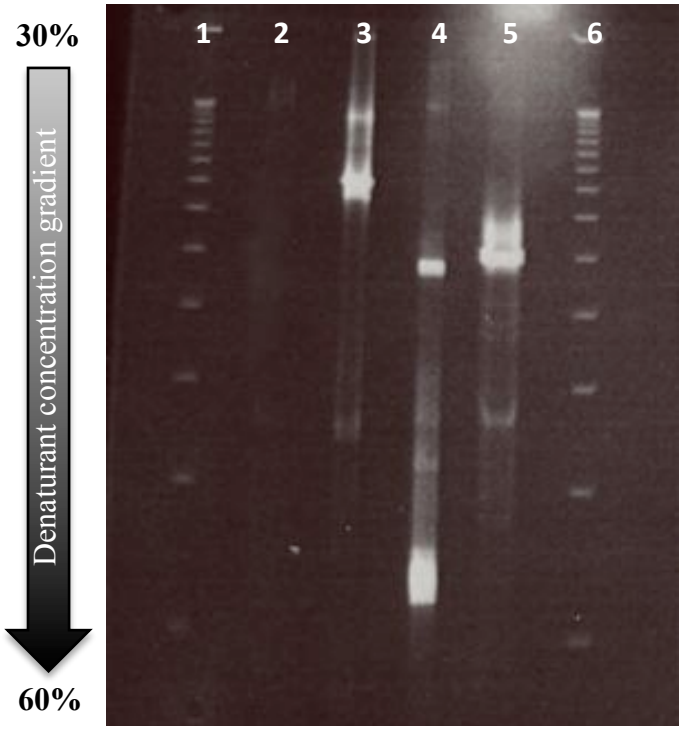

Figure 6.3: DGGE acrylamide gel (7.5%) with a denaturant concentration range of 30% to 60%. **Lanes 1,6**: 50bp, **lane 2**: -ve control, **lane 3**: *P.gingivalis* (ATCC 33277), **lane 4**: *S.mutans* (NCTC 10449), **lane 5**: *S. gordonii* (DL1).

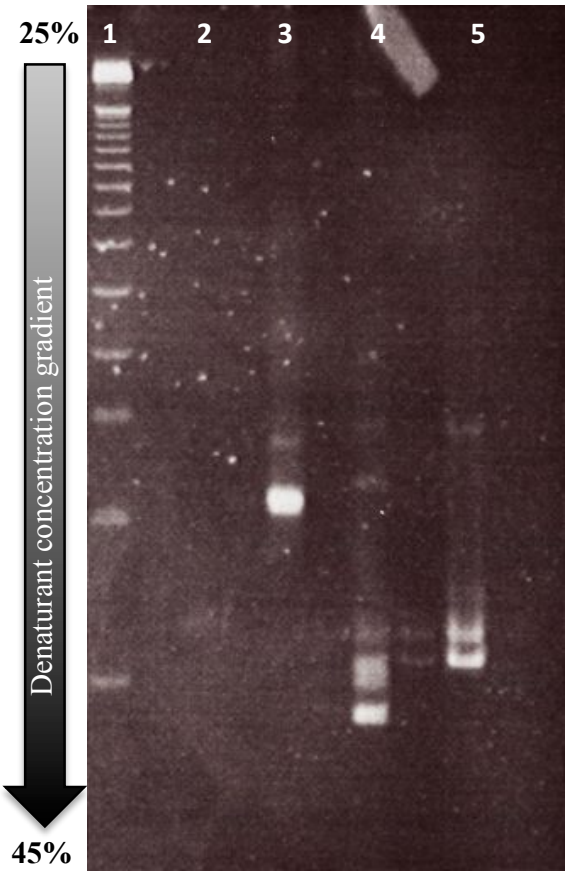

Figure 6.4: DGGE acrylamide gel (7.5%) with a denaturant concentration range of 25% to 45%. **Lane 1**: 50bp DNA marker, **lane 2**: -ve control, **lane 3**: *P.gingivalis* (ATCC 33277), **lane 4**: *S.mutans* (NCTC 10449), **lane 5**: *S. gordonii* (DL1).

The analysis was performed on three laboratory grown bacterial strains: *P.gingivalis* (ATCC 33277)*, S. mutans* (NCTC 10449) and *S. Gordonii* (DL1) (Figure 6.5). These bacteria are to be used as controls when analysing the red fluorescent plaque collected from the participants.

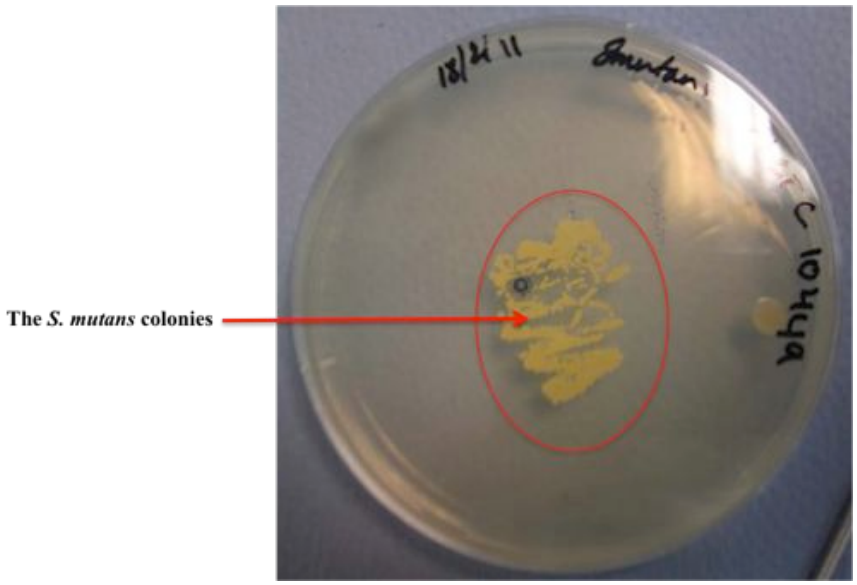

Figure 6.5: *S.mutans* (NCTC 10449) colonies after 72 hours of aerobic incubation at 37^0C on BHI agar.

Chapter 7

Molecular analysis of red fluorescent plaque

All the red fluorescent samples collected (n=80) from the 14 participants were analysed. The time frame of each plaque sample collected is presented in Table 7.1. T1 represents the base line sample collected prior to bracket placement. The following collected samples time frame is measured from the day of bracket placement. For example; participant 3 had two red fluorescent plaque samples identified and collected at base line and six months and ten days after bracket placement.

Total DNA was extracted first, then subjected to the first PCR amplification and then the second PCR amplification of the 16s rRNA genes and finally subjected to DGGE to separate the DNA fragments with the same bp size but with different base-pair sequence. The control samples were those of *P.gingivalis* (ATCC 33277)*, S. mutans* (NCTC 10449) and *S. gordonii* (DL1).

7.1 GC-clamp PCR

The fist PCR amplification DNA fragments were subjected to another PCR amplification reaction using the GC-clamp PCR primers. The resulting DNA fragment size was between 200 and 250 bp (Figures 7.1, 7.2, 7.3, 7.4, 7.5).

Table 7.1: The number and time (T) of visits for each participant and the plaque samples collected for any visit (S). Time for each visit is represented as months and days from bracket placement.

	Time in Months [Days]								
	T1	T2	T3	T4	T5	T6	T7	T8	T9
Participant 1	0	3^{6}	5^{6}	7^{6}	8^{17}	9^{29}	12^{8}	---	---
Plaque sample	S1	S2	S3	S4	S5	S6	S7	---	---
Participant 2	0	1^{11}	3^{0}	4^{11}	7^{24}	9^{20}	11^{7}	12^{8}	---
Plaque sample	S1	S2	S3	S4	S5	---	S7	S8	---
Participant 3	0	1^{11}	3^{6}	5^{1}	6^{10}	8^{5}	9^{5}	11^{10}	12^{14}
Plaque sample	S1	---	---	---	S5	---	---	---	---
Participant 4	0	1^{11}	2^{29}	4^{1}	7^{6}	8^{21}	10^{4}	11^{21}	13^{6}
Plaque sample	S1	S2	S3	S4	S5	S6	S7	S8	S9
Participant 5	0	1^{3}	3^{13}	4^{27}	8^{1}	---	---	---	---
Plaque sample	S1	---	S3	S4	S5	---	---	---	---
Participant 6	0	1^{11}	2^{22}	4^{1}	6^{17}	7^{28}	11^{11}	---	---
Plaque Sample	S1	S2	S3	S4	---	---	S7	---	---
Participant 7	0	2^{25}	4^{3}	6^{24}	8^{21}	11^{27}	---	---	---
Plaque sample	S1	S2	S3	S4	S5	----	---	---	---
Participant 8	0	3^{14}	5^{28}	10^{17}	11^{28}	---	---	---	---
Plaque sample	---	S2	---	---	---	---	---	---	---
Participant 9	0	1^{17}	2^{28}	5^{10}	6^{26}	8^{8}	9^{24}	10^{26}	12^{5}
Plaque sample	S1	S2	S3	S4	S5	S6	S7	S8	S9
Participant 10	0	1^{22}	4^{1}	5^{5}	6^{24}	9^{16}	11^{25}	---	---
Plaque sample	S1	S2	---	S4	S5	S6	S7	---	---
Participant 11	0	1^{14}	3^{12}	5^{14}	7^{29}	9^{8}	10^{13}	12^{8}	---
Plaque sample	S1	S2	S3	S4	S5	S6	S7	S8	---
Participant 12	0	1^{14}	2^{25}	4^{20}	5^{20}	8^{27}	10^{2}	11^{6}	12^{19}
Plaque sample	S1	S2	S3	S4	S5	S6	S7	---	S9
Participant 13	0	2^{0}	3^{8}	5^{9}	7^{1}	8^{5}	9^{17}	11^{16}	---
Plaque sample	S1	S2	S3	S4	S5	S6	S7	---	---
Participant 14	0	1^{22}	4^{0}	5^{2}	7^{11}	8^{24}	11^{4}	---	---
Plaque sample	S1	---	---	---	---	---	---	---	---

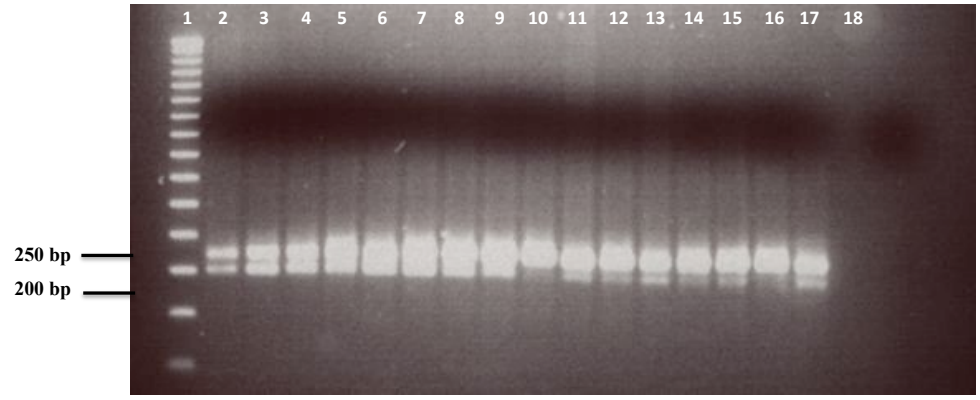

Figure 7.1: Agarose gel (2.5%) profile of GC-clamp 16s rRNA gene-PCR product stained with ethidium bromide. **Lane 1**: 50 bp marker, **lanes 2-8**: red fluorescent plaque samples 1-7 for participant 1, **lanes 9-15**: red fluorescent plaque samples 1-7 for participant 2, **lanes 16,17**: red fluorescent plaque samples 1-2 for participant 3, **lane 18**: -ve control.

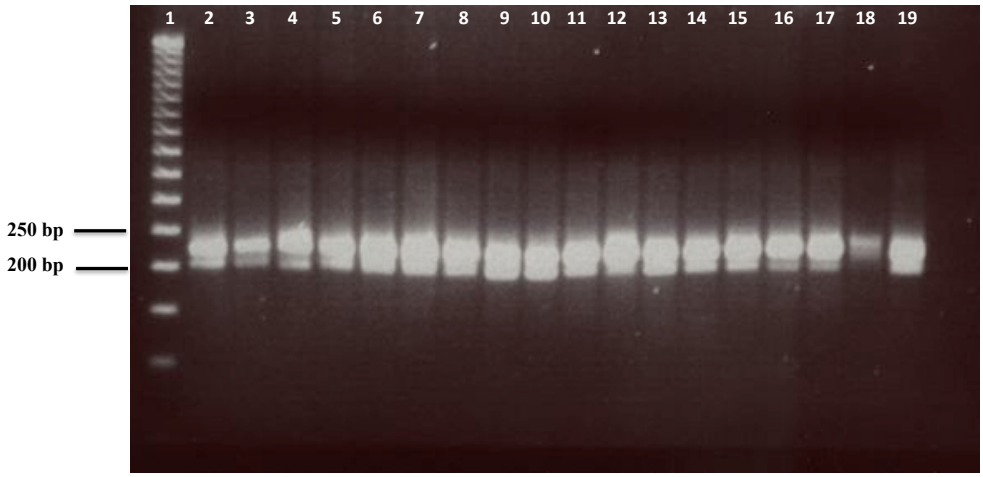

Figure 7.2: Agarose gel (2.5%) profile of GC-clamp 16s rRNA gene-PCR product stained with ethidium bromide. **Lane 1**: 50 bp marker, **lanes 2-10**: red fluorescent plaque samples 1-9 for participant 4, **lanes 11-14**: red fluorescent plaque samples 1-4 for participant 5, **lanes 15,19**: red fluorescent plaque samples 1-5 for participant 6.

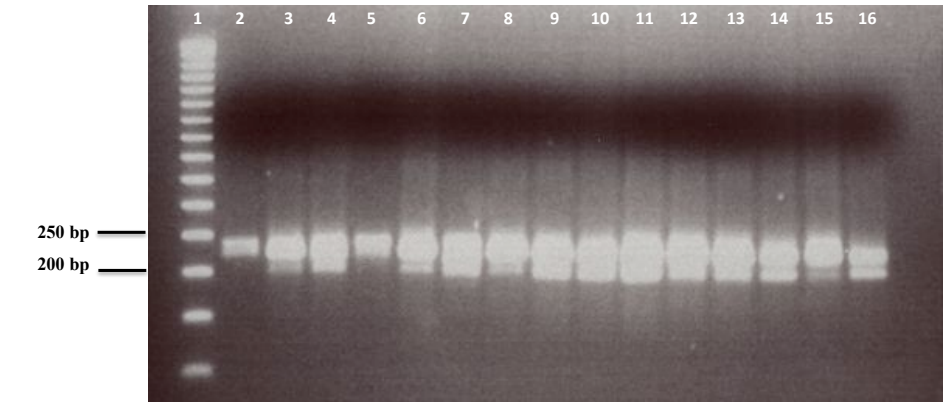

Figure 7.3: Agarose gel (2.5%) profile of GC-clamp 16s rRNA gene-PCR product stained with ethidium bromide. **Lane 1**: 50 bp marker, **lanes 2-6**: red fluorescent plaque samples 1-5 for participant 7, **lane 7**: red fluorescent plaque sample 1 for participant 8, lanes, **8-16**: red fluorescent plaque samples 1-9 for participant 9.

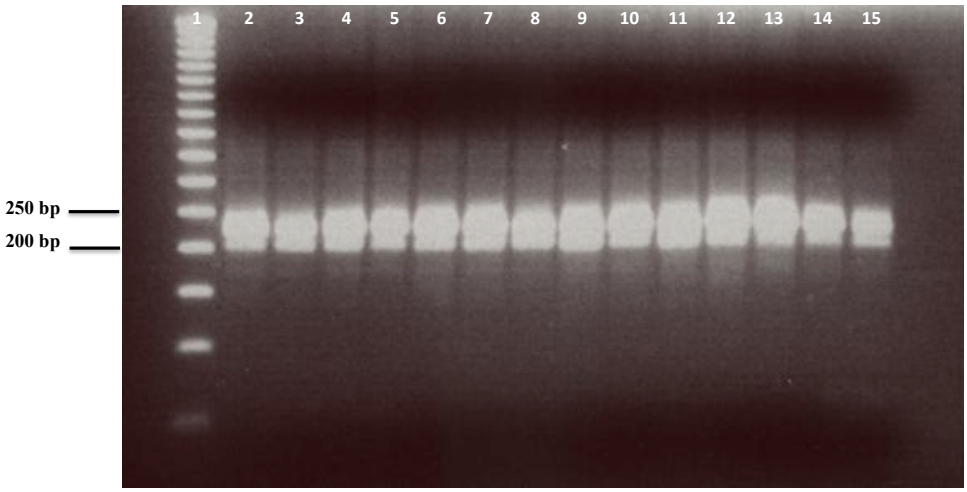

Figure 7.4: Agarose gel (2.5%) profile of GC-clamp 16s rRNA gene-PCR product stained with ethidium bromide. **Lane 1**: 50 bp marker, **lanes 2-7**: red fluorescent plaque samples 1-6 for participant 10, **lanes 8-15**: red fluorescent plaque sample 1-8 for participant 11.

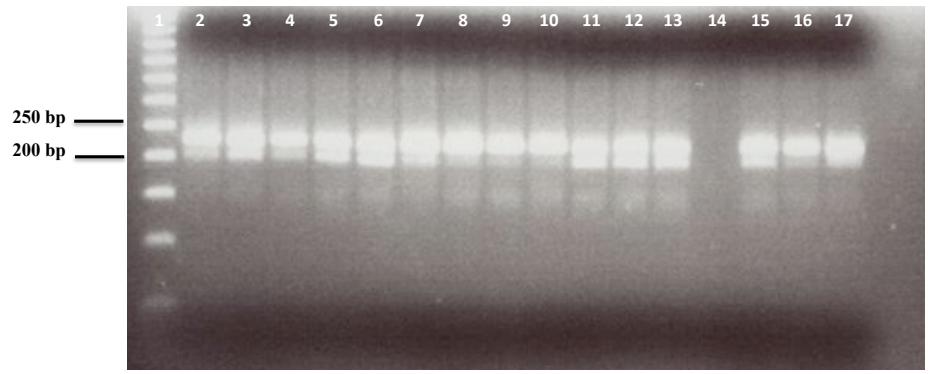

Figure 7.5: Agarose gel (2.5%) profile of GC-clamp 16s rRNA gene-PCR product stained with ethidium bromide. **Lane 1**: 50 bp marker, **lanes 2-9**: red fluorescent plaque samples 1-8 for participant 12, **lanes 10-16**: red fluorescent plaque sample 1-7 for participant 13, **lane 17**: red fluorescent plaque sample 1 for participant 14.

7.2 DGGE

The PCR amplification fragments were then subjected to DGGE analyses using the 25%-45% denaturing gradient. The gels were then stained with ethidium bromide and viewed under U.V. light (Figures 7.6, 7.7, 7.8, 7.9).

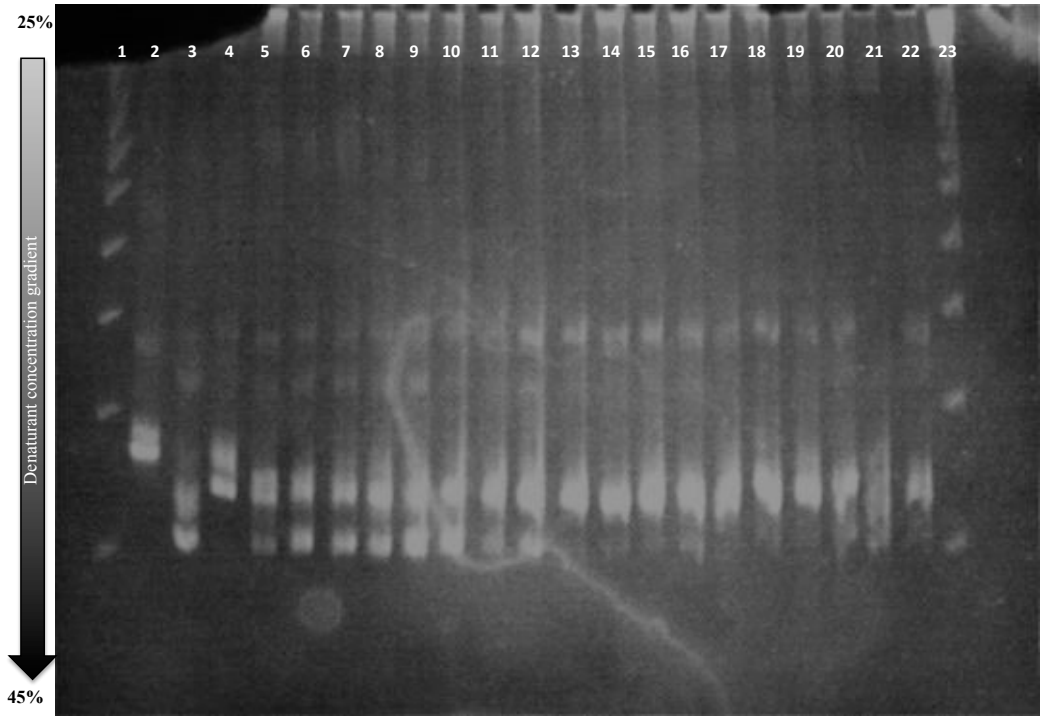

Figure 7.6: DGGE profiles from red fluorescent dental plaque samples and laboratory strains of oral bacteria stained with ethidium bromide. **Lanes 1,23**: 50 bp DNA marker, **lane 2**: *P. gingivalis* (ATCC 33277,) **lane 3**: *S.mutans* (NCTC 10449), **lane 4**: *S. gordonii* (DL1), **lanes 5-11**: red fluorescent plaque samples 1-7 for participant 1, **lanes 12-18**: red fluorescent plaque samples 1-7 for participant 2, **lanes 19-20**: red fluorescent plaque samples 1,2 for participant 3, **lane 21**: red fluorescent plaque sample 1 for participant 8 **lane 22**: red fluorescent plaque sample for participant 14.

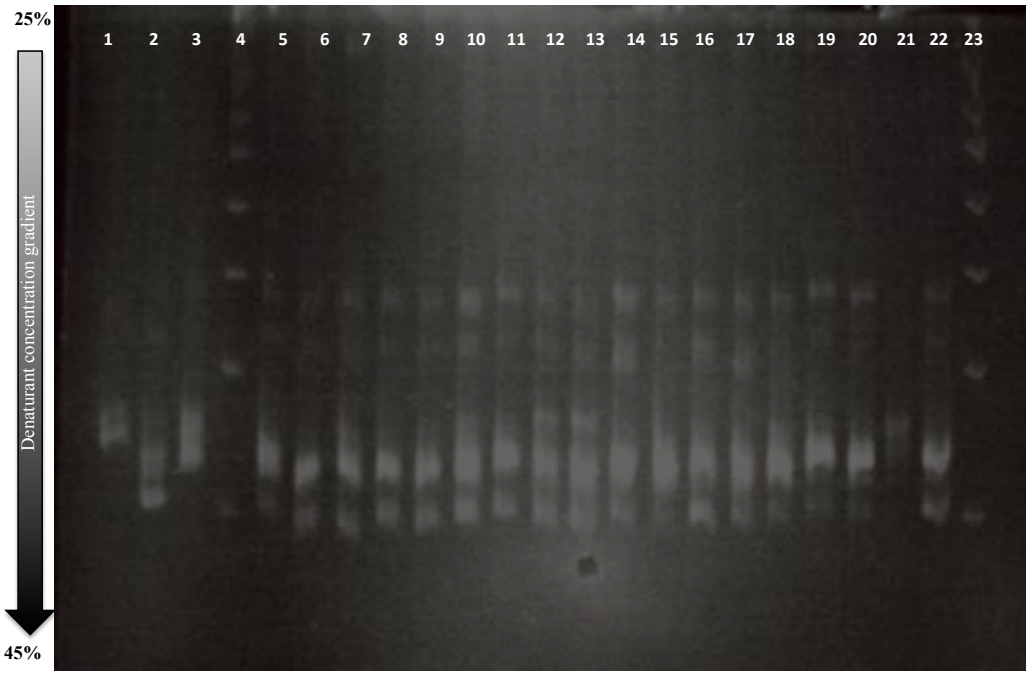

Figure 7.7: DGGE profiles from red fluorescent dental plaque samples and laboratory strains of oral bacteria stained with ethidium bromide. **Lane 1**: *P. gingivalis* (ATCC 33277), **lane 2**: *S.mutans* (NCTC 10449), **lane 3**: *S. gordonii* (DL1), **lanes 4,23**: 50 bp DNA marker, **lanes 5-13**: red fluorescent plaque samples 1-9 for participant 4, **lanes 14-17**: red fluorescent plaque samples 1-4 for participant 5, **lanes 18-22**: red fluorescent plaque samples 1-5 for participant 6.

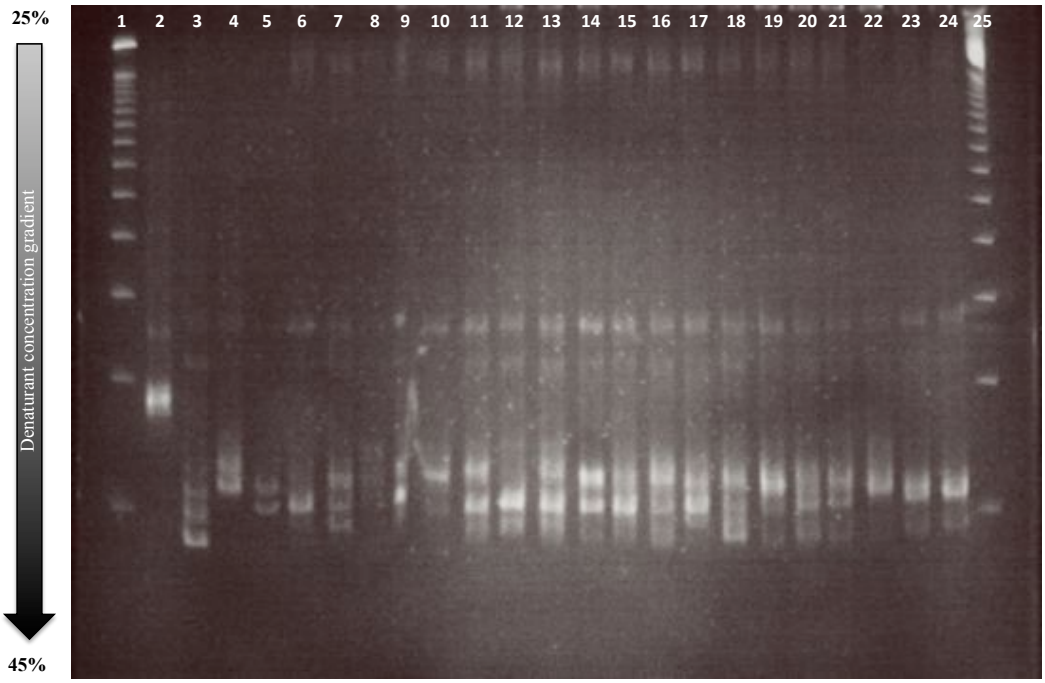

Figure 7.8: DGGE profiles from red fluorescent dental plaque samples and laboratory strains of oral bacteria stained with ethidium bromide. **Lanes 1,25**: 50 bp DNA marker, **lane 2**: *P. gingivalis* (ATCC 33277), **lane 3**: *S.mutans* (NCTC 10449), **lane 4**: *S. gordonii* (DL1), **lanes 5-9**: red fluorescent plaque samples 1-5 for participant 7, **lanes 10-18**: red fluorescent plaque samples 1-9 for participant 9, **lanes 19-24**: red fluorescent plaque samples 1-6 for participant 10.

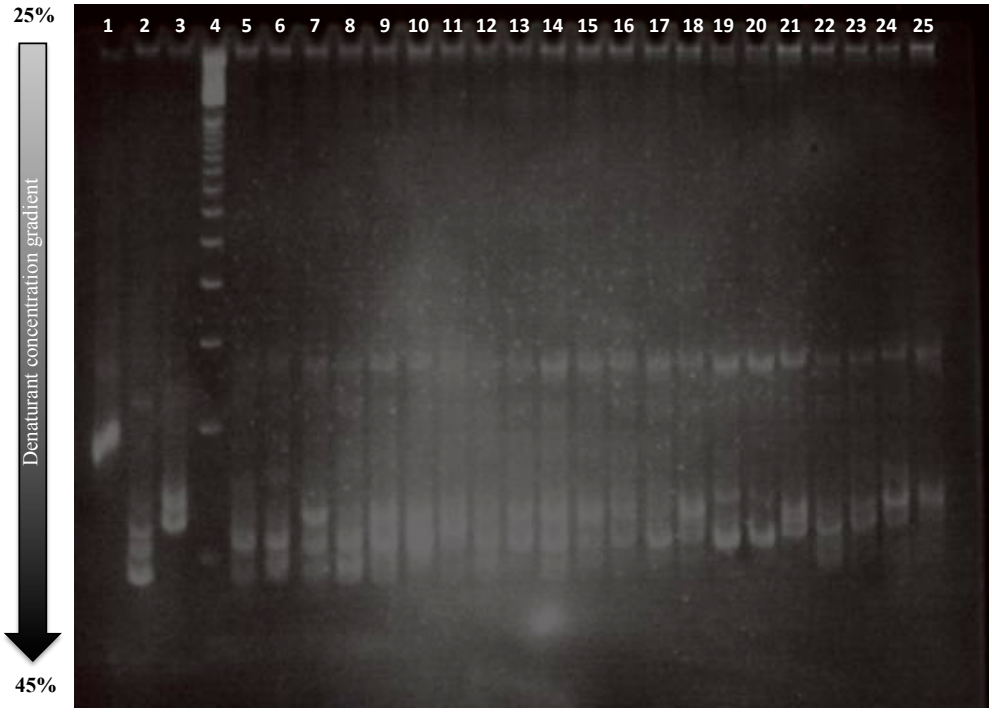

Figure 7.9: DGGE profiles from red fluorescent dental plaque samples and laboratory strains of oral bacteria stained with ethidium bromide. **Lane 1**: *P. gingivalis* (ATCC 33277), **lane 2**: *S.mutans* (NCTC 10449), **lane 3**: *S. gordonii* (DL1), **lane 4**: 50 bp DNA marker, **lanes 5-11**: red fluorescent plaque samples 1-7 for participant 11, **lanes 12-19**: red fluorescent plaque samples 1-8 for participant 12, **lanes 20-25**: red fluorescent plaque samples

7.2.1 The bacterial composition of red fluorescent supragingival dental plaque

The analysis of the red fluorescent dental plaque was based on the number of bands detected on the DGGE gel and the distances travelled by theses bands. The bands were then compared to the band profile of the three bacterial strains used as controls (*P.gingivalis* (ATCC 33277), *S.mutans* (NCTC 10449), *and S.gordonii* (DL1). Band identification and distances travelled were calculated using the TotalLab Quant Software, TotalLab Ltd., U.S.A (Figure 7.10). The volume of bands (corresponding to their concentration) was not compared because the loaded concentration was not known (Appendix H). The results will be presented for each participant separately (Tables 7.2, 7.3, 7.4).

7.2.1.1 Participant 1

Seven red fluorescent dental plaque samples were analysed. At base line, no bands corresponding to *P.gingivalis* were detected but bands corresponding to *S.mutans* and *S.gordonii* were detected. Eight months after fixed appliance placement when sample 5 was collected, a change in the band profile was detected as a new faint band (unknown band 1) appeared in the profile. This band was detected in the plaque samples collected during the remaining follow up period.

7.2.1.2 Participant 2

Seven red fluorescent dental plaque samples were analysed. At base line, no bands corresponding to *P.gingivalis* were detected but bands corresponding to *S.mutans* and *S.gordonii* were detected as well as an extra band. No changes in the band profile were detected during the follow up period.

7.2.1.3 Participant 3

Two red fluorescent dental plaque samples were analysed. At base line, no bands corresponding to *P.gingivalis* were detected but bands corresponding to *S.mutans* and *S. gordonii* as well as an extra band were detected. No changes in the bands profile were detected between the two samples collected at baseline and 6 months after bracket placement.

7.2.1.4 Participant 4

Nine red fluorescent dental plaque samples were analysed. At baseline, no bands corresponding to *P.gingivalis* were detected but bands corresponding to *S.mutans* and *S. gordonii* were detected. Three months after fixed appliance placement when sample 3 was collected, a change in the band profile was detected as a new faint band appeared in the profile (unknown band 1). Seven months after fixed appliance placement when sample 5 was collected another change in the band profile was detected, as another band was evident below the initial unknown band (unknown band 2). This band was not detected in the plaque samples collected during the remaining follow up period.

7.2.1.5 Participant 5

Four red fluorescent dental plaque samples were analysed. At base line, no bands corresponding to *P.gingivalis* were detected but bands corresponding to *S.mutans* and *S.gordonii* were detected as well as two unknown bands (unknown band 1 and 2) above the known bands. Three months after fixed appliance placement when sample 3 was collected the second unknown band was not detected, but was evident in the profile of the samples collected during the remaining follow up period.

7.2.1.6 Participant 6

Five red fluorescent dental plaque samples were analysed. At base line, no bands corresponding to *P.gingivalis* were detected but bands corresponding to *S.mutans* and *S.gordonii* were detected as well as unknown bands number 1 and 3. One month after fixed appliance placement when sample 2 was collected unknown band 3 was not detected. Four months after fixed appliance placement when sample 4 was collected only a band corresponding to *S.gordonii* was detected. Finally, after 11 months of fixed appliance placement when sample 7 was collected bands corresponding to both *S.mutans* and *S.gordonii* were detected.

7.2.1.7 Participant 7

Five red fluorescent dental plaque samples were analysed. At base line, no bands corresponding to *P.gingivalis* were detected but bands corresponding to *S.mutans* and *S.gordonii* were detected. Three months after fixed appliance placement when sample 2 was collected unknown band 1 was detected and no bands corresponding to *S. gordonii* were detected. Four months after fixed appliance placement when sample 3 was collected bands corresponding to *S. mutans* and *S. gordonii* were detected and unknown band 1 was lost. A final change was noted eight months after fixed appliance placement when unknown band 1 reappeared.

7.2.1.8 Participant 8

Only one red fluorescent plaque sample was collected and analysed where bands corresponding to *S.mutans* and *S. gordonii* were detected.

7.2.1.9 Participant 9

Nine red fluorescent dental plaque samples were analysed. At base line, no bands corresponding to *P.gingivalis* were detected but a band corresponding to *S.gordonii* and unknown band 1 were detected. One month after fixed appliance placement a band corresponding to *S. mutans* was detected. Three months after fixed appliance placement when sample 3 was collected the band corresponding to *S. gordonii* was not detected and unknown band 3 was detected. One visit later when sample 4 was collected, unknown sample 3 was not detected whereas *S. gordonii* was evident. Unknown band 3 was detected again in samples 7 and 9, 10 and 12 months following fixed appliance placement correspondingly.

7.2.1.10 Participant 10

Six red fluorescent dental plaque samples were analysed. At base line no bands corresponding to *P.gingivalis* were detected but bands corresponding to *S.mutans*, *S. gordonii*, unknown band 1 and unknown band 3 were detected. Seven months after fixed appliance placement when sample 4 was collected, unknown bands 1 and 3 were not detected, but were detected again nine months after fixed appliance placement when sample 6 was collected.

7.2.1.11 Participant 11

Eight red fluorescent dental plaque samples were analysed. At base line no bands corresponding to *P.gingivalis* were detected but bands corresponding to *S.mutans* were detected. When plaque sample 2 was collected almost 2 months following fixed appliance placement, a band corresponding to *S. gordonii* and unknown band 1 were detected. The band corresponding to *S. gordonii* was not detected five and eight months following fixed appliance placement when samples 4 and 5 were collected,

but was detected again when sample 6 was collected nine months after fixed appliance placement.

7.2.1.12 Participant 12

Eight red fluorescent dental plaque samples were analysed. At base line no bands corresponding to *P.gingivalis* were detected but bands corresponding to *S.mutans*, *S. gordonii* and unknown band 1 were detected. One month after fixed appliance placement when sample 2 was collected, unknown band 3 was detected. This band was not detected again. Five months after fixed appliance placement when sample 5 was collected, the band corresponding to *S. gordonii* was not detected, but was detected again the following visit nine months after fixed appliance placement when sample 6 was collected, but lost again after twelve months of fixed appliance placement when sample 9 was collected.

7.2.1.13 Participant 13

Six red fluorescent dental plaque samples were analysed. At base line no bands corresponding to *P.gingivalis* were detected but bands corresponding to *S.mutans* and unknown band 1 were detected. Two months later when sample 2 was collected, a band corresponding to *S.* gordonii was detected but lost again when sample 3 was collected three months after fixed appliance placement. Five months after fixed appliance placement no bands corresponding to *S. mutans* were detected when sample 4 was collected, unknown band 1, bands corresponding to *S. mutans* and *S. gordonii* were later detected eight months after fixed appliance placement when sample 6 was collected.

7.2.1.14 Participant 14

Only one red fluorescent plaque sample was collected at base line and analysed where bands corresponding to *S.mutans* and *S. gordonii* were detected.

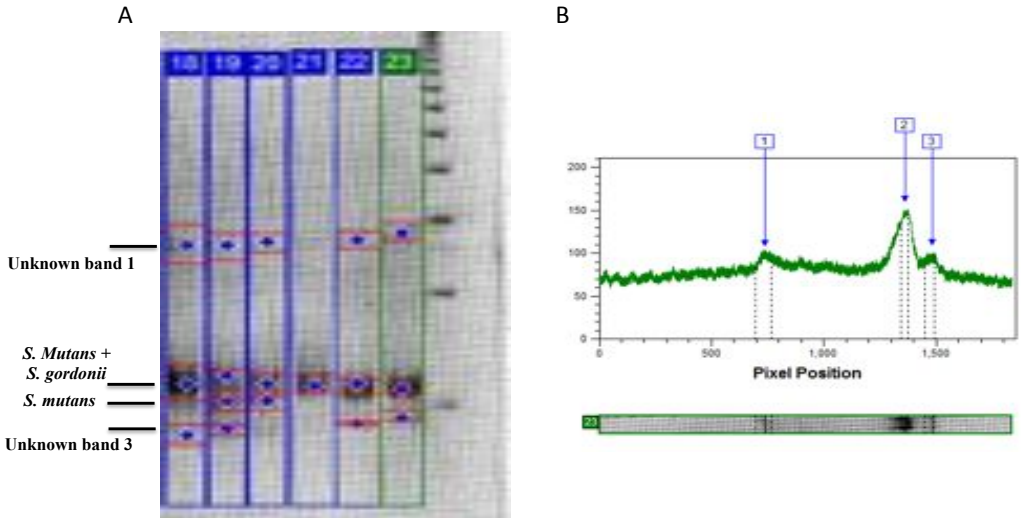

Figure 7.10: A: example of DGGE analysis performed with TotalLab Quant software for participant 10 on a negative view of the gel image. B: lane 23 analysis showing the bands and corresponding peaks.

When an association between the change in the composition of dental plaque and the development of white spot lesions was tested using chi-square test, there was no association between the two with a p value >0.05 (x^2= 2.53).

Table 7.2: The distribution of bands and their corresponding microorganisms according to the controls used and the incidence of white spot lesions (WSL) as detected with QLF for participants 1-5.

	Sample	*P. gingivalis ATCC33277*	*S. mutans NCTC 10449*	*S. gordonii DL1*	Unknown 1	Unknown 2	Unknown 3	WSL
Participant 1	1	-	+	+	-	-	-	-
	2	-	+	+	-	-	-	-
	3	-	+	+	-	-	-	-
	4	-	+	+	-	-	-	-
	5	-	+	+	+	+	-	-
	6	-	+	+	+	-	-	-
	7	-	+	+	+	-	-	+
Participant 2	1	-	+	+	+	-	-	-
	2	-	+	+	+	-	-	-
	3	-	+	+	+	-	-	-
	4	-	+	+	+	-	-	-
	5	-	+	+	+	-	-	-
	6	-	+	+	+	-	-	-
	7	-	+	+	+	-	-	+
3	1	-	+	+	+	-	-	-
	2	-	+	+	+	-	-	-
Participant 4	1	-	+	+	-	-	-	-
	2	-	+	+	-	-	-	-
	3	-	+	+	+	-	-	+
	4	-	+	+	+	-	-	+
	5	-	+	+	+	-	-	
	6	-	+	+	+	+	-	
	7	-	+	+	+	-	-	+
	8	-	+	+	-	-	-	
	9	-	+	+	-	-	+	+
Participant 5	1	-	+	+	+	+	-	-
	2	-	+	+	+	-	-	-
	3	-	+	+	+	+	-	-
	4	-	+	+	+	+	-	-

Table 7.3: The distribution of bands and their corresponding microorganisms according to the controls used and the incidence of white spot lesions (WSL) as detected with QLF for participants 6-10.

	Sample	*P. gingivalis ATCC 33277*	*S. mutans NCTC 10449*	*S. gordoii DL1*	Unknown 1	Unknown 2	Unknown 3	WSL
Participant 6	1	-	+	+	+	-	-	-
	2	-	+	+	+	-	-	-
	3	-	+	+	+	-	-	-
	4	-	+	+	-	-	-	+
	5	-	+	+	-	-	-	+
Participant 7	1	-	+	+	+	-	-	-
	2	-	+	+	+	-	-	-
	3	-	+	+	+	-	-	-
	4	-	+	+	+	-	-	-
	5	-	+	+	+	-	-	-
	6	-	+	+	+	-	-	-
	7	-	+	+	+	-	-	+
8	1	-	+	+	-	-	-	+
Participant 9	1	-	-	+	+	-	-	-
	2	-	+	+	+	-	-	-
	3	-	+	+	+	-	-	-
	4	-	+	+	+	-	-	-
	5	-	+	+	+	-	-	-
	6	-	+	+	+	-	-	-
	7	-	+	+	+	-	+	-
	8	-	+	+	+	-	-	-
	9	-	+	+	+	-	-	+
Participant 10	1	-	-	+	+	-	+	-
	2	-	+	+	+	-	+	-
	3	-	+	+	+	-	-	+
	4	-	+	+	-	-	-	
	5	-	+	+	+	-	+	+
	6	-	+	+	+	-	+	+

Table 7.4: The distribution of bands and their corresponding microorganisms according to the controls used and the incidence of white spot lesions (WSL) as detected with QLF for participants 11-14.

	Sample	*P. gingivalis ATCC 33277*	*S. mutans NCTC 10449*	*S. gordonii DL1*	Unknown 1	Unknown 2	Unknown 3	WSL
Participant 11	1	-	+	+	-	-	-	-
	2	-	+	+	+	-	-	-
	3	-	+	+	+	-	-	-
	4	-	+	-	+	-	-	-
	5	-	+	-	-	-	-	-
	6	-	+	+	+	-	-	-
	7	-	+	+	+	-	-	-
	8	-	+	+	+	-	-	-
Participant 12	1	-	+	+	+	-	-	-
	2	-	+	+	+	-	+	-
	3	-	+	+	+	-	-	+
	4	-	+	+	+	-	-	-
	5	-	+	-	+	-	-	-
	6	-	+	+	+	-	-	-
	7	-	+	+	+	-	-	+
	8	-	+	-	+	-	-	-
Participant 13	1	-	+	-	+	-	-	-
	2	-	+	+	+	-	-	-
	3	-	+	+	+	-	-	-
	4	-	-	+	+	-	-	+
	5	-	+	+	+	-	-	+
	6	-	-	+	+	-	-	+
14	1	-	+	+	+	-	-	-

Chapter 8

Identification and monitoring of white spot lesions

8.1 Method

8.1.1 Lesion identification

QLF images were obtained at base line (before fixed appliances placement) and every second orthodontic appointment. That was approximately every 12 weeks. Images of individual anterior segment teeth (canine to canine) were captured, stored and then analysed with the QLF Inspektor Pro software (Quantitative Light-Induced Fluorescence 1.93e, Inspektor Research Systems B.V., The Netherlands).

Early signs of enamel demineralisation were identified visually as darker areas of less fluorescence on the individual tooth image.

8.1.2 Lesion quantification

Demineralisation was quantified by measuring the fluorescence loss from the enamel surface. The value of fluorescence loss was reconstructed by interpolation of sound enamel fluorescence surrounding the demineralised enamel. The border of the enamel lesion adjacent to the orthodontic bracket was excluded from the analysis to eliminate the effect of the non-fluorescent bracket image on the analysis. The difference between original pixel values and the reconstructed sound pixel values of areas with fluorescence levels below the threshold of 95% gave the resulting fluorescence loss in the demineralised enamel. This difference was expressed as a

change in fluorescence radiance (ΔF, %). The programme also calculated the area of demineralised enamel in mm^2. The multiplication of these two variables results in a third metric output (ΔQ,%).

8.2 Results

A total of 14 participants agreed to take part in this study, 8 females and 6 males, age range 12^7-22 years (mean $14^9 \pm 3^2$ years). Following attainment of consent/assent, QLF images were obtained before the placement of the fixed appliances and at every other orthodontic appointment, and then stored for analysis (Table 8.1). Participants receiving a single arch fixed orthodontic appliance had QLF images taken of that arch only.

Table 8.1: Number of visits, QLF images collected and white spot lesions identified per participant.

Participant	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Number of visits	7	8	9	9	5	7	6	5	9	7	8	9	8	7
Number of visits QLF images were obtained	4	4	5	5	2	4	4	3	5	4	5	5	4	4
Total number of QLF images obtained	20	24	60	45	10	48	16	36	60	48	50	60	48	48
Number of sites with identified white spot lesions	1	1	0	2	0	4	3	1	2	3	0	2	5	0

A total of 573 QLF images were obtained for the 14 participants, four of which had upper fixed appliances only. One participant moved from Liverpool and had only one set of QLF images taken following the placement of the fixed appliance.

The images taken before the placement of the fixed appliances were used as a baseline to detect any signs of enamel opacities or demineralisation that might have been present before orthodontic treatment. The following images were taken after the placement of the orthodontic brackets. During analysis, the area covered by the orthodontic bracket was excluded from the analysis, as the software might record it as demineralisation due to the lack of fluorescence (Figure 8.1).

Another effect of the bracket was the reduction in the fluorescence of the surrounding tooth structure. That led to the images being significantly darker when compared to the baseline images, especially for the lower incisors. The brightness of the images had to be adjusted prior to any analysis. To ensure that the adjustment of the brightness had no effect on the analysis of fluorescence, a number of images were analysed before and after adjustment and no difference in the fluorescence value was detected.

Four out of the fourteen participants (28.6%) did not show any signs of demineralisation throughout the observation period. Out of the ten remaining participants, six (60%) showed signs of demineralisation at the first QLF recording and four showed signs of demineralisation at the last QLF recording. The participants that had early signs of demineralisation had larger lesions on a larger number of sites, whereas the participants that developed demineralisation later on during their treatment had smaller lesions on a single site (Table 8.2).

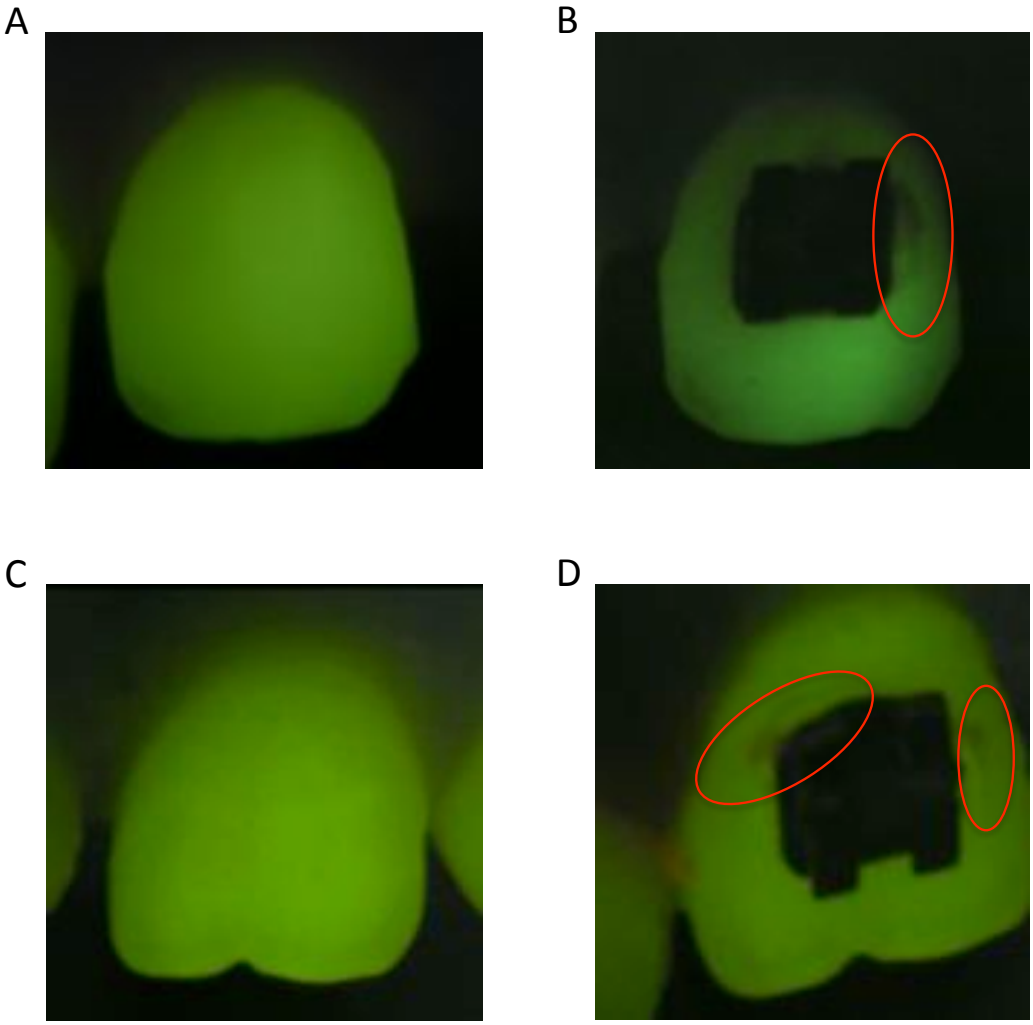

Figure 8.1: QLF images of an upper right lateral incisor at base line (A) and 5 months after bracket placement (B) for participant 13 and of an upper right central incisor at base line (C) and five months (two visits) after bracket placement (D) for participant number 4. Signs of demineralisation are circled red.

Table 8.2: The distribution of white spot lesions detected with QLF, their average size in mm^2 and the time when first signs of demineralisation was detected for each participant.

Participant	First sign of demineralisation	Average white spot lesion area mm^2											
		UR3	UR2	UR1	UL1	UL2	UL3	LL3	LL2	LL1	LR1	LR2	LR3
1	UL2 at 12 months	-	-	-	-	2.29	-	-	-	-	-	-	-
2	UL3 at 12 months	-	-	-	-	-	0.99	-	-	-	-	-	-
3	None	-	-	-	-	-	-	-	-	-	-	-	-
4	UR1 at 3 months	-	0.53	1.05	-	-	-	-	-	-	-	-	-
5	None	-	-	-	-	-	-	-	-	-	-	-	-
6	UR2 at 4 months	1.06	0.96	-	-	-	0.25	-	0.23	-	-	-	-
7	UR2 at 6 months	-	1.19	0.22	1.63	-	-	-	-	-	-	-	-
8	UR2 at 12 months	-	0.27	-	-	-	-	-	-	-	-	-	-
9	UR 3,1 at 12 months	0.13	-	1.02	-	-	-	-	-	-	-	-	-
10	UR3, 1, UL3 at 5 months	1.00	-	1.61	-	-	0.34	0.23	-	-	-	-	-
11	None	-	-	-	-	-	-	-	-	-	-	-	-
12	UR3, 2 at 3 months	0.75	0.88	-	-	-	-	-	-	-	-	-	-
13	UR3, 2, 1 at 5 months	0.58	2.64	0.43	0.71	0.59	-	-	-	-	-	-	-
14	None	-	-	-	-	-	-	-	-	-	-	-	-

The overall incidence of white spot lesions was 4.2%. The mean number of white spot lesions was 1.7 ± 1.5 per participant. The size of lesions ranged from 0.08mm^2 to 5.62mm^2, with a median value of 0.76mm^2. None of the lesions were identified clinically (Figure 8.2).

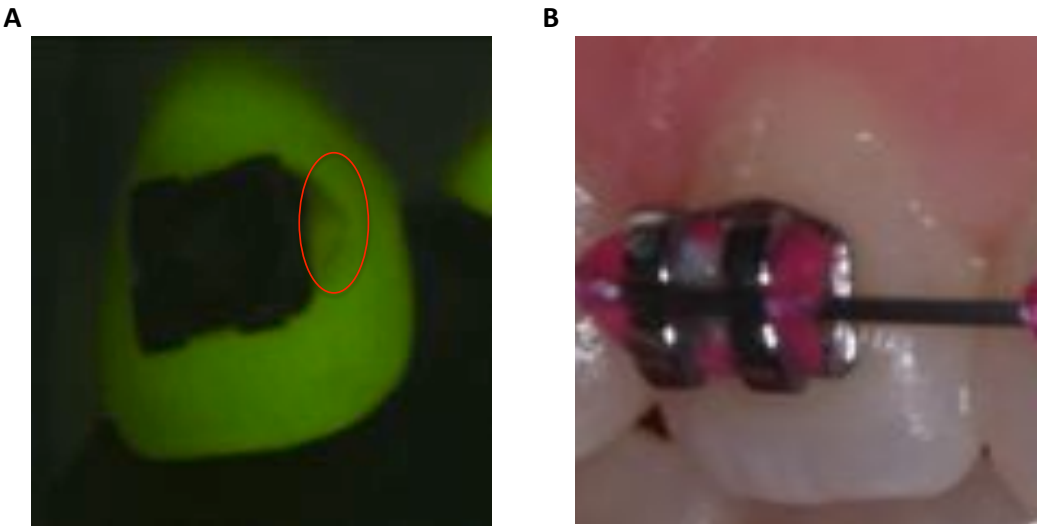

Figure 8.2: (A) QLF image of the upper right lateral incisor for participant number 6 where demineralisation was detected (ΔF= -22.2%, area= 1.19mm^2) and a clinical photograph of the same tooth with no evidence of demineralisation (B).

Chapter 9

Discussion

The present study was conducted to investigate the bacterial composition of supragingival plaque and any changes in its composition following the placement of fixed orthodontic brackets in adolescents. The QLF and Toothcare™ techniques were employed to identify the presence of red fluorescent plaque around the fixed orthodontic brackets. Molecular techniques were used to look into the bacterial composition of the identified red fluorescent plaque. The development of enamel white spot lesions on the labial surfaces of the anterior teeth during a period of twelve months of fixed orthodontic appliance treatment was also monitored.

9.1 Identification of red fluorescent plaque

Dental plaque contains a large variety of bacteria but only a few, especially mutans streptococci, are believed to be associated with the development of dental caries (Tanzer et al., 2001). The longer the plaque is retained on the tooth surface and the greater the quantity, the higher the chances that demineralisation will take place(Hadler-Olsen et al., 2011). Large quantities of mature plaque tend to fluoresce red or orange when viewed using QLF. This autofluorescence phenomenon is caused by the ability of certain oral microorganisms to synthesise endogenous metal-free fluorescent porphyrins (Coulthwaite et al., 2006; Pretty et al., 2005; van der Veen & de Josselin de Jong 2000). In this study, Toothcare™, which is a portable and easy to use hand-held device that is based on the principle utilised by QLF of detecting bacterial autofluorescence was use to identify supragingival red fluorescent plaque.

In this study, a single surface of any of the included teeth was inspected at a time to identify the presence of red fluorescent plaque. Pooling the plaque was avoided to eliminate any effect that different anatomical sites might have on the type of bacteria in the plaque. The lateral incisors were chosen to be the first teeth to be inspected, followed by the canines and finally the central incisors, as it was reported that the lateral incisors and canines were the most common teeth to show signs of demineralisation after orthodontic treatment (Mizrahi 1983).

The results showed that the upper right lateral incisor was the most common site for red fluorescent plaque to be identified and collected, accounting for 35% of the total samples collected, followed by the upper right canine and the lower left lateral. This needs to be interpreted with caution as only the site where the red fluorescent plaque was collected from was recorded according to the sequence of site inspection decided upon in this study. Other sites might have had red fluorescent plaque identified on them but were not recorded. Therefore, the surface distribution of red fluorescent plaque is only related to the sites where plaque was collected from and not the overall distribution in relation to the twelve teeth per participant included in the study.

The sites where red fluorescent plaque was collected from were correlated with the development of white spot lesions using chi square test statistical test. No significant relationship was established between the presence of red fluorescent plaque and the incidence of white spot lesions. At some surfaces, where red fluorescent plaque was present, no signs of demineralisation were detected (24.4%), and at other surfaces, identified white spot lesions (11.4%) did not have signs of red fluorescent plaque on presentation. One confounding factor that might play a role in

this is when the participants brush their teeth well just before their appointment, removing all red fluorescent plaque where white spot lesions are detected. Therefore, it cannot be definitely assumed that white spot lesions might develop in the absence of red fluorescent plaque and that the presence of red fluorescent plaque does not necessarily lead to white spot lesion development.

9.2 Identification of red fluorescent plaque bacterial composition

Molecular techniques were chosen to study the bacterial composition of the collected supragingival red fluorescent plaque. The culture-based methods have been traditionally used to identify plaque bacteria but they cannot identify non-cultivable species and bacteria present in small numbers. Molecular techniques, on the other hand, have proven to overcome the limitations associated with traditional methods of bacterial identification, as they do not rely on bacterial growth in the laboratory (Aas et al., 2008; Li et al., 2004).

PCR amplification of the hypervariable16s rRNA genes and DGGE analysis was performed on the bacterial genomic DNA isolated from the red fluorescent supragingival plaque samples collected. Each bacteria has a unique 16s rRNA gene sequence that can be identified on the DGGE as separate bands with a specific migration distances producing a fingerprint for each bacteria (Li et al., 2007).

The DGGE band profile of the collected red fluorescent supragingival plaque was compared to the band profile of three known bacterial species; *P.gingivalis* (ATCC 33277)*, S.mutans* (NCTC 10449) and *S.gordonii* (DL1). The initial DGGE analysis confirmed the presence of a number of different bacterial species in the

collected plaque samples, but accurate identification of the number of bands and the distances travelled by those bands was difficult. That was mainly due to the fact that the separation of the bands took place at the top end of the denaturing gel where the lower denaturant concentration is present. This resulted in the bands being so closely related to each other, which made it difficult to distinguish individual bands. The PCR primers used resulted in a large size amplicons, which were about 1,500 bp. These amplicons denatured at low denaturant concentration limiting the distance they can move down the gel.

From the initial analysis of red fluorescent plaque, no differences in the band separation profile was noted when comparing different plaque samples collected at different times following orthodontic bracket placement for the same participant, but different band separation profiles were noted to exist when comparing plaque sample profiles for different participants.

The initial DGGE profile analyses did not yield any valuable conclusions; therefore the method was modified by changing the PCR primers and conditions to yield more distinguishable PCR amplicons. Incorporating a GC-rich sequence (clamp at the 5' end of the primer) was reported to increase the resolution of fragments in the denaturing gradient (Muyzer et al., 1993). Therefore, a nested PCR reaction, where the amplicons obtained from the first PCR reaction were subjected to subsequent PCR amplification using the GC-rich primers, was used. A further modification was made by reducing the denaturing gradient concentration to allow the amplicons to travel longer distances and to make distinguishing of individual bands easier.

It was clear from the DGGE profile (figures 7.6, 7.7, 7.8, 7.9; pages 80-83) that each of the three different bacterial species had a unique band separation profile, which was used as a reference to compare the separation distances of the dental plaque sample bands. The modified PCR-DGGE method confirmed the initial finding that different participants might have different band profiles on the DGGE and therefore potentially different bacterial components.

None of the collected supragingival red fluorescent plaque samples showed bands corresponding to *P.gingivalis* (ATCC 33277) as expected, because *P.gingivalis* is a predominantly subgingival bacteria usually found in the gingival crevice (Fujimoto et al., 2003). When comparing the bands and the distance travelled down the denaturing gel for the collected plaque samples to the band and the distances travelled for *S.mutans* (NCTC 10449) and *S.gordonii* (DL1), it was found that for almost all the samples, the bands corresponding to both bacterial species could be identified. For the remaining samples, either *S.mutans* (NCTC 10449) or *S.gordonii* (DL1) bands were not detected, but they were not both absent at the same time. Therefore, for all the fourteen participants in this study where red supragingival plaque was collected, both *S.mutans* (NCTC 10449) and *S.gordonii* (DL1) bacterial species were detected in the collected samples.

S.gordonii (DL1) is one of the early plaque colonisers that facilitate the attachment of other bacterial to tooth surface. Therefore they are indirectly associated with dental caries as they encourage the adherence of more cariogenic bacteria (Kilic et al., 2004). Therefore detection of *S.gordonii* (DL1) in the plaque samples in this study was not surprising.

S.mutans is one of the bacteria that can coaggregate with *S.gordonii* and play a role in the development of older plaque (Palmer, Jr. et al., 2003). Moreover, *S.mutans* has been studied extensively in relation to cariogenesis and there have been numerous studies that attempted to link it to the development of dental caries. The presence of *S.mutans* in high concentrations had been associated with the development of caries in children (Alaluusua and Renkonen 1983; Choi et al., 1994; Kishi et al., 2009) and it was reported that suppression of *S.mutans* may inhibit dental caries formation (Tanzer et al., 1985).

Therefore, the presence of *S.mutans* in the plaque samples studied, suggests that red fluorescent plaque might have a pathogenic role in the development of dental caries in adolescent orthodontic patients. *S.mutans* were detected in plaque samples collected from all the participants in this study, but not all the participants developed dental caries or showed signs of demineralisation detectable with QLF or by clinical inspection. It was suggested in the literature that the concentration and the presence of certain strains of *S.mutans* that are resistant to proteases is the main causative factor of dental caries (Phattarataratip et al., 2011).

In this study, only the presence of one strain of *S.mutans* (NCTC 10449) was investigated without quantification. Therefore, presence of different strains and concentrations of *S.mutans* in the plaque cannot be ruled out. Therefore further work needs to be done to establish if the participants that developed white spot lesions have harboured different strains and/or concentrations of *S.mutans* in their plaque.

S.gordonii as an early coloniser is necessary for attachment and colonisation of other bacteria including *S.mutans,* but the presence of *S.gordonii* might inhibit the colonisation of certain *S.mutans* species (CSP and MB71) as suggested by Wang and co-workers (Wang et al. 2011). Therefore, the fact that *S.gordonii* were almost always detected in the plaque samples alongside *S.mutans* might have an inhibitory effect on *S.mutnas* and subsequently inhibit the development of dental caries and enamel demineralisation in some participants.

The other important finding was of the changes in the band profile seen when analysing different plaque samples for the same participants collected at different time points during the twelve months of fixed orthodontic treatment follow up period. Analysis of the red fluorescent plaque samples that showed changes in the DGGE profile and the development of white spot lesions, using chi square statistical test, showed no statistically significant association ($p>0.05$). This suggests that the development of white spot lesions is not caused by a change in the composition of supragingival dental plaque.

Three bands with a migration distance that did not match the migration distances of bands representing *P.gingivalis, S.mutans* or *S.gordonii* were detected. The most commonly found band was labelled number 1 as it travelled the least distance on the denaturant gel. Those three bands correspond to unknown bacterial species that might be implicated in the development of white spot lesions. Although no association was found between the presence of these bands and the development of white spot lesions, those bands need to be excised from the gel and sequenced so that the bacterial species can be identified.

9.3 Identification and monitoring of white spot lesions

Demineralisation and the development of white spot lesions is one of the major risks associated with orthodontic treatment. In this study, presence or absence of initial enamel demineralisation on the buccal surfaces and around fixed orthodontic brackets was identified with QLF. QLF is a method used to identify early signs of demineralisation that might not be evident by clinical inspection. This early identification allows earlier intervention and possible reversal of the demineralisation process at its initial stages (Benson et al., 2003; Buchalla et al., 2002; Lee et al., 2010; Mattousch et al., 2007).

Pre-existing demineralisation or enamel opacities were recorded prior to commencement of orthodontic treatment. None of the participants in this study showed signs of demineralisation at baseline. The prevalence of white spot lesions at the end of the twelve-month observation period was 4.2% of the labial surfaces of the twelve anterior teeth included in this study, where 60% of the participants had some signs of demineralisation on one or more surfaces of any of the twelve teeth, but no significant correlation was established between the development of white spot lesions and changes in the bacterial composition of red fluorescent supragingival dental plaque. This falls within the reported prevalence range on white spot lesions amongst orthodontic patients of 2-96% (Årtun & Brobakken 1986; Gorelick et al., 1982; Mizrahi 1982; Mizrahi 1983; Øgaard et al.,1988; Zachrisson & Zachrisson 1971). The available literature reported a wide range of white spot lesion prevalence due to the variability of orthodontic treatment length, the examination technique used and if lesions present prior to orthodontic treatment.

The medial value for the recorded white spot lesions was <1mm^2 (0.76mm^2), which is considerably small and subclinical. Therefore, none of the white spot lesions identified with QLF were visible clinically. This reinforces the sensitivity of QLF to detect enamel demineralisation at early stages where it is possible to remineralise the demineralised sections. All patients with signs of early enamel demineralisation were given further oral hygiene instructions and advised to use a daily sodium fluoride mouth wash to attempt to reduce the occurrence and severity of enamel white spot lesions and possibly remineralise the demineralised sections as suggested in the literature (Benson et al., 2005).

9.4 Limitations of the study

9.4.1 Sample

A search of the available literature on the study of dental plaque composition in adolescent orthodontic patients did not result in any related studies to be used as guidance for sample size calculation. An independent statistician suggested that a sample size of 30 would be acceptable. Due to time limitations and availability of suitable orthodontic patients prior to the commencement of this study, only fourteen participants that fulfilled the inclusion criteria agreed to take part. This resulted in a relatively smaller sample size and may have had an effect on the robustness of the study. One participant withdrew from the study because they moved away from the area and were transferred to another orthodontist. Thirteen participants completed the 12 months follow up period of the study.

Including a wide age range in the study can have an effect on the type of supragingival plaque. The different eating and diet habit between an eleven and a twenty three year old, especially the sugar content of the diet, can have a significant effect on the type and amount of bacteria present in the plaque. A follow up of the diet habits during the period of the study can overcome this problem and help subdivide the participants into two groups; one with high sugar intake and one with low sugar intake.

9.4.2 Analysis of red fluorescent plaque

Red fluorescent supragingival plaque was preferred for analyses over non-fluorescent plaque because it was believed that red fluorescent plaque is older and more pathogenic when compared to non-fluorescent plaque. Red fluorescent plaque from only one site of any of the twelve teeth included in this study was collected for further analyses. Differences in the degree of protection from oral environment factors and in the gradients of any chemical factors exists between different sites on different teeth and might have an effect on the types of bacteria present in the plaque at any particular site. This resulted in very small volumes of collected plaque, which lead to difficulties in isolation of bacterial genomic DNA. The amount of isolated genomic DNA was too small; therefore no attempts were made to quantify it. Instead, the entire recovered DNA was used for molecular analyses. As a consequence of that, the results were descriptive but not quantitative.

The DGGE analyses of the plaque samples were limited with only three bacterial species as controls. The extra bands detected in the gels were not identified and need to be excised, cleaned and sequenced..

The DGGE profile for *P.gingivalis* (ATCC 33277) had one distinctive band, but for *S.mutans* (NCTC 10449) and *S.gordonii* (DL1) had more than one band, which is considered to be one of the draw backs of DGGE. This might suggest that either more than one strain were present in the grown culture or that multiple copies of the 16s rRNA genes are present for a single species, which is more likely to be the case as reported elsewhere (Machado de Oliveira et al. 2007). Another limitation of DGGE is the fact that it is based on the assumption that DNA is equally extracted from all bacterial species present in the plaque (Siqueira, Jr. & Rocas 2005; Suzuki & Giovannoni 1996), only bacteria present in high concentrations in the plaque might be represented on the gel (Muyzer & Smalla 1998) and that multiple copies of the 16s rRNA gene might be present for a single species of bacteria. Therefore, the extra bands detected on the gel of the collected plaque samples might be related to different bacteria or to the same bacteria, and unless the bands are excised and sequenced for the exact DNA component, the fact that different bacteria might be detected at different time frames cannot be excluded.

9.4.3 Obtaining QLF images

QLF images were obtained at base line and at every second visit. The base line images were obtained easily. Difficulties in obtaining images suitable for analysis were however encountered once the orthodontic brackets were placed. The brackets obscured most of the labial surface of the teeth, which led to a reduction in the amount of fluorescence of the teeth. The images were therefore dark and difficult to analyse, with the lower incisor teeth being the most difficult to analyse. In addition, severely malaligned teeth proved to be difficult to image at the start of treatment. One participant had severely rotated upper canines and one participant's upper canine was very distally positioned in the mouth and was in contact with the first molar, this made positioning the hand piece at the correct angle very difficult. These problems were resolved once the teeth were aligned.

The darker images were adjusted for brightness prior to analysis to facilitate distinguishing between sound enamel and demineralised enamel. This proved to be helpful in enabling the successful analysis of the teeth.

QLF is a very sensitive method of detecting early signs of demineralisation, but a number of confounding factors play a role in the process (Pretty et al., 2002). Firstly, determining the areas of sound enamel and demineralised enamel can be very subjective. Secondly, determining the borders of the demineralised lesion has an element of subjectivity. Finally, the presence of obscuring factors around or on the tooth surface such as moisture, calculus, orthodontic brackets, wires and any other orthodontic auxiliaries can affect the amount of fluorescence of the tooth detected by the QLF and the positioning of the QLF hand piece.

9.4.4 Statistical analysis

Due to the observational nature of this study and the categorical data collected, the only statistical analysis conducted was a chi square test to check for associations between red fluorescent plaque, and white spot lesions development and between dental plaque composition change and the development of white spot lesions. Further statistical tests could have been conducted if the results were more quantitative, particularly in terms of the concentration of the DNA samples and therefore the concentration of the specific bacterial species present in the plaque samples.

Chapter 10

Conclusions

The development of white spot lesions on the labial surfaces of teeth with bonded orthodontic brackets remain to be a frequent and undesirable effect of orthodontic treatment affecting 4.2% of the total surfaces of teeth included in this study and 78.6% of the participants.

The presence or absence of red fluorescent plaque is not related to the development of white spot lesions ($p>0.05$).

Over a period of one year of fixed orthodontic appliance therapy, some changes can be detected in the composition of red fluorescent plaque, but cannot be related to the development of white spot lesions ($p>0.05$). Not until DNA sequencing and larger volumes of plaque collected to be analysed do identify the unknown bacteria species, a definitive conclusion cannot be drawn regarding the effect of plaque composition on the risk of white spot lesion development.

Chapter 11

Recommendations for future work

The detection of red fluorescent plaque using Toothcare™ is a quick and easy method of detecting red fluorescent plaque compared to using the Inspector Pro QLF machine. The drawback of Toothcare™ is the fact that it cannot store images of the red fluorescent plaque that has been detected and it relies on the subjectivity of the operator using it at a specific time. A recent development is the introduction of QLF-D, which is a digital camera capable of recording white light images as well as recording and storing the images recorded with Toothcare™ . This will have the effect of reducing the subjectivity of red plaque recording because the images can be stored and analysed by more than one operator at different times and direct comparisons can be made between the clinical picture and the red fluorescent areas.

The oral microflora is a complex ecosystem involved in a number of oral diseases particularly dental caries. Accurate analysis of dental plaque is required to fully understand the process of dental demineralisation and its causative bacteria, so that demineralisation can be prevented and treated and the process of remineralisation precisely targeted. Culture independent techniques such as 16srRNA gene PCR and DGGE have been able to isolate bacterial species that were not detected in plaque with conventional culture based methods. The main contribution of this study to the literature, is the establishment of the first 16s rRNA V3 hypervariable region DGGE profile for red fluorescent supragingival plaque in adolescents undergoing orthodontic treatment. Further work can be directed towards

using PCR-DGGE molecular analysis techniques to investigate the bacterial component of non-red fluorescent supragingival plaque and compare it to that of red fluorescent plaque. A more detailed analysis can be undertaken by comparing the plaque DGGE profile to that of a large number of known bacteria. Another improvement would be to quantify the amount of each bacterium present and to sequence the unidentified bands for a more accurate analysis. A larger sample size would also be beneficial to increase the power of the study.

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Appendix A
Information sheet

UNIVERSITY OF LIVERPOOL

Department of Clinical Dental Sciences
Liverpool University Dental Hospital and School of Dentistry
Pembroke Place, Liverpool, L3 5PS

Participant Information Sheet

The effect of fixed orthodontic brackets on the bacterial composition of dental plaque in adolescents.

You are being invited to participate in a research study. Before you decide whether to participate, it is important for you to understand why the research is being done and what it will involve.

During fixed orthodontic treatment, the fixed orthodontic brackets act as traps for food and plaque. This leads to an increase in plaque collection and in turn renders the teeth more susceptible to loss of mineral that may appear as white or brown marks. In this study plaque will be identified by Tooth Care ™ and mineral loss will be identified by Quantitative light fluorescence (QLF).

Tooth Care ™ is a hand held device which emits light. It facilitates the viewing of dental plaque when it cannot be seen by direct eye vision.

Quantitative light fluorescence (QLF) is a similar device which captures images of the teeth and allows their transmission and storage onto a computer for later analysis for any mineral loss.

What is the purpose of the study?
The presence of fixed orthodontic brackets makes tooth brushing more difficult. This in term leads to increased plaque accumulation on the teeth around the orthodontic brackets. This study will be looking at the composition of the dental plaque and whether the presence of the brackets has any effect on the bacterial constituents of plaque as well as its quantity.

Who will be conducting the study?
The study s being run by Prof. Susan Higham (Professor of Oral Biology), Prof. Neil Pender (Professor of Orthodontics and Consultant in Orthodontics), Dr. Sabeel Valappil (Lecturer in Dental Science(), and Mrs Amal Sadeq (Postgraduate in Orthodontics)

Why have I been chosen to take part?
You have been asked to take part because we need healthy volunteers who are about to receive fixed orthodontic treatment.

Do I have to take part?
No, your participation is voluntary.

Version 1.2
October 2009

UNIVERSITY OF LIVERPOOL

Department of Clinical Dental Sciences
Liverpool University Dental Hospital and School of Dentistry
Pembroke Place, Liverpool, L3 5PS

What will happen if I take part?
Plaque on the teeth will be visualised with the aid of Tooth Care ™ and a sample of plaque will be collected and sent to the laboratory for analysis. The teeth will then be cleaned to remove any remaining plaque residues and images of the teeth using QLF will be taken to detect any marks on the teeth.

How long will the study last?
You will be monitored for a maximum period of twelve months. The plaque sample and data collection for the study will take place during your normal appointment adding approximately 20 minutes to the normal appointment time.

What if I am unhappy or if there is a problem?
You can withdraw from the study at any time without the need to give reasons. Your orthodontic treatment will continue as normal.

Will my participation be kept confidential?
Yes, all information which identifies you will be removed and replaced by a code number. The person responsible for security and access to your data is Professor Neil Pender (the Chief investigator of the study). All information about you will be processed and analysed by the research team of the study. Data will be stored for ten years.

Who can I contact if I have further questions?
If you have any questions or problems you can speak to any of the dentists in the hospital who are running this study.

Thank you for taking the time to read this.

Version 1.2
October 2009

Appendix B
Under 16s information sheet

UNIVERSITY OF LIVERPOOL

Department of Clinical Dental Sciences
Liverpool University Dental Hospital and School of Dentistry
Pembroke Place, Liverpool, L3 5PS

Information Sheet for children under 16

The effect of fixed orthodontic brackets on the bacterial composition of dental plaque in adolescents.

We are asking you to help us in our research project. Before you decide whether to take part or not, it is important that you understand why the project is being done and what you need to do

Why are we doing this project?

Once you have your fixed "train-track" braces fitted, you will find that brushing your teeth will be more difficult. The brace will trap food and plaque around it. Plaque is a clear material that sticks to your teeth and contains bacteria. These bacteria can produce acid that will eat away your tooth causing holes known as cavities.

In this project we want to know if braces on your teeth cause more of the usual plaque and bacteria to collect on the teeth or they can help in trapping different kind of plaque and bacteria that can be more harmful to your teeth.

Why ask for your help?

Because we need healthy young people who are about to have fixed braces fitted on their teeth.

Do you have to take part?

No, you can choose not to take part

Version 1.1
October 2009

UNIVERSITY OF LIVERPOOL

Department of Clinical Dental Sciences
Liverpool University Dental Hospital and School of Dentistry
Pembroke Place, Liverpool, L3 5PS

What will happen if you take part?

We will use a special blue light to see the plaque on your front teeth. Then we will take some of that plaque and put it in a small tube to study it.

We will also use a special camera that is attached to a computer to take pictures of your front teeth to help us look for any marks or cavities that might appear on your teeth

How long will the project last?

Everything will be done during your regular orthodontic appointment. It means that you will have slightly longer appointments than usual. The project will not take more than one year.

What if I am not happy or have a problem?

You can stop taking part in this project at any time. Your brace treatment will continue as normal.

If you have any questions, feel free to ask and I will do my best to answer them.

Thank you for taking the time to read this.

Version 1.1
October 2009

Appendix C
Parent/guardian information sheet

UNIVERSITY OF LIVERPOOL

Department of Clinical Dental Sciences
Liverpool University Dental Hospital and School of Dentistry
Pembroke Place, Liverpool, L3 5PS

Participant Parent/Guardian Information Sheet

The effect of fixed orthodontic brackets on the bacterial composition of dental plaque in adolescents.

Your child is being invited to participate in a research study. Before you decide whether to participate, it is important for you to understand why the research is being done and what it will involve.

During fixed orthodontic treatment, the fixed orthodontic brackets act as traps for food and plaque. This leads to an increase in plaque collection and in turn renders the teeth more susceptible to loss of mineral that may appear as white or brown marks. In this study plaque will be identified by Tooth Care ™ and mineral loss will be identified by Quantitative light fluorescence (QLF).

Tooth Care ™ is a hand held device which emits light. It facilitates the viewing of dental plaque when it cannot be seen by direct eye vision.

Quantitative light fluorescence (QLF) is a similar device which captures images of the teeth and allows their transmission and storage onto a computer for later analysis for any mineral loss.

What is the purpose of the study?
The presence of fixed orthodontic brackets makes tooth brushing more difficult. This in term leads to increased plaque accumulation on the teeth around the orthodontic brackets. This study will be looking at the composition of the dental plaque and whether the presence of the brackets has any effect on the bacterial constituents of plaque as well as its quantity.

Who will be conducting the study?
The study s being run by Prof. Susan Higham (Professor of Oral Biology), Prof. Neil Pender (Professor of Orthodontics and Consultant in Orthodontics), Dr. Sabeel Valappil (Lecturer in Dental Science(), and Mrs Amal Sadeq (Postgraduate in Orthodontics)

Why has my child been chosen to take part?
Your child has been asked to take part because we need healthy volunteers who are about to receive fixed orthodontic treatment.

Does my child have to take part?
No, your child' s participation is voluntary.

Version 1.2
October 2009

UNIVERSITY OF LIVERPOOL

Department of Clinical Dental Sciences
Liverpool University Dental Hospital and School of Dentistry
Pembroke Place, Liverpool, L3 5PS

What will happen if my child takes part?
Plaque on the teeth will be visualised with the aid of Tooth Care [TM] and a sample of plaque will be collected and sent to the laboratory for analysis. The teeth will then be cleaned to remove any remaining plaque residues and images of the teeth using QLF will be taken to detect any marks on the teeth.

How long will the study last?
Your child will be monitored for a maximum period of twelve months. The plaque sample and data collection for the study will take place during your normal appointment adding approximately 20 minutes to the normal appointment time.

What if my child is unhappy or if there is a problem?
Your child can withdraw from the study at any time without the need to give reasons. Their orthodontic treatment will continue as normal.

Will my child's participation be kept confidential?
Yes, all information which identifies your child will be removed and replaced by a code number. The person responsible for security and access to your data is Professor Neil Pender (the chief investigator of the study). All information about your child will be processed and analysed by the research team of the study. Data will be stored for ten years.

Who can I contact if I have further questions?
If you have any questions or problems you can speak to any of the dentists in the hospital who are running this study.

Thank you for taking the time to read this.

Version 1.2
October 2009

Appendix D
Consent form

CONSENT FORM

Title of Research Project:

The effect of fixed orthodontic brackets on the bacterial composition of dental plaque in adolescents

	Please initial box
1. I confirm that I have read and have understood the information sheet dated October 2009 [V 1.2] for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.	☐
3. I understand that the data collected during the study will be analysed by the study investigators. I give permission for these individuals to have access to my records.	☐
4. I agree to take part in the above study.	☐

Name of Person giving consent	Date	Signature
Relation to Participant	Date	Signature
Researcher	Date	Signature

The contact details of lead Researcher (Principal Investigator) are:

Professor Neil Pender
Liverpool University Dental Hospital, Liverpool Dental School, Pembroke place, L3 5PS
Tel. 0151 7065030

Appendix E
Assent form

UNIVERSITY OF LIVERPOOL

ASSENT FORM FOR CHILDREN UNDER 16

Title of Research Project:

The effect of fixed orthodontic brackets on the bacterial composition of dental plaque in adolescents

Please circle YES or NO

1. I have read the information sheet dated October 2009 (V 1.1).	Yes	No
2. The study has been explained to me	Yes	No
3. I had the chance to ask questions and they were answered in a way that I understand.	Yes	No
4. I understand what this study is all about	Yes	No
5. I understand it's okay to stop taking part at any time	Yes	No
6. I am happy to take part	Yes	No

If you are happy to take part please write your name below

Name Date

Name of parent or guardian

Name Date Signature

Name of researcher

Name Date Signature

The contact details of lead Researcher (Principal Investigator) are:

Professor Neil Pender
Liverpool University Dental Hospital, Liverpool Dental School, Pembroke place, L3 5PS
Tel: 0151 7065030

Appendix F

Acrylamide denaturing stock solution preparation

50x TAE Buffer (1L)

Reagent	Amount/volume
Tris Base	242.0 g
Glacial acetic acid	57.1 ml
0.5 M EDTA, pH 8.0	100.0 ml
dH_2O	600.0 ml

Mixed and added dH_2O to 1 litre then autoclaved for 20-30 minutes and stored at room temperature.

Denaturing solution (100 ml)

Reagent	Amount/volume
40% Acrylamide/Bis (37.5:1)	18.8 ml
50 x TAE buffer	2.0 ml
dH_2O	79.2 ml

De-gased for about 10-15 minutes then stored at 4^oC in a brown bottle (to protect from ambient light) for up to one month.

Denaturing solution per 100 ml solution

Reagent	30%	60%
Formamide	12 ml	24 ml
Urea	12.6 g	25.2 g

10% Ammonium Persulfate (1 ml)

Reagent	Amount/volume
Ammonium persulfate	0.1 g
dH_2O	1.0 ml

Mixed and stored at -20^{o}C for about a week.

Appendix G
Silver staining reagents and protocol

Reagents

Reagent	Amount/volume
Fixing solution (1 L)	90% (v/v) dH_2O, 10% 9v/v) ethanol, 25 ml acetic acid
0.1 % (w/v) silver stain	1L dH_2O, 1 g silver nitrate
1.5 % (w/v) sodium hydroxide	15 g sodium hydroxide, 1L dH_2O
0.75% (w/v) calcium carbonate	7.5 g calcium carbonate, 1L dH_2O
38% (w/v) formaldehyde	1.5 ml

Protocol

1. Placed gels in fixing solution for 10 minutes.
2. Rinsed with dH_2O.
3. Placed in silver staining solution for 20 minutes.
4. Prepared 1 litre of developer solution.
5. Rinsed gels in dH_2O. The staining solution can be used up to 20 times.
6. Added 1.5 ml of formaldehyde to the developer.
7. Poured a small amount of the developer on the gels to remove any silver stain remaining in the gel tray.
8. Rinsed with dH_2O.
9. Submerged gels in developer for 20 minutes or until the bands appeared.
10. Poured away the solution and submerged in calcium carbonate for 5 minutes.

Appendix H
TotalLab Quant Software analysis

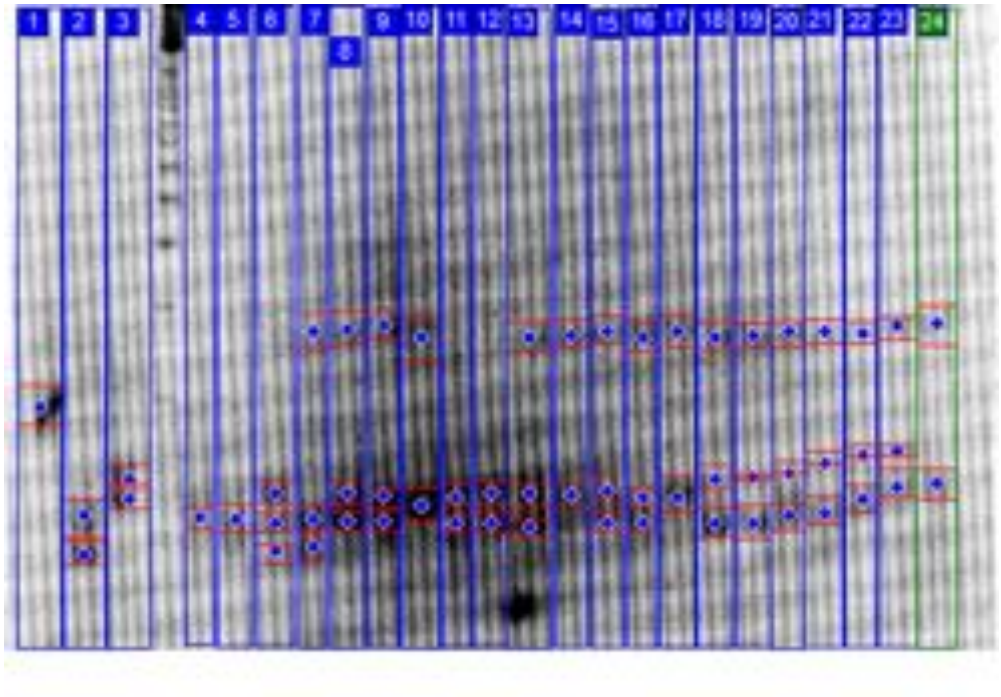
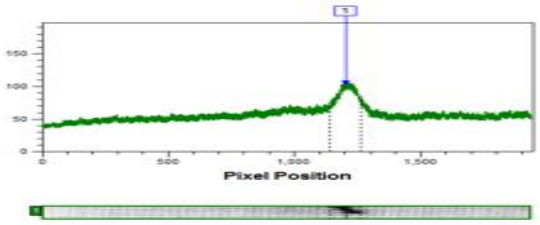
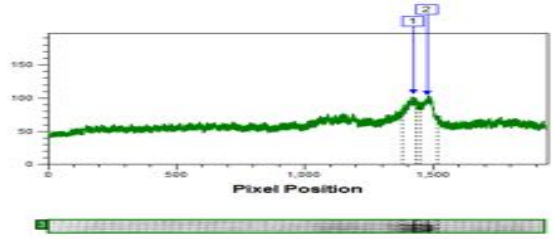
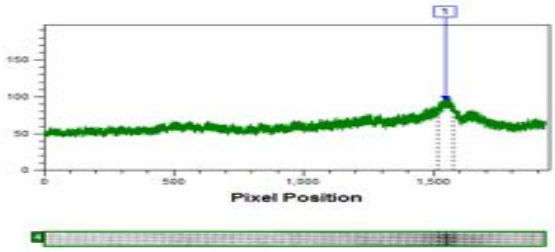


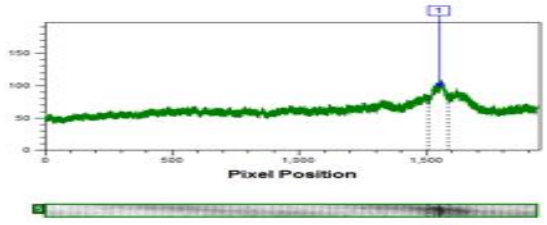
Figure H-1: DGGE profiles and TotalLab Quant software analysis of red fluorescent dental plaque samples and laboratory strains of oral bacteria stained with ethidium bromide. **Lane 1**: *P.gingivalis* (ATCC 33277), **lane 2**: *S.mutans* (NCTC 10449), **lane 3**: *S.* gordonii (DL1), **lane 4**: 50 bp DNA marker, **lanes 5-11**: red fluorescent plaque samples 1-7 for participant 11, **lanes 12-19**: red fluorescent plaque samples 1-8 for participant 12, **lanes 20-25**: red fluorescent plaque samples 1-6 for participant 13.

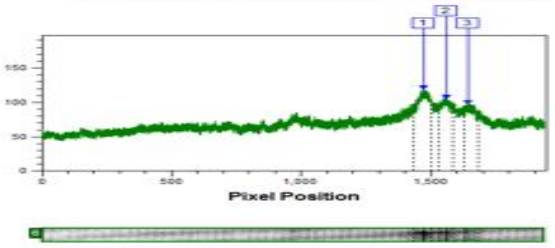
Lane 1		
Band No	Volume	Rf
1	1444304.00	0.623
Lane 2		
Band No	Volume	Rf
1	1213616.00	0.792
2	919766.00	0.852
Lane 3		
Band No	Volume	Rf
1	669399.00	0.734
2	757026.00	0.764
Lane 4		
Band No	Volume	Rf
1	465840.00	0.799
Lane 5		
Band No	Volume	Rf
1	723768.00	0.799
Lane 6		
Band No	Volume	Rf
1	808293.00	0.759
2	584350.00	0.805
3	569927.00	0.850
Lane 7		
Band No	Volume	Rf
1	701601.00	0.505
2	662199.00	0.797
3	551933.00	0.840

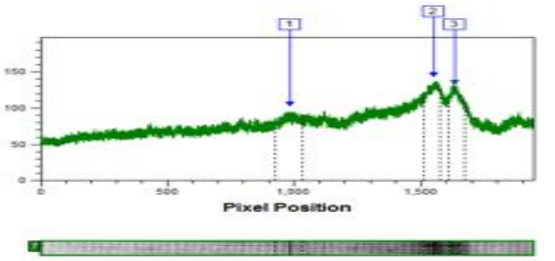




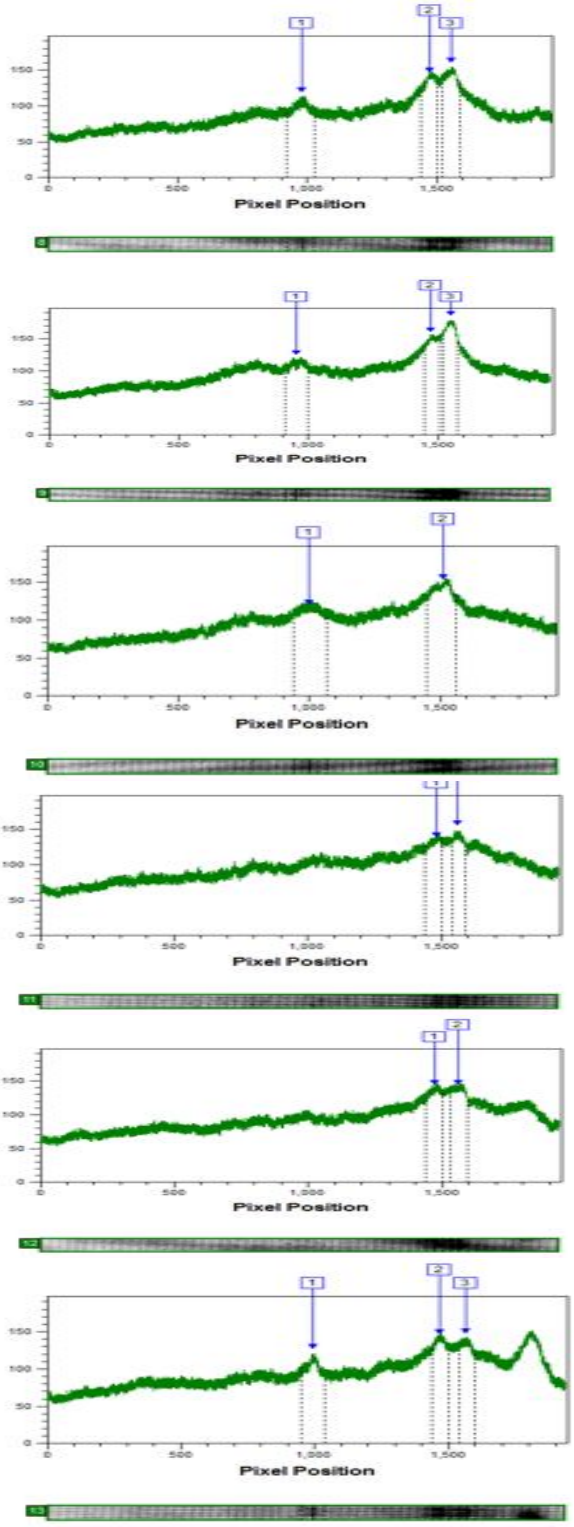




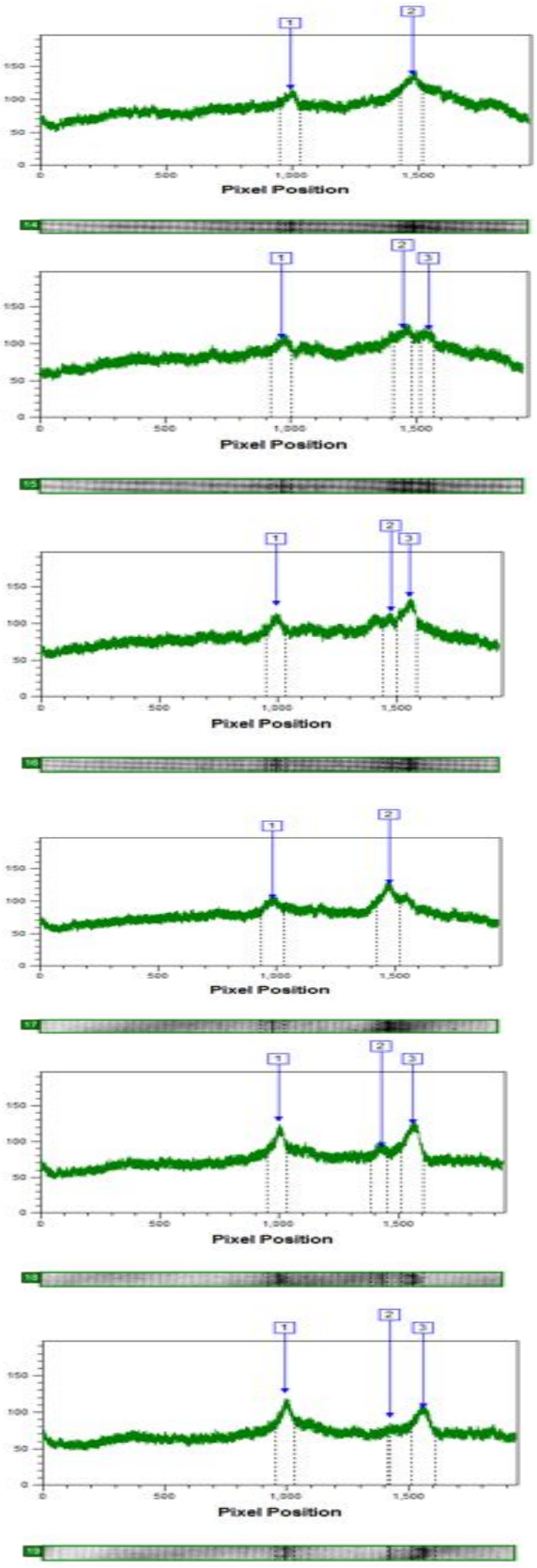




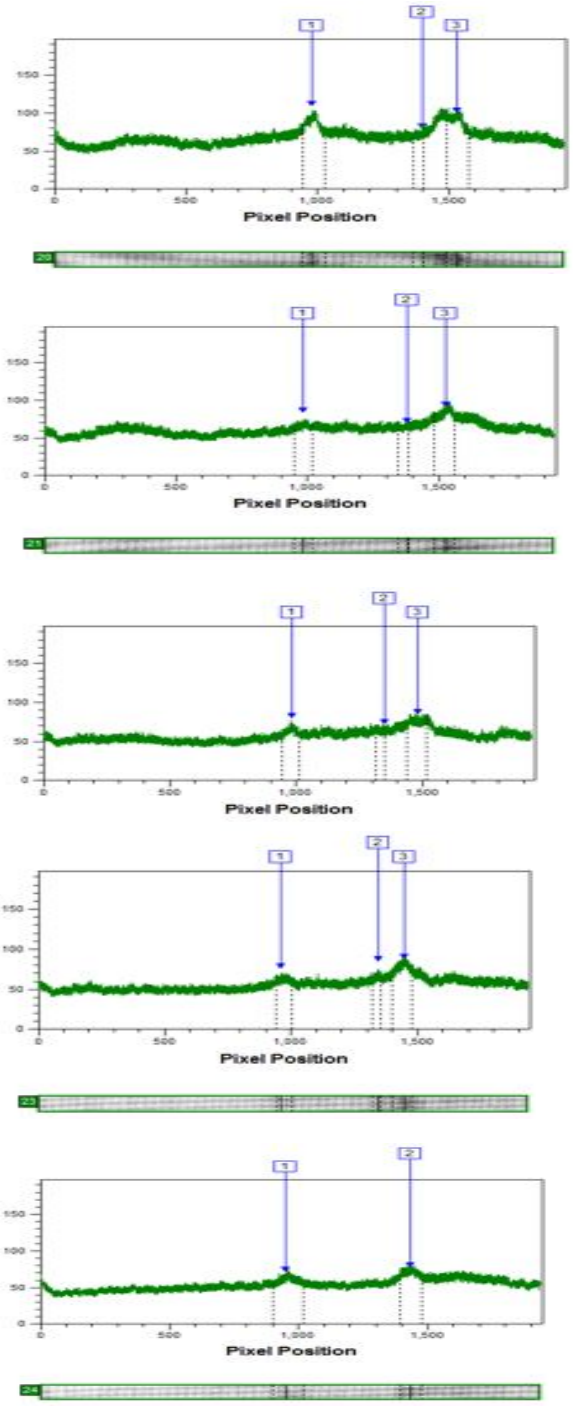
Lane 8		
Band No	**Volume**	**Rf**
1	1035383.00	0.503
2	868028.00	0.757
3	944606.00	0.800
Lane 9		
Band No	**Volume**	**Rf**
1	965959.00	0.496
2	767292.00	0.762
3	918264.00	0.802
Lane 10		
Band No	**Volume**	**Rf**
1	1643701.00	0.515
2	1741476.00	0.777
Lane 11		
Band No	**Volume**	**Rf**
1	654104.00	0.766
2	523992.00	0.807
Lane 12		
Band No	**Volume**	**Rf**
1	861476.00	0.759
2	873195.00	0.805
Lane 13		
Band No	**Volume**	**Rf**
1	1228293.00	0.514
2	1060528.00	0.757
3	1042974.00	0.809



Lane 14		
Band No	Volume	Rf
1	803402.00	0.514
2	1110492.00	0.762
Lane 15		
Band No	Volume	Rf
1	787011.00	0.502
2	794750.00	0.754
3	605728.00	0.804
Lane 16		
Band No	Volume	Rf
1	770861.00	0.514
2	500367.00	0.764
3	839307.00	0.805
Lane 17		
Band No	Volume	Rf
1	994872.00	0.507
2	1213635.00	0.764
Lane 18		
Band No	Volume	Rf
1	910802.00	0.517
2	653376.00	0.736
3	1065863.00	0.805
Lane 19		
Band No	Volume	Rf
1	796079.00	0.514
2	99008.00	0.734
3	990434.00	0.805



Lane 20		
Band No	**Volume**	**Rf**
1	737889.00	0. 504
2	287751.00	0.724
3	760213.00	0.792
Lane 21		
Band No	**Volume**	**Rf**
1	509540.00	0.507
2	287847.00	0.711
3	734741.00	0.789
Lane 22		
Band No	**Volume**	**Rf**
1	450144.00	0.509
2	222431.00	0.698
3	605631.00	0.769
Lane 23		
Band No	**Volume**	**Rf**
1	505162.00	0.497
2	265007.00	0.694
3	751057.00	0.749
Lane 24		
Band No	**Volume**	**Rf**
1	855925.00	0.491
2	735411.00	0.739



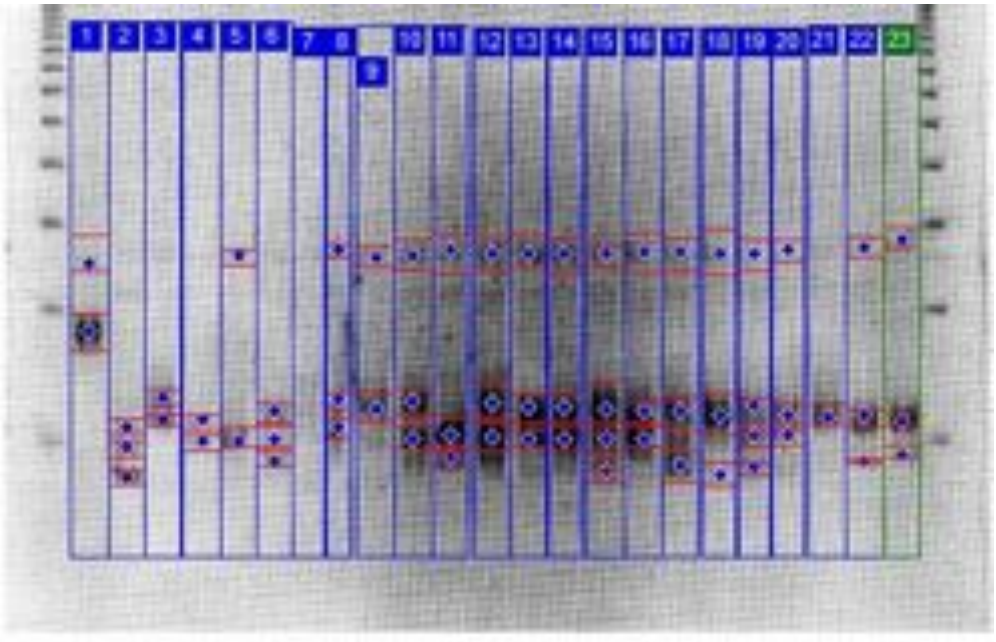
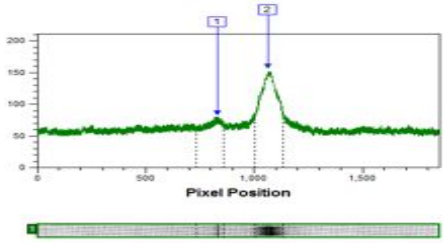
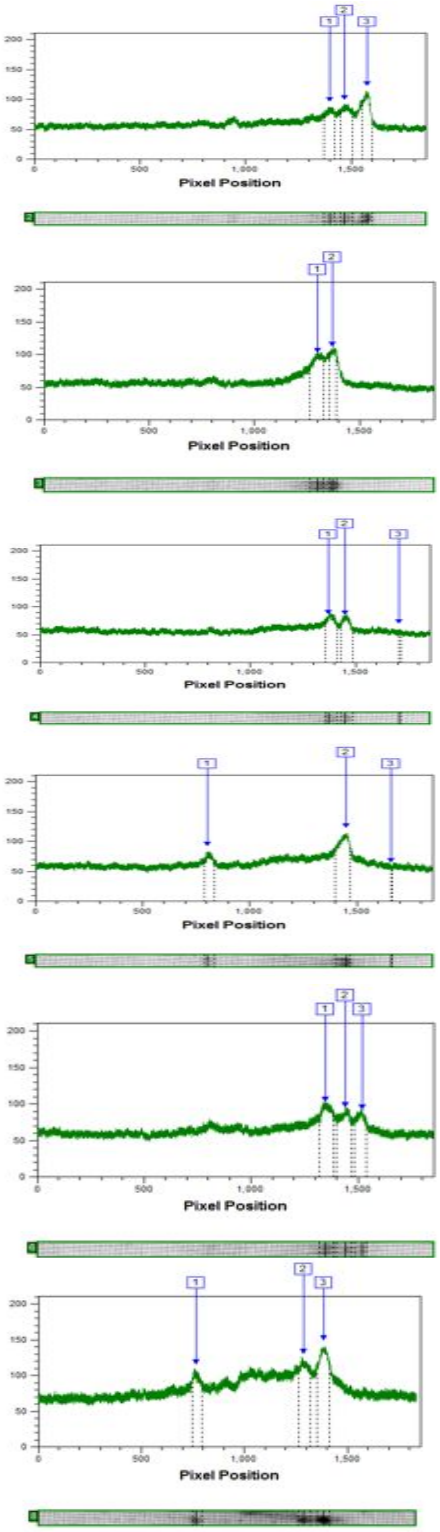


Figure H-2: DGGE profiles and TotalLab Quant software analysis of red fluorescent dental plaque samples and laboratory strains of oral bacteria stained with ethidium bromide. **Lanes 1,25**: 50 bp DNA marker, **lane 2**: *P. gingivalis* (ATCC 33277), **lane 3**: *S.mutans* (NCTC 10449), **lane 4**: *S. gordonii* (DL1), **lanes 5-9**: red fluorescent plaque samples 1-5 for participant 7, **lanes 10-18**: red fluorescent plaque samples 1-9 for participant 9, **lanes 19-24**: red fluorescent plaque samples 1-6 for participant 10.

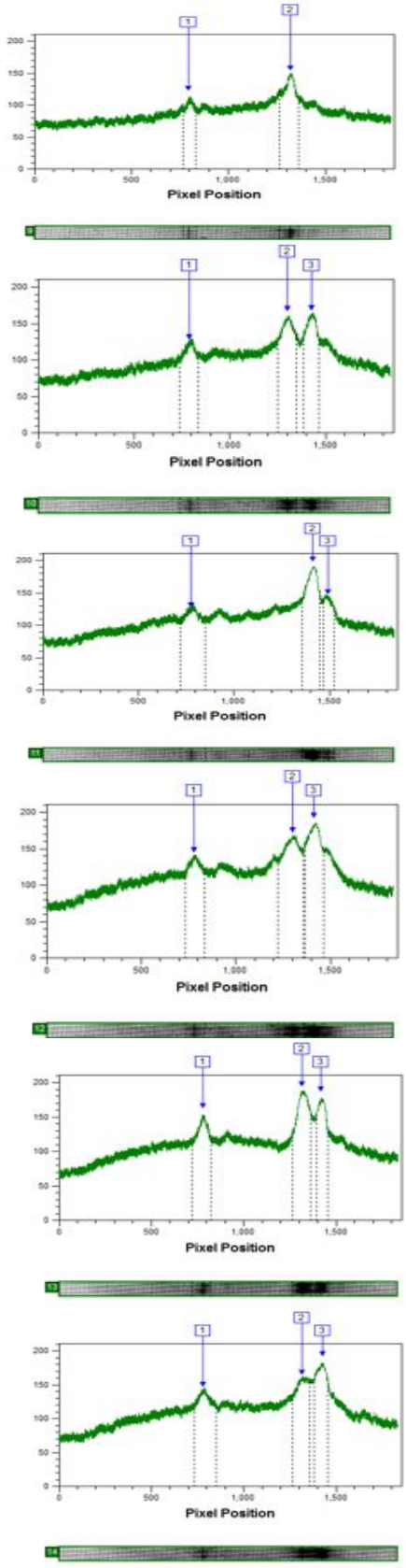
Lane 1			
Band No	**Volume**	**Rf**	**Width**
1	1078111	0.45	126
2	1980441	0.576	132



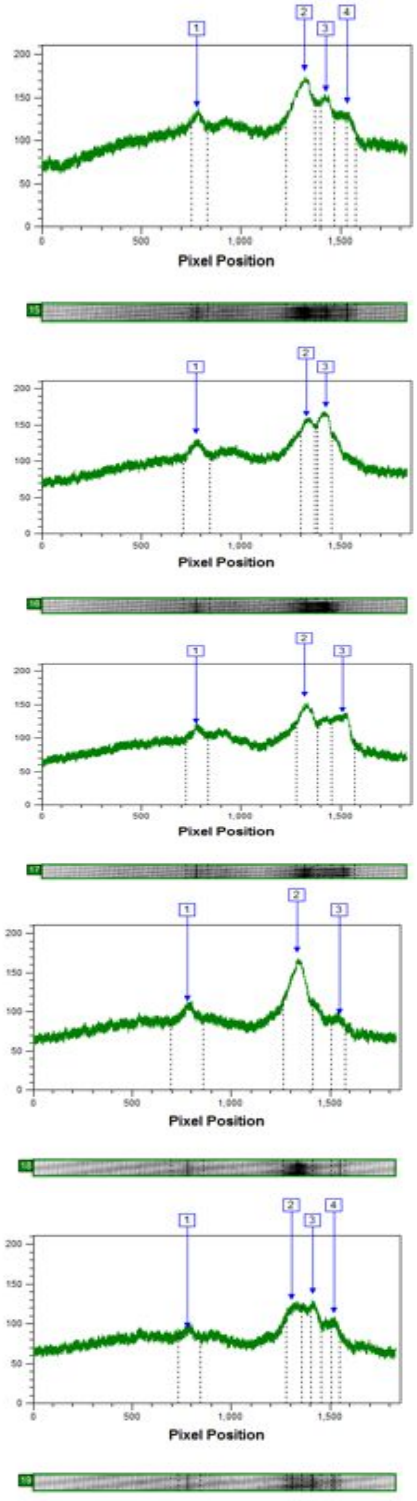
Lane 2			
Band No	**Volume**	**Rf**	**Width**
1	480947	0.756	48
2	621583	0.794	59
3	540373	0.85	44
Lane 3			
Band No	**Volume**	**Rf**	**Width**
1	719135	0.701	61
2	432003	0.741	33
Lane 4			
Band No	**Volume**	**Rf**	**Width**
1	518977	0.741	52
2	516478	0.782	55
Lane 5			
Band No	**Volume**	**Rf**	**Width**
1	467676	0.435	51
2	888489	0.782	70
Lane 6			
Band No	**Volume**	**Rf**	**Width**
1	800957	0.727	69
2	777610	0.779	74
3	591180	0.82	57
Lane 8			
Band No	**Volume**	**Rf**	**Width**
1	403021	0.417	51
2	485117	0.701	52
3	656661	0.755	63



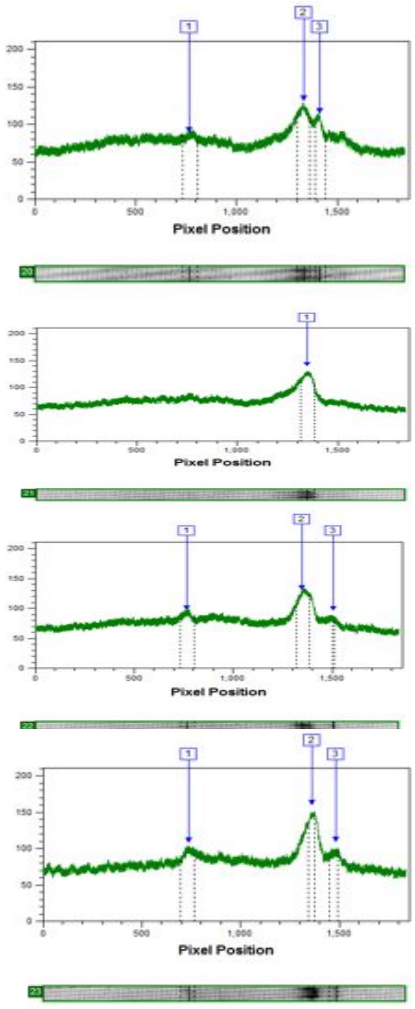
Lane 9			
Band No	**Volume**	**Rf**	**Width**
1	885462	0.433	69
2	1618447	0.719	98
Lane 10			
Band No	**Volume**	**Rf**	**Width**
1	1431581	0.43	98
2	1748916	0.707	94
3	1501127	0.777	78
Lane 11			
Band No	**Volume**	**Rf**	**Width**
1	2015210	0.421	135
2	2014394	0.77	93
3	1040550	0.811	59
Lane 12			
Band No	**Volume**	**Rf**	**Width**
1	1704540	0.427	107
2	2482168	0.709	131
3	1984441	0.772	94
Lane 13			
Band No	**Volume**	**Rf**	**Width**
1	1743439	0.425	107
2	2064394	0.717	101
3	1182883	0.775	58
Lane 14			
Band No	**Volume**	**Rf**	**Width**
1	1947412	0.425	121
2	1814406	0.717	97
3	1590218	0.778	75



Lane 15			
Band No	**Volume**	**Rf**	**Width**
1	1204209	0.425	75
2	2822423	0.719	144
3	1338589	0.778	73
4	705209	0.836	45
Lane 16			
Band No	**Volume**	**Rf**	**Width**
1	2004776	0.422	135
2	1364176	0.724	70
3	1410840	0.778	69
Lane 17			
Band No	**Volume**	**Rf**	**Width**
1	1603103	0.422	116
2	1871384	0.722	107
3	1775635	0.826	112
Lane 18			
Band No	**Volume**	**Rf**	**Width**
1	1786751	0.427	159
2	2397804	0.729	149
3	733528	0.844	71
Lane 19			
Band No	**Volume**	**Rf**	**Width**
1	1151326	0.427	112
2	1142387	0.714	83
3	688835	0.77	51
4	512907	0.829	45



Lane 20			
Band No	**Volume**	**Rf**	**Width**
1	765232	0.419	79
2	882426	0.729	65
3	621800	0.77	54
Lane 21			
Band No	**Volume**	**Rf**	**Width**
1	1074943	0.734	70
Lane 22			
Band No	**Volume**	**Rf**	**Width**
1	795363	0.416	70
2	1024046	0.732	65
3	135702	0.818	13
Lane 23			
Band No	**Volume**	**Rf**	**Width**
1	796153	0.4	66
2	679349	0.742	37
3	454155	0.808	38



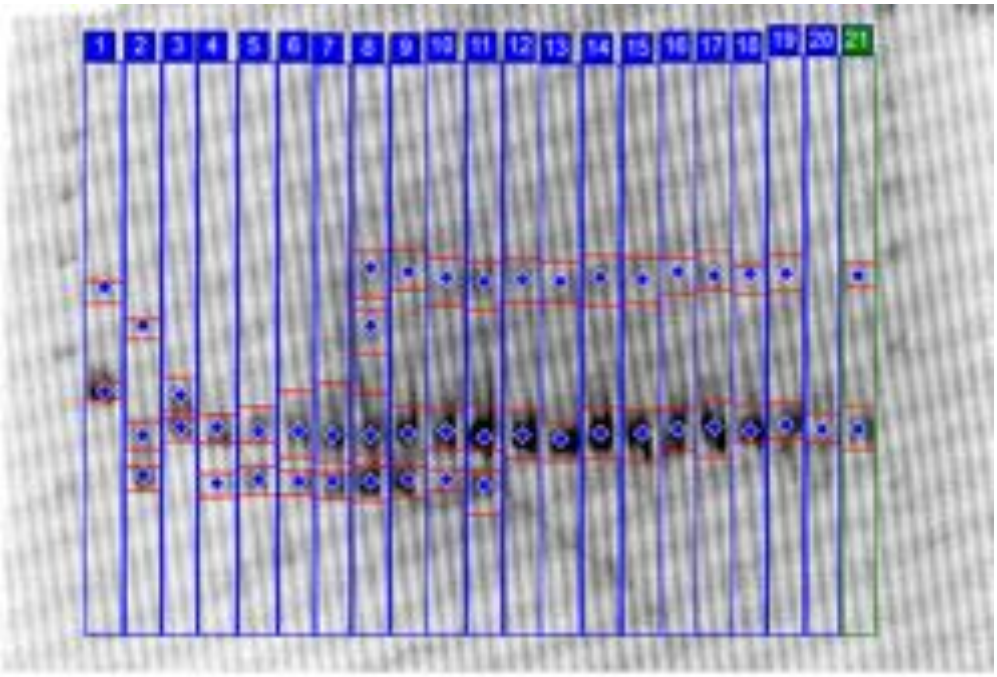
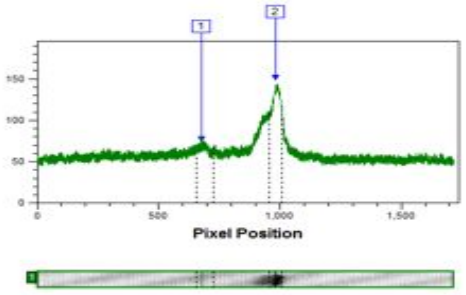
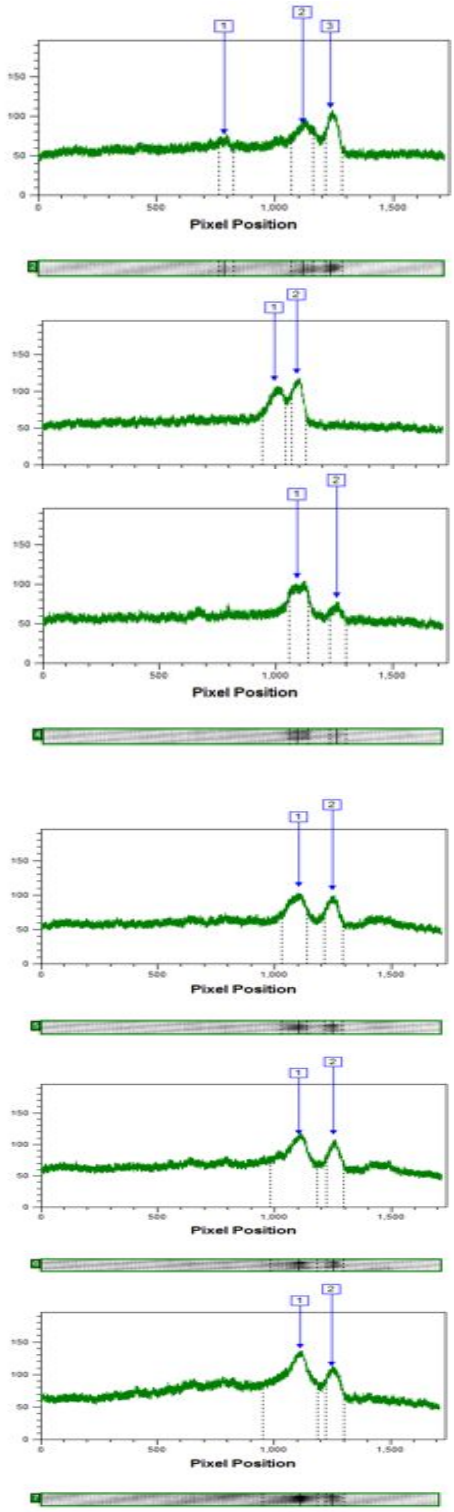


Figure H-3: DGGE profiles and TotalLab Quant software analysis of red fluorescent dental plaque samples and laboratory strains of oral bacteria stained with ethidium bromide. **Lanes 1,23**: 50 bp DNA marker, **lane 2**: *P. gingivalis* (ATCC 33277), **lane 3**: *S.mutans* (NCTC 10449), **lane 4**: *S. gordonii* (DL1), **lanes 5-11**: red fluorescent plaque samples 1-7 for participant 1, **lanes 12-18**: red fluorescent plaque samples 1-7 for participant 2, **lanes 19-20**: red fluorescent plaque samples 1,2 for participant 3, **lane 21**: red fluorescent plaque sample 1 for participant 8 **lane 22**: red fluorescent plaque sample for participant 14.

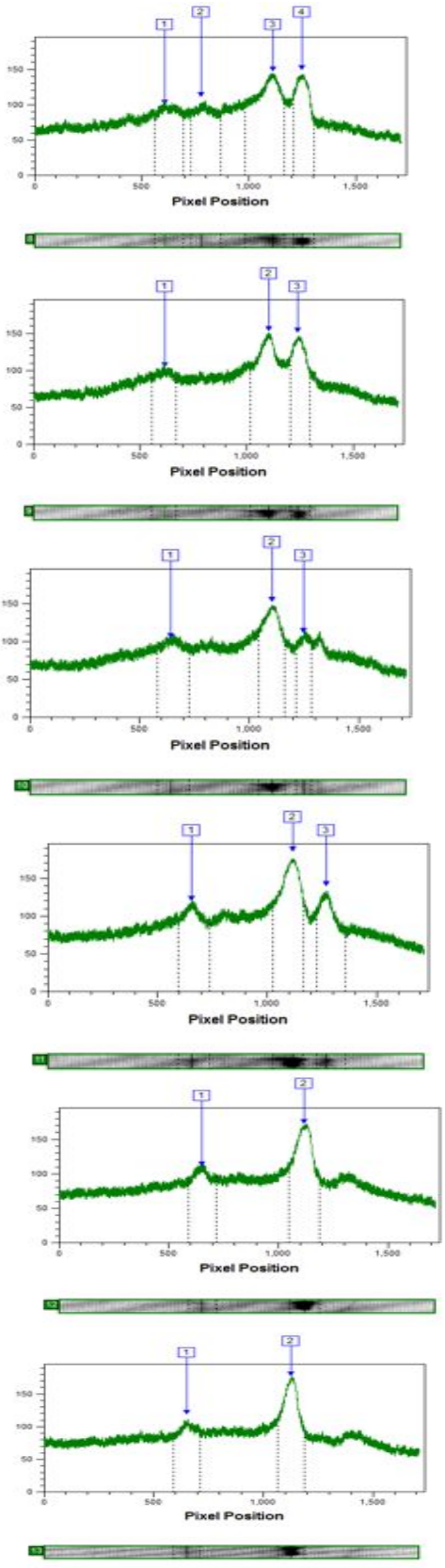
Lane 1			
Band No	Volume	Rf	Width
1	483195	0.394	66
2	727494	0.574	52



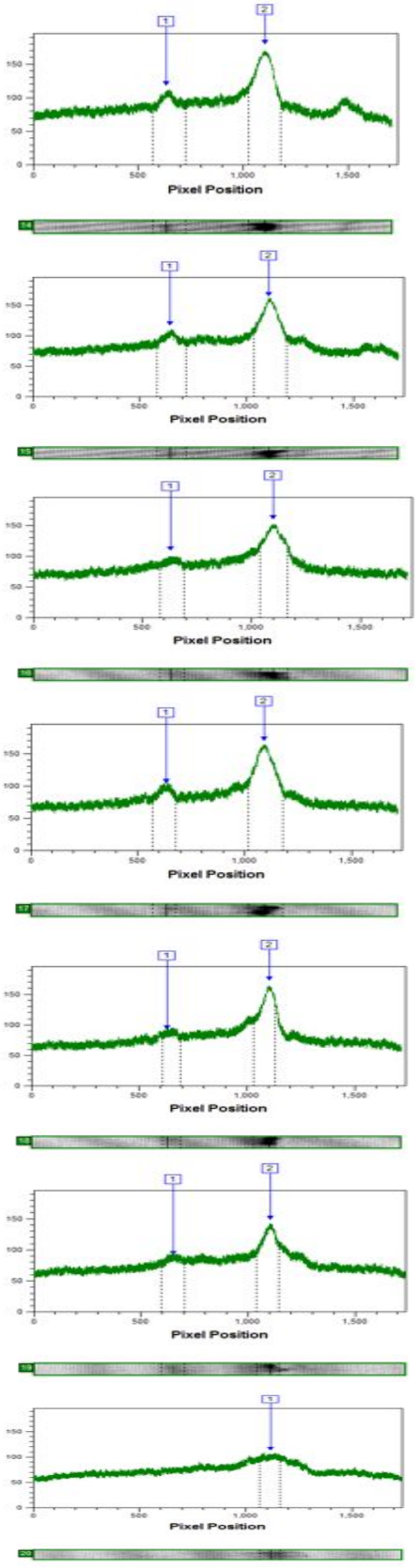
Lane 2			
Band No	Volume	Rf	Width
1	453060	0.457	61
2	819986	0.65	88
3	687462	0.721	68
Lane 3			
Band No	Volume	Rf	Width
1	1043623	0.582	104
2	712194	0.635	63
Lane 4			
Band No	Volume	Rf	Width
1	875752	0.638	79
2	526729	0.734	68
Lane 5			
Band No	Volume	Rf	Width
1	1154535	0.643	111
2	782056	0.729	80
Lane 6			
Band No	Volume	Rf	Width
1	2103632	0.643	200
2	785857	0.731	75
Lane 7			
Band No	Volume	Rf	Width
1	2704588	0.65	234
2	858595	0.732	80



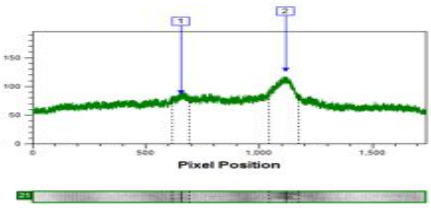
Lane 8			
Band No	**Volume**	**Rf**	**Width**
1	1423660	0.357	138
2	1396403	0.456	136
3	2394608	0.65	179
4	1337348	0.729	98
Lane 9			
Band No	**Volume**	**Rf**	**Width**
1	1245668	0.362	117
2	2542234	0.645	188
3	1330819	0.726	93
Lane 10			
Band No	**Volume**	**Rf**	**Width**
1	1559300	0.374	144
2	1705130	0.643	118
3	753979	0.729	66
Lane 11			
Band No	**Volume**	**Rf**	**Width**
1	1685532	0.383	148
2	2276318	0.653	138
3	1568635	0.74	132
Lane 12			
Band No	**Volume**	**Rf**	**Width**
1	1392856	0.379	126
2	2194300	0.65	143
Lane 13			
Band No	**Volume**	**Rf**	**Width**
1	1372024	0.38	121
2	1978290	0.658	120



Lane 14			
Band No	**Volume**	**Rf**	**Width**
1	1728581	0.372	153
2	2537485	0.645	154
Lane 15			
Band No	**Volume**	**Rf**	**Width**
1	1523066	0.375	135
2	2343039	0.645	150
Lane 16			
Band No	**Volume**	**Rf**	**Width**
1	988615	0.366	105
2	1780501	0.64	128
Lane 17			
Band No	**Volume**	**Rf**	**Width**
1	1068399	0.371	113
2	2267450	0.638	165
Lane 18			
Band No	**Volume**	**Rf**	**Width**
1	714454	0.368	80
2	1468981	0.643	104
Lane 19			
Band No	**Volume**	**Rf**	**Width**
1	940985	0.375	105
2	1338895	0.636	105
Lane 20			
Band No	**Volume**	**Rf**	**Width**
1	968417	0.644	92



Lane 21			
Band No	**Volume**	**Rf**	**Width**
1	631839	0.379	74
2	1414455	0.644	134



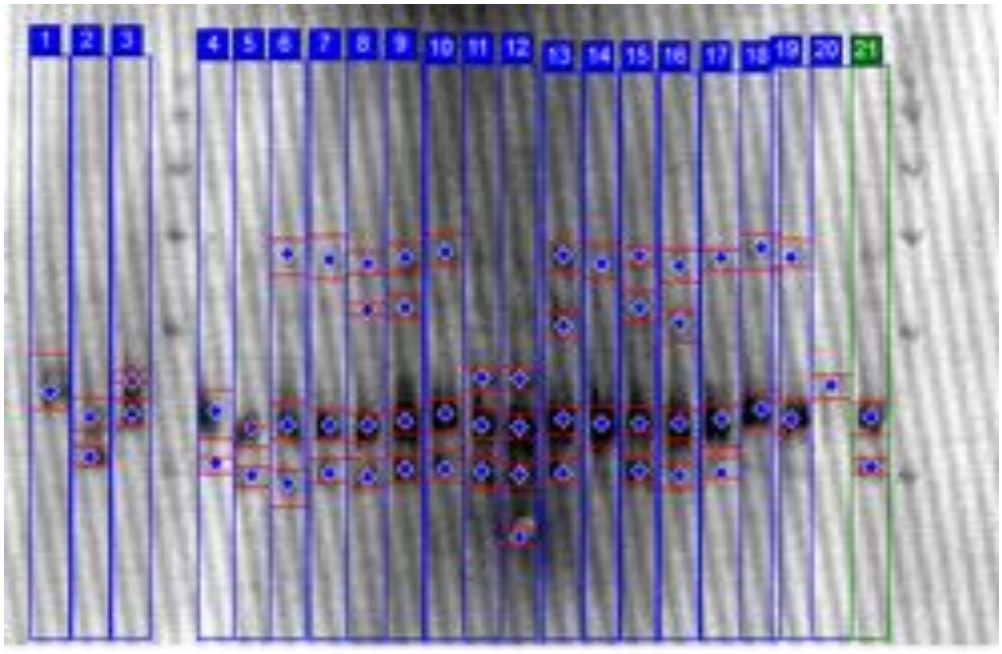
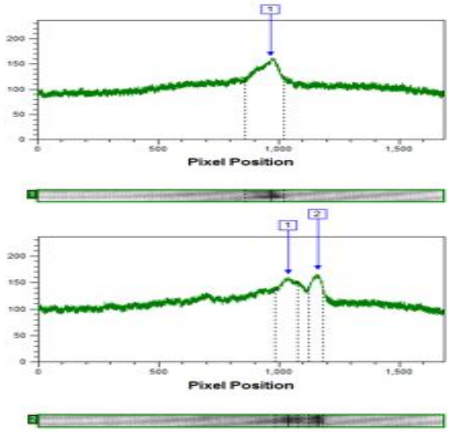
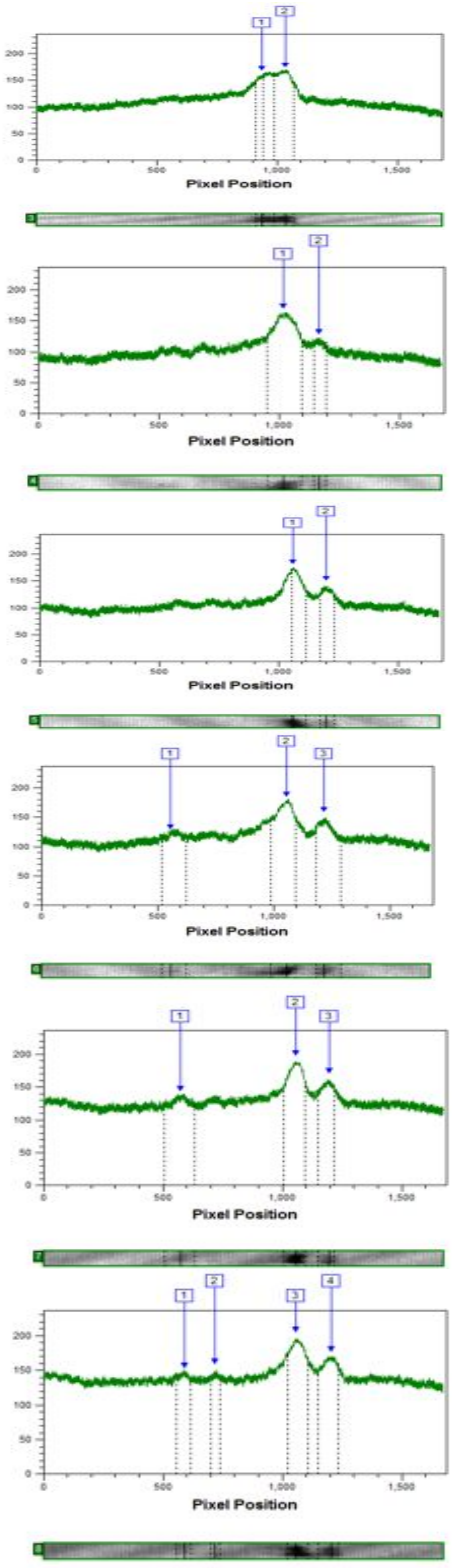


Figure H-4: DGGE profiles TotalLab Quant software analysis of red fluorescent dental plaque samples and laboratory strains of oral bacteria stained with ethidium bromide. **Lane 1**: *P. gingivalis* (ATCC 33277), **lane 2**: *S.mutans* (NCTC 10449), **lane 3**: *S. gordonii* (DL1), **lanes 4,23**: 50 bp DNA marker, **lanes 5-13**: red fluorescent plaque samples 1-9 for participant 4, **lanes 14-17**: red fluorescent plaque samples 1-4 for participant 5, **lanes 18-22**: red fluorescent plaque samples 1-5 for participant 6.

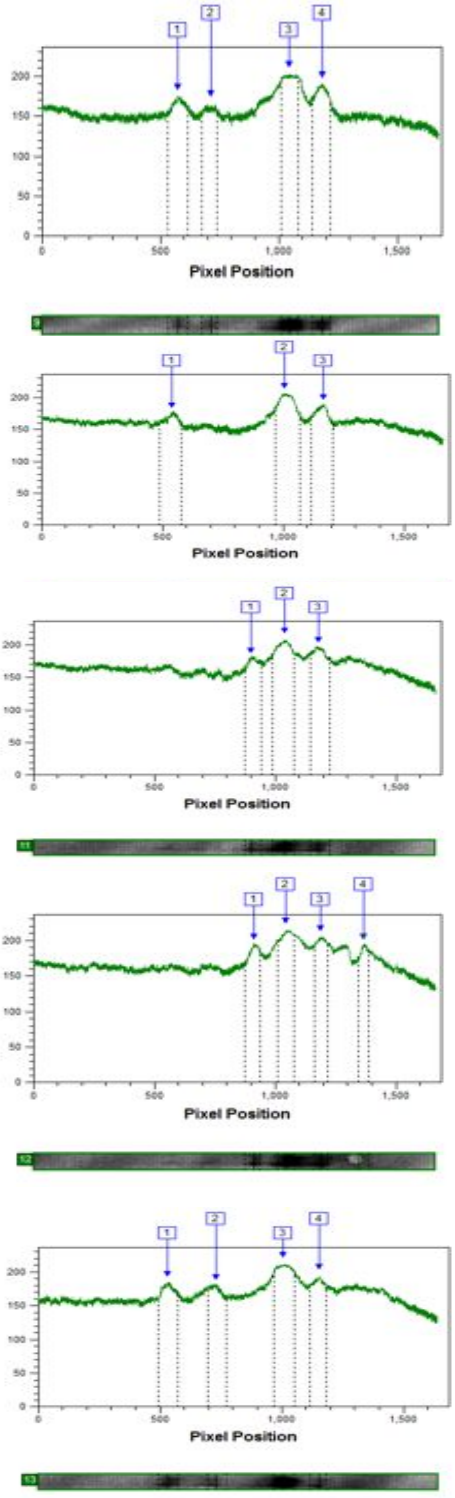
Lane 1			
Band No	**Volume**	**Rf**	**Width**
1	2566126	0.576	161
Lane 2			
Band No	**Volume**	**Rf**	**Width**
1	1584338	0.618	93
2	1040173	0.689	59



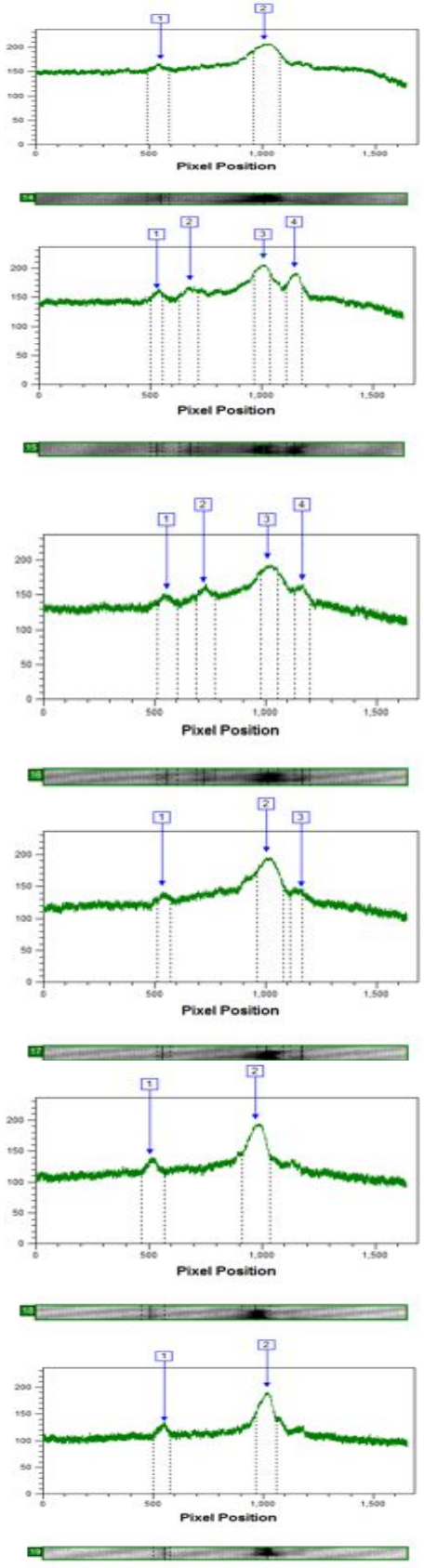
Lane 3			
Band No	**Volume**	**Rf**	**Width**
1	524540	0.558	30
2	1418246	0.614	77
Lane 4			
Band No	**Volume**	**Rf**	**Width**
1	2174302	0.608	144
2	581527	0.696	49
Lane 5			
Band No	**Volume**	**Rf**	**Width**
1	954615	0.635	59
2	809203	0.716	59
Lane 6			
Band No	**Volume**	**Rf**	**Width**
1	1314923	0.334	106
2	1797985	0.63	106
3	1490082	0.729	109
Lane 7			
Band No	**Volume**	**Rf**	**Width**
1	1824869	0.344	130
2	1639530	0.63	85
3	1137699	0.714	68
Lane 8			
Band No	**Volume**	**Rf**	**Width**
1	983365	0.352	63
2	623445	0.43	40
3	1716308	0.63	85
4	1455817	0.721	82



Lane 9			
Band No	**Volume**	**Rf**	**Width**
1	1576924	0.342	87
2	1149386	0.425	66
3	1579387	0.625	72
4	1531026	0.706	77
Lane 10			
Band No	**Volume**	**Rf**	**Width**
1	1658176	0.324	92
2	2149612	0.606	102
3	1717655	0.703	90
Lane 11			
Band No	**Volume**	**Rf**	**Width**
1	1296551	0.543	69
2	1904870	0.627	89
3	1711211	0.709	84
Lane 12			
Band No	**Volume**	**Rf**	**Width**
1	1329551	0.548	67
2	1529244	0.629	68
3	1184837	0.716	55
4	903442	0.823	45
Lane 13			
Band No	**Volume**	**Rf**	**Width**
1	1472541	0.323	78
2	1425989	0.445	76
3	1813653	0.613	81
4	1280114	0.705	64



Lane 14			
Band No	**Volume**	**Rf**	**Width**
1	1720468	0.336	101
2	2550729	0.617	118
Lane 15			
Band No	**Volume**	**Rf**	**Width**
1	927242	0.323	55
2	1491274	0.414	86
3	1473158	0.617	68
4	1417661	0.703	72
Lane 16			
Band No	**Volume**	**Rf**	**Width**
1	1477795	0.339	89
2	1523421	0.442	85
3	1742517	0.617	80
4	1232717	0.711	68
Lane 17			
Band No	**Volume**	**Rf**	**Width**
1	974741	0.326	63
2	2563710	0.613	122
3	968994	0.708	59
Lane 18			
Band No	**Volume**	**Rf**	**Width**
1	1473542	0.31	101
2	2401120	0.594	119
Lane 19			
Band No	**Volume**	**Rf**	**Width**
1	1076902	0.333	80
2	1843500	0.614	97



Lane 20			
Band No	**Volume**	**Rf**	**Width**
1	921064	0.557	68
Lane 21			
Band No	**Volume**	**Rf**	**Width**
1	1554611	0.611	89
2	596626	0.698	41