Oral Microbiome From Birth to Adolescence

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Dedicated to

My parents and my husband for their endless love and

support

Declaration

I hereby certify that the work embodied in this thesis is the result of my own investigations, except where otherwise stated in the aknowledgments; the contents of this thesis are entirely my work. This has not been submitted, in part or full, for a degree of this or any other university or examination board.

Acknowledgements

Abstract

The oral cavity is one of the most complex microbial habitats across the human body. A balance of endogenous and exogenous factors controls this environment; any disturbance of that balance will lead to diseases, such as caries and periodontal diseases. Therefore a comprehensive assessment study of the oral microbiome acquisition and development from birth to adolescent age will provide valuable baseline data and better understanding of the microbial ecology. Analysing the depth of oral microbiome ecology and the impact of chronological factors such as teeth eruption and puberty by means of Next Generation Sequencing was the main aim of this study.

Supragingival plaque samples were collected from 80 subjects (aged 1 month to 17 years), divided chronologically in to four groups: edentulous infants, deciduous dentition, prepuberty and post puberty. Microbial composition of plaque was assessed by barcoded pyrosequencing of the V5-V6 hypervariable regions of the 16S rRNA. Pyrosequencing reads represented 8 phyla and 102 higher taxa (genus or more inclusive taxa when sequences could not be confidently classified to the genus level) of these 31 unclassified yet. Four phyla (Firmicutes, Actinobacteria, Prtoteobacteria and Bacteroidetes) predominated all age groups.

In the infant cohort, formula feeding was found to be an important factor in altering the oral microbiome, since exclusively formula-fed infants showed higher prevalence of *Prevotella melaninogenica, Neisseria polysaccharea,* and *Granulicatella adiacens.* However, there was no significant difference between the two groups with respect to phyla level.

Moreover, no conclusion could be drawn regarding the correlation with dmft/DMFT in the pre-puberty and post-puberty groups due to the discrepancy in the sample size. However, at genera level *Abiotrophia* (mainly A. *Defectiva*) and *Capnocytophaga* (mainly *C. gingivalis)* were detected more frequently in the healthy group. Other major sequences involve (*Streptococcus, Neisseria, Lautropia, Fusobacterium, Leptotrichia,*

Porophyromonas, Prevotella, Actinobaculum, Actinomyces, Corynebacterium, Rothia) were all recorded to be higher in dmft/DMFT group.

Conclusion: Using next-generation sequencing we obtained the first basic map of microbial acquisition and maturation from birth to adolescent. Our results demonstrated that plaque microbiome of children (1 month to 17 years) is still in the process of maturation and that prepubertal stage characterized by the highest level of Gram negative anaerobes, and the least richness expressed in edentulous infants. This study indicates the need for further studies involving larger population, at the depth of next generation sequencing, to understand oral microbiome ecology and provide essential intervention.

Contents

List of Tables

List of Figures

List of Definitions and abbreviations

Term	abbreviation	Definition
Taxonomy	-	The science of organism classification.
Classification	-	Ordering organisms in to groups (taxa) on the basis of their phylogenetic relationships.
Phylogeny	-	The process in which lineage of organisms evolved by separation from a common ancestor.
Identification	-	The act and result of assigning unidentified organisms to a particular rank in a previously made classification.
Species	-	A taxonomic group below the genus level in the taxonomic hierarchy.
Operational Taxonomic Unit	OTU	Unit that is typically used for rDNA sequence and a percent similarity threshold for classifying microbes within the same, or different, OTUs".
Biovar	-	Group of strains within a species can sometimes be distinguished by special characteristics.
Quantitative Polymerase Chain Reaction	qPCR	Quantitative monitoring of the formation of PCR product in real time by using special fluorescent dyes.
PCR-denaturing gradient gel electrophoresis	PCR-DGGE	Electrophoretic separation of PCR amplified 16S rDNA fragments through polyacrylamide gel with increasing gradient of denaturants
Polymerase Chain Reaction	PCR	Thermo-cycling amplification of the target DNA template.

Term	abbreviation	Definition
Next Generation Sequencing	NGS	Term for the range of the new technologies that allow for profiling of the microbiomes and metagenomes at unprecedented depths.
Papillary bleeding index	PBI	It is a periodontal index that based upon the actual bleeding tendency of the gingival papillae.
Human Oral Microbiome Identification Microarray	HOMIM	One of the targeted molecular approaches to for rapid determination of bacterial profiles of clinical samples from the human oral cavity (Dewhirst *et. al.,*2010).
Reduced Transport Fluid	RTF	One of the viable transport medium for oral samples.
Oxidation-reduction potential	Eh	The assessment of the tendency of chemical molecules to acquire electrons and thereby be reduced.
secretory immunoglobulin A	S-IgA	The predominant immunoglobulin isotype in infant's mouth, as a first line of defence against pathological microbial adherence.

Chapter One: Literature Review

1   Introduction

The oral cavity is one of the most complex microbial habitats in the human body. The terminology used to describe microorganisms in the oral cavity refers to the oral microflora, oral microbiota or more recently, the oral microbiome, a term coined by Joshua Lederberg to signify "the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space and have been all but ignored as determinants of health and disease" (Lederberg and McCray, 2001). This term has been adopted by the Human Microbiome Project and considered as the favoured nomenclature to define the complex oral bacterial community, their genetic elements and environmental interactions, which may be involved in disease (Dewhirst *et al.*, 2010).

These residential symbionts of the oral microbiome are essential in performing several physiological processes such as digestion and pathogen resistance (Costello *et al.*, 2009). Oral microbiome balance is controlled by the interaction of three main elements: bacteria, host and environmental factors (Kononen, 2000). Pathological conditions such as caries and periodontal disease occur when this synergy is disturbed (Kononen, 2000). It has been demonstrated recently that the pathological impact of the oral microbiome may extend to involve other systems such as respiratory diseases (Raghavendran *et al.*, 2000) and cardiac diseases (Ordovas *et al.*, 2006).

Therefore, a comprehensive assessment of healthy oral microbiome acquisition and structure is critical in order to understand the normal base line and how changes can lead to disease.

1.1   Ecology of the oral microbiome

An ecosystem consists of the microbial community living in a defined habitat and the surroundings composed of physical and chemical elements. The oral ecosystem is composed of a fluctuating microbial community that constantly interacts with, and is modified by, surrounding factors. The relationship

between the oral environment and the microbial community is dynamic. Although the components of the habitat determine the colonising organisms at certain sites; the metabolism of the microbial community can alter the physical and chemical properties of the surrounding environment (Pflughoeft and Versalovic, 2012). Oral ecology is the science that assesses the interaction between the oral microbiome and its habitat (Atlas and Bartha, 1992). The regulatory forces influencing the oral ecosystem can be divided into four major categories: physiochemical, host-related, microbe-related, and external factors (Marcotte and Lavoie, 1998).

1.1.1 Physiochemical factors

The physicochemical factors represent the specific environmental parameters that result from the combined action of host, microbial and external factors. In all *in vivo* and *in vitro* models, the growth of microorganisms is controlled by five crucial variables: temperature, pH, availability of water, availability of nutrients, and oxidation-reduction potential (Atlas and Bartha, 1992).

1.1.1.1 Temperature

The oral cavity is kept relatively constant temperature (35 to 36°C), which gives proper conditions for the growth of a wide range of microorganisms. During food intake, microorganisms colonizing different sites in the oral cavity are exposed to intermittent fluctuations in temperature. However, no information about the influence of this periodic temperature variation on oral bacteria exists (Marcotte and Lavoie, 1998). Although temperature has been proved to not be a fixed variable in different oral sites even within the same individual, a strong correlation was detected between periodontal pathological conditions and localised increase in temperature. For example, periodontal pockets with active disease have a higher mean temperature (38 to 39°C) compared to healthy sites (Fedi and Killoy, 1992). Moreover, temperature in pockets tends to increase with increasing pocket depth (Mukherjee, 1981). On a molecular level, this increase in temperature can interfere with gene

expression of some pathological species such as *Porphyromonas gingivalis* by hindering important proteases of this micro-organism (Percival *et al.*, 1999). In contrast, raising the temperature up regulates the synthesis of superoxide dismutase, which is an important mediator in neutralization of harmful oxygen metabolites (Amano *et al.*, 1994).

1.1.1.2 pH

Microorganisms generally require a neutral pH and they are sensitive to extreme acidic or alkali pH values. pH in the oral cavity is maintained at close to neutrality (6.7 to 7.3) and regulated by saliva (Edgar and Higham, 1996). After sugar consumption the plaque pH drops below 5 predominantly due to lactic acid production by bacteria, then the pH returns slowly to the base line value via two salivary regulatory mechanisms. Firstly, the salivary mechanical flow removes carbohydrates and washes away the acids produced by bacteria. Secondly, the buffering effect of saliva constantly neutralise the acidity from external nutritional sources and from bacterial metabolic products drinks and foods (Jensen and Schachtele, 1983). The buffering effect of saliva depends mainly on bicarbonate, but other components are also involved, such as peptides and proteins. Many of the commensal plaque species that are associated a healthy oral condition can tolerate a minor transient drop to acidic pH. However, prolonged acidic pH results in inhibition of acid-sensitive species and the selective growth of organisms with an aciduric ability, such as *Streptococci mutans* and *Lactobacilli spp,* which can subsequently result in caries. This has clearly been demonstrated in individuals with a high sugar containing diet (Harper and Loesche, 1984; Bradshow *et al.*, 1989). In contrast to mucosal and dental sites, the healthy gingival crevice has a neutral pH (6.9) which can raise during inflammatory conditions to about 7.2 (Eggert *et al.*, 1991). Such a localised increase in pH can alter the microbial community of the gingival crevice. *In vitro* testing of the effect of high pH medium on Gram-negative anaerobes showed that a rise in pH from 7.0 to 7.5 can correlate with the proportional increase of periodontal pathogens such as *Porphyromonas gingivalis* (Marsh, 1993).

1.1.1.3 Oxidation-reduction potential

Oxidation-reduction potential is the assessment of the tendency of chemical molecules to acquire electrons and thereby be reduced. It is measured in volts (V), or millivolts (mV) (Theilade, 1990). In the oral environment many metabolic reactions are of the oxidation-reduction type and the proportion of oxidized to reduced molecules is the redox potential (Eh). Molecular oxygen is the main determinant factor that influences redox potential. The variety of oxidation-reduction potentials present in the mouth creates an excellent habitat for the growth of aerobic, facultative anaerobic and anaerobic species (Theilade, 1990). Oxidation-reduction potential has been assessed during stages of plaque formation in 7 day period, on clean tooth surfaces. Early colonisers utilise oxygen and produce carbon dioxide with initial (Eh) positive values 1294 mV, later colonisers consume the oxygen and produce reducing agents such as hydrogen and sulphur containing compounds with a drop in Eh to negative -141 mV (Kenny and Ash, 1969).

1.1.1.4 Nutrients.

The nutrient is a substance that is available in the environment and used by an organism to live and grow. In the oral cavity nutrients can be divided according to the source into endogenous and exogenous types(De Jong, 1987).

1.1.1.4.1 Endogenous nutrients

Endogenous nutrients provided by the host have proved to be the primary source of nutrients for succession and diversity of the oral microbiome (Beighton and Hayday, 1986). The major source of endogenous nutrients is saliva which components such as peptides, proteins, glycoproteins and vitamins (Scannapieco, 1994). In addition, the gingival crevice is bathed with gingival crevicular fluid (GCF) which provides an additional source of novel nutrients such as albumins and glycoproteins (Cimasoni, 1983). The difference in the endogenous nutrients of the GCF is one of the significant

factors for the dental microbial variations compared to other oral sites (Cimasoni, 1983). Nutritional dynamic interactions also occur between microorganisms. For example, subgingival microbiome species have the ability to degrade proteins and glycoproteins such as albumin and haemoglobin (Carlsson *et al.*, 1984, Sundqvist *et al.*, 1985).

1.1.1.4.2 Exogenous nutrients

In addition to the endogenous nutrients, the oral cavity is characterized by exposure to exogeneous nutrients through daily repetitive ingestion of food and beverages. Despite the dietary complexity, researchers found that fermentable carbohydrate is one of the main elements that significantly affect oral microbiome ecology. Oral bacterial enzymes glycocosyltransferases and fructosyltranseraferases perform the first break down step of fermentable carbohydrates such as sucrose to produce polymers such as glucans which provide a principle substrate for strong bacterial attachment, and fructans that act as extracellular nutrients compounds (Kuramtsu, 1993, Russell, 1994). It has been demonstrated that frequent consumption of fermentable carbohydrate with associated repetitive drop to acidic pH throughout the day results in increased levels of species such as *Streptococcus mutans* and *Lactobacilli* spp. and decrease in the acid sensitive species such as *Streptococcus sanguis* and *Streptococcus gordonii* (Bradshow *et al.*, 1989; Losche, 1989).

In contrast to carbohydrate ingestion, dairy products such as milk are found to be protective for teeth against caries due to the buffering capacity of milk proteins. Moreover milk protein casein can be adsorbed onto dental surfaces and subsequently interfere with the S.*mutans* adhesion process (Schupbach *et al.*, 1996).

The effect of diet on the human gut microbiome was recently investigated on two groups of children at different geographic locations. The first group were from a village in the West African country of Burkina Faso, while the second group were from Florence, Italy. Although two bacterial phyla, Bacteroidetes

and Firmicutes, dominated the microbiota of the population in each environment, there was a dramatic shift in terms of the relative percentages of these two dominant phyla: 73% and 12%, respectively, in Burkina Faso and 27% to 51%, respectively, in Italy (De Fillippo *et al.*, 2010). This finding suggests that a high fibre diet enhances intestinal microbiome shift to Bacteroidetes which allows maximum energy intake from fibres and improve protection against non-infectious colonic inflammatory diseases.

1.1.2 Host-related factors

1.1.2.1 Host immunity

Host immunity is an important factor in the establishment and development of the oral microbiome. The natural innate immunological defence of the supragingival environment is moderated mainly by the constant salivary flow, which is enhanced by muscular actions which wash bacteria from hard and soft oral surfaces (Tenovuo *et al.*, 1987). Other nonspecific defence elements include mucins, salivary glycoproteins, lactoferrin, lysozyme and peroxidise, which play an important role in inhibiting microbial adhesion and growth (Tenovuo *et al.*, 1987).

Mucins are high-molecular-weight glycoproteins secreted by submandibular, sublingual, and minor salivary glands. Saliva has two types of mucins, MG1 and MG2. The MG1 mucin, is of high molecular weight, and is involved mainly in hard and soft tissue coating, to protect teeth from acid demineralization. MG2, which has a low molecular mass, can act as a barrier to hinder streptococci aggregation and adherence (Tenovuo *et al.*, 1987).

Lysozyme is a small cationic protein of saliva that can hydrolyse glycosidic linkages in some bacterial cell walls (Mandel, 1987).

IgG, IgM, and IgA antibodies have been detected in GCF (Ebersole *et al.*, 1985; Luo *et al.*, 1988; Kent *et al.*, 1992). These antibodies may impact on the oral microbiome picture by hindering the mechanism of bacterial adherence or interfering with metabolism (Smith *et al.*, 1994). Furthermore,

complement or opsonization has been found to be enhanced by IgG antibodies which accelerate phagocytosis and killing of oral microorganisms (Scully and Lehner, 1979; McArthur *et al.*, 1993).

The specific immune mechanism in an infant's oral cavity is presented mainly in secretory immunoglobulin A (S-IgA), which constitutes the predominant immunoglobulin isotype. As a first line of defence against pathogens the main function of S-IgA is to interfere with microbial adherence as well as penetration of foreign antigens into the mucosa by neutralizing enzymes, toxins and viruses (Marcotte and Lavoie, 1998). Tooth eruption is found to correlate with salivary antimicrobial agents. Tenovuo et al (1987) analyzed differences in the salivary antimicrobial agents pre and post tooth eruption and compared these findings with adult saliva. Interestingly, salivary lysozyme and peroxidise concentrations were found to be at the adult level at the time when the primary teeth erupt. On the other hand, immunoglobulins (IgA, IgG and IgM), lactoferrin and thiocyanate concentrations were significantly lower in children than in adults. Children with teeth had more IgG and protein in whole saliva than edentulous children (Tenovuo *et al.*, 1987).

Other immunological components such as IgG are delivered to the oral cavity via GCF especially after teeth emergence (Tenovuo *et al.*, 1987).

1.1.2.2 Hormones

The major hormonal fluctuations in humans are during puberty and pregnancy. These chronological changes are associated with high plasma levels of steroid derivatives as well as in the main body fluids, including GCF and saliva (Evans *et al.*, 1984, Lachelin and McGarrigle, 1984).

It has been shown that these periods are associated with increased gingivitis and increased production of gingival exudates (Zachariasen, 1993). Gingival inflammation may be due to microbial changes in the gingival crevice secondary to hormonal alterations (Kornman and Loesche 1980; Jensen *et al.*, 1981; Zachariasen, 1993). As such periods of hormonal increase may be

considered for growth of certain subgingival species such as *Prevotella intermedia* and Gram negative anaerobes that utilise hormones as growth factors (Kornman and Loesche 1980).

1.1.2.3 Genetics

Beside microbial transmission, genetic susceptibility of an individual may play a significant role in influencing the colonisation of certain species over others. It has been shown that specific genes may well mediate the colonisation of the oral cavity by certain species (Moore *et al.*, 1993).

The impact of ethnicity on the oral microbiome was recently assessed by analysis of the oral microbiome in children from China and United States using Polymerase Chain Reaction-denaturing gradient gel electrophoresis (PCR-DGGE). Interestingly, species of the genus *Granulicatella* were more abundant in the plaque samples of high caries children from the United States (Kanasi *et al.*, 2010) and less abundant in a similar population from China (Ling *et al.*, 2010).

The composition of the subgingival microbiome was investigated using a culturing technique in a twin study by Moore et al. (1993); a group of 10 monozygotic and 10 dizygotic twins were followed longitudinally and sampled over a three years period (age 11 to 14 years). Subgingival microbiome components in monozygotic twins (11 to 14 years of age) showed more similarity than in dizygotic twins (Moore *et al.*, 1993).

Moreover, the heritability factor was investigated to determine the abundance of oral microbial species in caries-free twins relative to caries-active twins. Corby et al. (2007) study included 204 pairs of twins (80 monozygotic and 124 dizygotic) with age range 24 to 36 months. Using the reverse capture checkerboard hybridization assay and genetic analysis, the 10 most abundant species among caries-free twins had been identified to be moderate to highly heritable; these speices were in descending order, *S. parasanguinis, Abiotrophia defectiva, Gemella haemolysans, S. mitis/oralis,*

S. sanguinis, S. cristatus, Streptococcus sp. clone CH016*, Eubacterium sp.* clone DO016*, Gemella morbillorum* and *S. salivarius.*

1.1.3 Bacterial factors

The first step of microbial colonisation process is adhesion to available soft and hard tissue surfaces. Adherence is crucial to resist the washing effect by salivary flow, and it is mediated by specific adhesins structures on the bacterial surface and complementary receptors on different oral surfaces or salivary components. Bacterial adhesins consist of polysaccharides and carbohydrate-binding proteins which are located cell wall components or capsules (Gibbons *et al.*, 1988; Scannapieco, 1994; Rudney *et al.*, 1995). In addition bacteria may adhere to other bacteria, in a process called co aggregation (Kolenbrander, 1993; Kolenbrander *et al.*, 1993).

Oral microbiome components live in beneficial synergy to maintain the homeostasis of the oral environment. An example of a positive interaction is oxygen utilisation by facultatively anaerobic bacteria which reduces the oxygen concentration and the Eh to levels that allow the colonization of anaerobic bacteria (Theilade, 1990). Another example of bacterial synergy is co-aggregation, which permits indirect adherence of some bacteria on oral surfaces. Moreover, it has been demonstrated that co-aggregated cells were more resistant to phagocytosis and killing by neutrophils *in vitro* and *in vivo* (Ochiai *et al.*, 1993).

Beside the pathological effect of the oral microbiome, bacteria favourably interact with immunological components in the human oral cavity (Kononen, 2000). For example, it was proven that bacteria influence infant's oral immunity significantly through their colonisation, and initial colonising species were found to play a beneficial role in infant's oral immunity and were mediated mainly by antibodies of the IgA1 subclasses (Kononen, 2000). Two oral streptococcal species are known to produce IgA1 proteases: all strains of *S. oralis* and *S. sanguis,* especially *S. mitis* biovar 1. Some other bacterial species colonizing in the oral cavity in childhood, such as anaerobic

Prevotella spp, also produce IgA1 protease enzyme (Kononen, 2000); this enzyme cleaves the IgA1 antibodies which are known to be the infant's first line of defence against pathological species adherence and colonisation (Tenovuo *et al.*, 1987; Kononen, 2000).

1.1.4 Environmental factors

The composition of the oral microbiome in dental plaque varies with time and all the related environmental factors such as teeth eruption, changes in dietary habits, oral hygiene and hormones (Bennett 1987; Winkelhoff *et al.*, 1988; Balmer and Wharton 1989).

1.1.4.1 Oral hygiene and antimicrobial agents

Mechanical and chemical intervention by oral hygiene methods is one of the most viable factors in the maintenance of oral health. Mechanical plaque removal by tooth brushing and flossing can significantly protect against caries and periodontal diseases (Stecksen-Blicks and Gustafsson. 1986; Mathiesen *et al.*, 1996). At the microbial level antimicrobial agents aid in protection by interfering with bacterial adhesion to the tooth surface, reducing the growth of microorganisms and plaque accumulation, by selectively supressing the expression of some virulence features, such as acid production or protease activity (Marsh, 1992). One of the most common caries preventing agents in oral hygiene products is fluoride. The caries prevention effect of fluoride is due to formation of fluoroapatite and calcium fluoride, which strengthen enamel against demineralization (Bradshaw *et al.*, 1990). Moreover, fluoride has a direct affect on bacterial physiology through reducing the sugar transport, glycolytic activity, and acid tolerance of many gram-positive species (Bradshaw *et al.*, 1990; Marsh, 1991). Other preventive agents that have been formulated for oral hygiene products include chlorhexidine, quaternary ammonium compounds, and phenolic compounds (Cummins and Creeth, 1992; Marsh and Bradshaw, 1993).

Other factors such as pathological conditions or medications that result in salivary hypofunction and subsequent increase of caries, periodontal diseases and candidosis will alter conditions in the oral cavity (Zachariasen, 1993). Oral or systemic antibiotics may reach the oral cavity via saliva and GCF and alter the microbiome balance (Sanders *et al.*, 1982, Sanders and Sanders, 1984). Other extra-oral factors such as wearing dentures (Marsh *et al.*, 1992) smoking and oral contraceptives usage (Zachariasen, 1993) may affect the oral microbiome.

1.2   Oral Microbiome and dental plaque

The oral microbiome is closely associated with dental plaque. Historically, dental plaque used to be viewed as a condensed layer of bacteria and bacterial by-products (Marsh, 1991; Spratt, 2003). With the introduction of the ecological plaque hypothesis (Marsh, 1991), dental plaque became acknowledged as three-dimensional biofilm structure composed of complex interacting microbial communities established in a matrix of polymers of bacterial and salivary origin (Spratt, 2003). Dental plaque formation is a highly ordered process performed by a range of organisms. Early colonisers adhere to saliva coated tooth surfaces which provide an initial net for the attachment of later colonisers in a process called co-aggregation (Gibbons, 1989). Studies have shown that the initial colonizers of newly cleaned dental surfaces constitute a highly selected part of the oral microbiome, mainly *S. sanguinis, S. oralis,* and *S. mitis* 1 (Nyvad and Kilian, 1987), but other genera, such as *Actinomyces,* are also present (Li *et al.,* 2004; Dige *et al.*, 2009). Microbes form biofilms on all available surfaces in the oral cavity and exhibit a range of different properties, these surfaces include:

1.2.1   Teeth

On hard dental surfaces plaque forms at the areas protected from mechanical shear forces, such as interdental spaces, the subgingival area and the pits and fissures of the occlusal surfaces. The plaque microbial composition

varies in each of these sites due to local environmental conditions (Marcotte and Lavoie, 1998). The availability of host-derived substances varies in different positions on the tooth surface; for example, In the subgingival area, bacteria are in close contact with nutrients and host defence molecules from blood circulation through GCF which enhance growth of obligatory anaerobic bacteria, most of which are Gram negative such as anaerobic *Streptococci* (now *Granulicatella* spp) and *Spirochaetes* (Slots, 1977). In contrast, bacteria growing on supragingival tooth surfaces have easy access to saliva as a major source of host nutrients. Gram-positive and facultativly anaerobic spieces are found to be predominant in supragingival dental plaque, particularly Streptococci and *Actinomyces* spp. (Nolte, 1982; Marsh and Martin, 1992). Gram-negative species such as *Veillonella*, *Haemophilus*, and *Bacteroides* are found in lower proportions (Bowden *et al*., 1975; Theilade, 1990).

1.2.2 Mucosal surfaces

There is little data available regarding microbiome structure of the oral mucosal surfaces (Kononen, 2000). The oral mucosa of the gingiva, palate, cheeks, and floor of the mouth are colonized with few microorganisms and predominated mainly by *S. oralis* and *S. sanguis* (Kononen, 2000). These two species have been found to possess a definite capacity to adhere to epithelial cells (Gibbons *et al*., 1971). On the other hand, tongue mucosa showed a significant and rich microbiome due to presence of filliform papilla (Kazor *et al*., 2003) which provide a suitable habitat for high density growth of *Streptococcus* species (mainly *S. salivarius, S. mitis*) and *Veillonella* spp. Other major groups isolated include *Peptostreptococcus* spp. (now mostly *Parvimonas*). Gram-positive rods (mainly *Actinomyces* spp*)* and *Bacteroides* spp (Kazor *et al*., 2003).

Interaction of these factors plays a significant role in bacterial acquisition and micriobiome development.

1.3   Stages of acquisition of the oral microbiome

1.3.1   Early acquisition of oral microbiome and *''Window of Infectivity''*

Development of the microbial habitat in the neonate sterile oral cavity is a dynamic process that starts during or within a few hours after birth (Cephas *et al.*, 2011). Initial acquisition of transient microbial colonisers occurs via passive transmission of micro-organisms from several sources such as milk, food, and saliva (particularly from the mother) (Alteras and Aryeli, 1980; Hopsu-Havu *et al.*, 1980, Redmo-Emanuelsson *et al.*, 1998, Cephas *et al.*, 2011).

Early studies investigating the impact of vaginal flora on oral microbiome formulation in the first days after birth revealed limited significance of transmission. Yeasts were recovered from the oral cavities one to four and seven days after birth, respectively, of only 7% and 2% out of infants, although 14% and 24% of the mothers, respectively, positively showed yeasts in their vaginal flora just before delivery (Alteras and Aryeli, 1980; Hopsu-Havu *et al.*, 1980).

However, more recently a direct correlation was found between the route of delivery and the nature of the infant oral microbiome using 16S rRNA gene sequencing. It was demonstrated that vaginally delivered infants acquire communities resembling their own mother's vagina, including *Lactobacillus* species*, Prevotella* species, and *Sneathia* species. On the other hand, infants born via Caesarean section acquire a microbial community dominated by skin bacteria such as *Staphylococcus* species, *Propionibacterium* species and, *Corynebacterium* species (Dominguez-Bello *et al.*, 2010).

The presence of the high turnover epithelial mucosal surfaces prevents the vast majority of bacterial species from colonising the oral cavity of infants; few of these will remain on the mucosal surfaces to establish the primitive oral microbiome (Cephas *et al.*, 2011). It has been demonstrated that the predominant initial colonisers during the first few days of life include mainly aerobic and facultativley anaerobic species with a high affinity to mucosal surfaces (Gibbons, 1989). This group of first microorganisms to colonise oral

mucosa are called pioneer species, and collectively make a pioneer community, predominated by *Streptococcus mitis*, *S. salivarius* and *S. oralis* (Smith *et al.*, 1993; Pearce *et al.*, 1995). Significant levels of *S. mitis* Biovar 1 has been shown in predentate infants (Smith *et al.*, 1993). *S. salivarius* was isolated in the first few hours after birth (Pearce *et al.*, 1995). Growth of pioneer species is modified by environmental factors such as pH, saliva, and nutrient requirements (Smith *et al.*, 1993) and this community will continue to flourish until environmental resistance is encountered through physical barriers (desquamation and salivary flow) or chemical factors (Eh, pH and antimicrobial properities of saliva). Over time, metabolic activity of the pioneer community modifies the oral environment and consequently provides conditions optimum for colonisation by succession of other species. Further maturation of the oral microbiome will occur gradually by acquisition of more species such as Gram-negative anaerobes. Eventually a stable situation of highly dynamic microbial diversity is achieved; which is referred to as climax community.In a study analysing the oral microbiome acquisition process in 30 infants at age (age 1 to 7 months), several Gram negative anaerobic species were identified, *Prevotella melaninogenica* was the predominant anaerobic species followed *by Fusobacteriutn nucleatum* and *Veillonella* species; and to a lesser extent *Capnocytophaga* species, *Prevotella loescheii* and *Prevotella intermedia* (Kononen *et al.*, 1992). In order to evaluate the effect of teeth eruption on the anaerobic composition of the oral microbiome Kononen et al. (1994) had followed this group longitudinally during teeth eruption. Gram negative anaerobes were present at a higher percentage with broader diversity from plaque samples around the gingival margin of newly erupted teeth. For example, some species with non-significant levels prior to teeth eruption (Kononen *et al.*, 1992), such as *Prevotella denticola* and *Capnocytophaga* species were found in all dentate children as demonstrated in (Table1-1) which confirm the ecological impact of tooth eruption on the oral microbiome.

Table 1-1 The effect of tooth eruption on the composition of the oral microbiome in young children (Kononen *et al.*, 1992, 1994)

Bacteria	Percentage isolation frequency at 3 months mean age	Percentage isolation frequency at 32 months mean age
Prevotella melaninogenica	76	100
Prevotella losecheii	14	90
Prevotella intermedia	10	67
Prevotella denticola	Not detected	71
Fusobacterium nucleatum	67	100
Selenomonas species	Not detected	43
Capnocytophaga species	19	100
Leptotrichia species	24	71
Campylobacter species	5	43
Eikenella corrodens	5	57
Veillonella species	63	63

The relation between primary dentition eruption and the acqusition of two other members of indigenous biota (*S. sanguinis* and *S. mutans*) which show high adherence properties to saliva coated non-shedding teeth surfaces, had been confirmed four decades earlier using the traditional culturing technique (Berkowitz *et al.*, 1975; Carlsson *et al.*, 1975; Stiles *et al.*, 1976; Caufield *et al.*, 1993, 2000). In a study by Berkowitz *et al.* (1975) it was shown that

S.mutans is strongly related to tooth eruption as *S. mutans* were found in 12 (30%) of 40 subjects with only erupted primary incisors and directly related to the number of emerged teeth (Caufield *et al.*, 1993). Table 1-2 shows the predominant taxa associated with tooth eruption.

Table 1-2 Summary of oral species in dentate infants

Facultative	Anaerobic
S. mitis Bioivar 1 & 2	*Actinobacillus spp*
S. salivarius	*Actinomyces spp*
S. oralis	*Camplylobacter spp*
S. sanguis	*Fusobacteruim spp*
S. gordonii	*Lactobacillus spp*
S. anginosus	*Leptotrichia spp*
Capnocytophaga spp	*Peptostreptococcus spp*
Eikenella spp	*Prevotella spp*
	Selenomonas spp
	Veillonella spp

Window of infectivity is a term given to the well-delineated age range (18 to 26 months) during which initial acquisition of *S. mutans* occurs in an infant (Caufield *et al.*, 1993). Vertical transmission of *S. mutans* from mothers to their children has been suggested by many studies using culturing methods (Van Houte *et al.*, 1981; Kohler *et al.*, 1984; Caufield *et al.*, 1988). In those studies isolates of *S. mutans* harboured by mothers and their children exhibit similar or identical bacterial profiles (Berkowitz and Jordan, 1975; Davey and Rogers, 1984) and identical plasmid or chromosomal DNA patterns (Caufield

et al., 1982, 1985, 1986, 1988; Caufield and Walker, 1989; Hagan *et al.*, 1989; Kulkarni *et al.*, 1989).

S. mutans is known to be a potent cariogenic bacterium as it has several virulence factors. For example, *S. mutans* has the ability to bind to tooth surfaces in the presence of sucrose by the formation of water-insoluble glucans, a polysaccharide that aids in binding to hard non-shedding tooth surface (Loesche, 1986). The most important virulence feature, however, is the acidophilicity of *S. mutans*. Unlike the majority of oral microorganisms, *S.mutans* thrives under acidic conditions and becomes the dominant bacterium in cultures with permanently reduced pH (Loesche, 1986).

S. mutans colonisation was investigated thoroughly by longitudinal monitoring of *S. mutans* levels in saliva samples of newly born infants and their mothers for three years. The initial acquisition of *S. mutans* occurred at the median age of two years old. *S. mutans* recovered from 75% of a group of 38 infants at three years age, compared to only 25% of the same group at age 19 months when tooth eruption started (Caufield *et al.*, 1993).

Several individual parameters may impact the initial acquisition of *S. mutans* in infants, including high maternal *S. mutans* levels and caries status, low infant birth-weight, obturators for management of cleft palate, early tooth emergence and low salivary IgA antibody levels (Klein, 1946; van Houte *et al.*, 1981; Kohler *et al.*, 1988; Smith *et al.*, 1998; Milgrom *et al.*, 2000; Wan *et al.*, 2001, 2003). Tooth enamel defects induced by maternal factors during pregnancy are significantly associated with early colonization by *S. mutans* in 3 to 5 year-old children (Li *et al.*, 1994). Moreover, pre-term infants are 4.4 times more likely to be colonized by *S. mutans* than are normal-term babies (Wan *et al.*, 2003).

In addition, mode of delivery was found to be a strong influencing factor on the rate of acquisition. In a four-year follow-up study of a newly-born infants cohort (127 vaginal and 29 Caesarean section deliveries) and their mothers, although the percentage of infants with detectable *S. mutans* was similar between the two groups (31.0% for Caesarean section *vs.* 36.7% for vaginal),

the Caesarean section infants acquired *S. mutans* at a younger age compared with the vaginally delivered infants (Caufield *et al.*, 2005).

S. mutans also appears capable of horizontal transmission. Horizontal transmission of *S. mutans is* the term used to describe the non-maternal acquisition of *S. mutans* by infants from other close members in the family or school. For example, children who had no detectable *S. mutans* levels until the age of five often shared strains with both mother and father when the bacterium was finally acquired (Loveren *et al.*, 2000). Moreover, identical salivary *S. mutans* strains were detected in salivary samples of a group of unrelated nursery schools children ranging in age from two months to four years (Mattos-Graner *et al.*, 2001; Tedjosasongko and Kozai, 2002; Berkowitz 2003; Liu *et al.*, 2007). Similar positive findings of identical *S.mutans* genotypes in an older age group (five to six years old) was demonstrated by analysing 96 subjects in three public schools; three unrelated pairs of children were identified (Domejean *et al.*, 2010).

During the last decade, the oral microbiome constitution in young population was re-examined using culture-independent DNA-based methods, which revealed a greater richness and diversity of the microbial communities in both edentulous and dentate infants than was previously thought (Aas *et al.*, 2008; Gezani *et al.*, 2009; Lazarevic *et al.*,2009; Ling *et al.*, 2010; Cephas *et al.*, 2011). The infant oral and body microbiome in the first few hours after birth found to be homogeneously distributed across the body with no specific differentiated site specific picture across skin, oral, nasopharyngeal, and gut habitats regardless of delivery mode (Dominguez-Bello *et al.*, 2010).

Cephas et al. (2011) analysed the salivary bacterial microbiome in a an older group of five infants (two to six months mean age) and their mothers using high throughput 454 pyrosequencing. The results demonstrated that the salivary microbiome of infants prior to tooth eruption had a highly variable population with over 99% of all sequences being members of the following phyla: *Firmicutes, Proteobacteria, Actinobacteria, Fusobacteria, Spirochaetes* and *Bacteroidetes* (Cephas *et al.*, 2011). The predominant genera were *Streptococcus* (62%), *Neisseria* (4.9%), *Rothia* (3.2%), *Veillonella* (15.3%),

Granulicatella (1.7%), *Prophyromonas* (2.9%), *Haemophilus* (2.6%) and *Prevotella* (0.72%). Interestingly, the ''window of infectivity'' was not proven and no legacy effect was detected between infants and mothers in *S. mutans* microbial count (Cephas *et al.*, 2011). Similar prevalence of these bacterial genera was demonstrated by Ling et al using high-throughput barcoded pyrosequencing and PCR-DGGE to examine bacterial diversity of the oral microbiome in saliva and supragingival plaque from 60 Chinese children aged three to six years old with and without dental caries. These results suggest that children aquired most of the species needed to generate a mature biofilm before tooth eruption, and all available soft tissue sites may act as essential reservoirs (Ling *et al.*, 2010; Cephas *et al.*, 2011).

In comparison to adult saliva (Keijser *et al.*, 2008), the microbial population in the primary dentition showed   higher proportions of Firmicutes and Actinobacteria and lower proportion of Bacteroidetes, Fusobacteria and Spirochaetes (Crielaard *et al.*, 2011)

Selected cariogenic bacteria (Streptococci, Lactobacilli, Actinomyces) at different soft tissue sites in this young population were comprehensively investigated by Gezani et al.(2009) using checkerboard DNA-DNA hybridization in 93 children (3 to 12 years). Samples from five different sites were analysed: saliva, supragingival plaque, subgingival plaque, tongue dorsum and buccal mucosa. The proportions of *S. mutans* isolated from soft tissue and *S. sanguinis* from soft tissue, subgingival plaque and saliva samples increased significantly with age, whereas the opposite was seen for *L. acidophilus*. Interestingly, cariogenic bacteria were present in almost all children though virtually no caries was observed (Gezani *et al.*, 2009). This relationship between high *S. mutans* levels and severity of caries in children has also been doubted by others. For example, Aas et al. (2008) investigated the microbial diversity in supragingival plaque samples collected from 39 healthy control subjects and 51 subjects with severe caries. Using 16S rRNA gene cloning and sequencing in a reverse-capture checkerboard assay, samples were analysed for 110 prevelant bacterial species. Results demonstrated that 10% of subjects with rampant caries have no detectable

level of *S. mutans* and other species of genera *Veillonella*, *Lactobacillus*, *Bifidobacterium*, and *Propionibacterium*, low-pH non-*S. mutans* streptococci, *Actinomyces* species and *Atopobium* species. These are likely play important roles in caries progression (Aas *et al.*, 2008). In addition, other species such as *Veillonella, Actinomyces, Granulicatella, Leptotrichia* and *Thiomonas* have been found in plaque and shown to be significantly associated with caries lesions in children and young adults (Ling *et al.*, 2010).

The concept of a specific healthy core microbiome and a disease core microbiome in children has been suggested by recent research among group of Chinese children with severe early childhood caries (ECC) (Jiang *et al.*, 2013). Supragingival plaque samples were obtained from 40 subjects (ages three to six years).   454 pyrosequencing demonstrated that three genera (*Streptococcus, Granulicatella*, *Actinomyces*) exhibited a relatively increased abundance in severe ECC subjects, whereas caries-free subjects exhibited a relatively increased abundance of *Aestuariimicrobium* (Jiang *et al.*, 2013).

 The sampling site is a significant factor noted in the oral microbial ecological studies. Many studies investigating microbiome site variation using both culturing and molecular techniques (Liljemark & Gibbons, 1971, 1972; Mager *et al.*, 2003; Aas *et al.*, 2005; Zaura *et al.*, 2009) have revealed that the bacterial microbiome greatly varies among different intraoral surfaces. However, because most studies have pooled their data by site, it is not clear if inter-subject variability is greater than the variability among sites within the same subject. One more example of this site somponents are findings using DNA-DNA hybridisation by Gezani et al. (2009); *Streptococcus spp.* made up a large proportion of the total DNA in bacterial counts, for the soft tissues as well as saliva, tongue, and supragingival samples. Specifically, *S. mitis* and *S. oralis* were found in high proportions in the soft tissue samples, *S. salivarius* was found in high proportions in saliva, soft tissue and tongue samples while *Actinomyces* species were found most frequently in supragingival and subgingival plaque samples (Gezani *et al.*, 2009).

1.3.2 Oral microbiome in puberty

During their transition to adolescence, children exhibit major physiological and hormonal changes which directly affect the nature of the oral microbiome (Mombelli *et al.*, 1989). Hormonal fluctuations are thought to have a direct effect on the periodontium micro-circulation by increasing vascular permeability which produces a corresponding shift in plaque microbiome structure (Kornman and Loesche, 1982). In addition, saliva and GCF of a child during puberty revealed high levels of steroids (Evans *et al.*, 1984, Lachelin and McGarrigle, 1984), which can become a nutrient source for some microbial species. For example, certain steroidal hormones have been found to be utilized by *Bacteroides* (Kornman and Loesche, 1982).

It has been demonstrated that these changes are associated with increased gingivitis and more production of gingival exudates (Zachariasen, 1993). A significant correlation between pubertal physiologic changes and the shift in oral microbiome was demonstrated in a longitudinal five-year study monitoring the gingival clinical inflammatory sign Papillary Bleeding Index (PBI) in relation to physiologic parameters of puberty between the ages of 11 and 15 years (Mombelli *et al.*, 1989). In a group of 22 boys and 20 girls, pubertal and skeletal development as well as plaque and gingival indices were assessed and correlated with PBI yearly between age 11 and 15. The bleeding percentage of all dental spaces of the dentition showed a direct relationship with the onset of puberty and reached the peak of 100% around age 14 years of age. A significant decrease of PBI after that age was noted in both gender groups when pubertal hormones start to decline to the regular physiologic ranges (Mombelli *et al.*, 1989).

A similar longitudinal study was later performed by Gusberti *et al.*(1990), in order to investigate the structure of the oral microbiome by analysing subgingival composition of individuals passing through puberty stage (22 boys, 20 girls) annually for five years. Subgingival microbial samples were obtained 10 times in five year period from a specific intraoral site (mesial to the upper first permanent molar). Microscopic analysis and culturing techniques were employed to assess the microbiome composition.

Actinomyces odontolyticus showed a significant time trend of increase among the Gram-positive bacteria and showed a direct relationship with puberty indices in both genders such as testes growth in boys and Tanner score for breast development in girls. In the Gram negative group, *Capnocytophaga spp. Bacteroidetes* were the predominant species. The shift to Gram-negative anaerobic bacteria (including Bacteroides) was found to be significant during the period of the mixed dentition (Wojcicki *et al.*, 1987). For example, *Prophyromonas gingivalis* has been isolated from the plaque of 80% of children during and after puberty (Watson *et al.*, 1991). *V. atypica, P. denticola and P. melaninogenica* were among the species that contributed most to changes in subgingival microbial composition during puberty (De Araujo and Macdonald 1964, Frisken *et al.*, 1987, Wojcicki *et al.*, 1987, Van Oosten *et al.*, 1988; Gusberti *et al.*, 1990).

This maturation of the oral microbiome during puberty was confirmed by high-throughput sequencing (Gezani *et al.*, 2009; Lazarevic *et al.*, 2009; Ling *et al.*, 2010; Crielaard *et al.*, 2011). In the study of Cirelaard et al.(2011); plaque samples from eleven adolescent subjects (ages 11 to 18 years) were analysed using PCR amplification of the 16S rDNA and high throughput 454 pyrosequencing. Results revealed that this age group experienced increased amounts of species from the *Bacteroidetes* (mainly *Prevotella*), *Veillonellaceae* and *Spirochaetes.*

1.3.3 Adult oral microbiome

The core oral microbiome in a healthy adult population was comprehensively investigated using culture-dependent and culture-independent methods. Approximately 280 bacterial species from the oral cavity have been isolated by culture and formally named (Dewhirst, 2008). Estimates of oral biodiversity have implicated the presence of more than 700 different microbial species (Socransky *et al.*, 1998; Kroes *et al.*, 1999; Paster *et al.*, 2001, 2006, Aas *et al.*, 2008, Dewhirst, 2008) of which half these are yet to be cultivated. Recently, several studies employing DNA-based technologies revealed great richness of the healthy core oral microbiome, either via cloning and

sequencing approaches of microbial 16S rDNA (Aas *et al.*, 2008; Preza *et al.*, 2008; Riggio *et al.*, 2008) or by revolutionary next generation sequencing methods (Keijser *et al.*, 2008; Zaura *et al.*, 2009).

Six major phyla were dominant in the healthy individual oral microbiome, *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, *Spirochaetes*, and *Fusobacteria*. At a genera level, 15 bacterial genera observed in higher counts in healthy individuals; *Neisseria*, *Cardiobacterium*, *Haemophilus*, *Campylobacter* (Proteobacteria) *Streptococcus*, *Granulicatella*, *Veillonella* (Firmicutes), *Fusobacterium* (Fusobacteria), *Rothia*, *Actinomyces*, *Corynebacterium Atopobium* (Actinobacteria); *Prevotella*, *Capnocytophaga* and *Bergeyella* (Bacteroidetes). The most predominant bacterial genera were *Streptococcus, Gemella, Abiotrophia, Granulicatella, Rothia, Neisseria,* and *Prevotella* (Dewhirst *et al.,* 2008; Keijser *et al.*, 2008; Zaura *et al.*, 2009). In contrast to the healthy microbiome, inflammation within gingival and periodontal tissues are associated with characteristic periodontal pathogens including *P. gingivalis* and *Aggregatibacter actinomycetemcomitans.* These species alter the transcriptional profile of epithelial cells and aggravate inflammatory process (Handfield *et al.*, 2005; Mans *et al.*, 2009).

In the elderly population several physiological processes are shown to be altered gradually with age, specifically the immune system (Marcotte and Lavoie 1998), masticatory force (Miura *et al.*, 2005) and salivary gland function (Amerongen *et al.*, 2002). Other age-related factors that can alter the oral microbial composition include dentures, hormones, long-term medication, diet and reduced oral hygiene (Hughes *et al.*, 2001).

Most studies of the oral microbiome have focused on young and middle-aged adults (Socransky *et al.*, 1998; Kroes *et al.*, 1999; Paster *et al.*, 2001,2006, Aas *et al.*, 2008, Dewhirst *et al.*, 2008; Gezani *et al.*, 2009; Crielaard *et al.*, 2011; Cephas *et al.*, 2011). A limited number of studies in the literature have investigated the microbiome of the elderly. Most of these studies used culture-based methods of single isolated sites or were limited to a few species. For example in an interesting study evaluating the microbiological shift with age by analysing saliva and plaque samples from four different age

groups in a wide age range (20 to 80 years), the prevalence of *S. mutans* and *Spirochetes* in plaque and saliva were similar in all age groups. Significant increases in Lactobacilli and yeasts were found among those subjects aged 70 years and above (Percival *et al.*, 1991). Similar results were found by Kaklamanos et al. (2005) among a samples of elderly Greeks.

Recently the site and subject specificity of the elderly oral microbiome was investigated using the pyrosequencing (Preza *et al.*, 2009). Plaque samples were collected from the tongue dorsum, buccal mucosa, hard palate, supragingival and subgingival plaque from the same roots for 30 subjects (age range 73 to 93 years). Using a 16S rRNA gene-based microarray technique, it was shown that the diversity in the elderly oral microbiome was site rather than subject specific. Cheek and palate represented the highest diversity, with *Streptococcus* spp, *Veillonella* spp and *Fusobacterium* spp*.* being predominant.

1.3.4   Modifying acquisition

The complex human microbiome represents approximately 90% of the cell count in and on the human body (Gill *et al.*, 2006). The relationship between the body's microbiome and their habitats is reciprocal; microbiomes at different body sites play a substantial role in controlling individual traits such as: nutrition, pathogen resistance, and immune system functions (Blaser and Falkow, 2009; Costello *et al.*, 2009). The composition and the amount of species in each microbiome is regulated by local environmental factors and the biology of each body habitat (Reid *et al.*, 2011, Spor *et al.*, 2011). Therefore, a human microbiome can be identified as an active changeable environment of genes and gene products, which makes it an interesting target for different local and systemic therapeutic measures or modifications using dietary changes hygienic practice and antibiotics (Pflughoeft and Versalovic, 2012).

Early efforts to modify dental plaque focused on eliminating *S. mutans* or replacing it with a mutant of *S. mutans* that lacked the acid production

capacity but had other advantageous properties (Hillman, 1978). Modification of the environment via application of direct preventive measures such as dietary counselling, professional oral hygiene instructions and fluoride treatment to the mother during the window of infectivity period was found to reduce the mother's *S. mutans* level and subsequently delay the onset of caries in the child (Kohler *et al*., 1983, 1984).

The emerging knowledge of bacterial physiology in a biofilm habitat is providing a new understanding that might be used in the design of novel antimicrobial strategies. Most common oral diseases such as caries and periodontal infections result from balance disturbance of resident microbial communities mediated by the interaction of microorganisms, behavioural habits and the immune system of the host (Marsh, 2003). A better understanding of the composition and ecological events that drive changes in the structure, from health to pathology, of the oral microbial communities is the first step in the development of preventive methods to maintain oral health (Diaz *et al*., 2012).

Nowadays, research is directed towards developing novel antimicrobial approaches, particular in the field of bacterial interactions and by-products interventions (Kaplan *et al*., 2011). For example, the development of an effective antibacterial experimental method called Selectively Targeted AntiMicrobial Peptides (STAMP) which is based on selective delivery of antimicrobial compounds in high concentrations that effectively kills *S. mutans* while leaving other bacteria in the environment unaffected (Kaplan *et al*., 2011).

Application of the novel non-culturing methods such as high-throughput 454 pyrosequencing will provide a better understanding of the composition of oral microbiome dimensions, ultimately leading to potentially effictive prevention methods.

1.4 Available methods to analyse the composition of the oral microbiome

1.4.1 The culture-based method

Traditionally identification of the species in any given sample was achived by growing it *in vitro* on suitable media. Culturing can be done on selective and non-selective media. Blood agar is a common non-selective medium as it allows growth of a broad spectrum of organisms (Samaranayake, 2002). More specific media include Gram negative anaerobic medium supplemented with vancomycin to selectively allow growth of Gram negative anaerobic rods while inhibiting Gram positive bacteria (Samaranayake, 2002). Another example of a selective medium is *Staphylococcus* spp*.* isolation on mannitol salt medium, as fermentation of this salt by *Staphylococcus aureus* will turn the medium from pink to yellow (Samaranayake, 2002).

Laboratory culturing under special conditions and using a range of media has allowed isolation of a diverse range of bacteria. However, it is well recognised that the main drawback of this method is its narrow spectrum. It has been estimated that 50% to 60% of distinct extant bacterial phyla in oral cavity still have no cultivable representatives (Kolenbrander, 2000; Vartoukian *et al.*, 2010; Siqueria *et al.*, 2013). Moreover, the culture-dependent technique is expensive, sensitive and needs a highly skilled individual.

There is a growing need for developing improved methods to cultivate and characterize the as-yet-uncultivated portion of the oral microbiome so as to unravel its role in health and disease (Siqueria *et al.*, 2013). Theoretically, all bacteria can grow under proper nutritional and physicochemical conditions (Clarridge *et al.*, 2004). However, development of new improved culture media is still a challenging goal and is mainly due to the highly diverse microbial community present with each member having different nutritional requirements (Tian *et al.*, 2010). Siqueria et al. (2013) has recently suggested a list of recommendations in order to cultivate the yet-uncultivated bacteria, such as the use of culture media with little or no added nutrients and addition of specific growth factors in the culture media. A very interesting strategy to ensure the availability of natural growth factors is to perform incubation in the

natural environment using special devices (Kaeberlein *et al.*, 2002; Gavrish *et al.*, 2008; Sizova *et al.*, 2012) such as a diffusion chamber (Kaeberlein *et al.*, 2002; Bollmann *et al.*, 2007) or a hollow fiber membrane chamber (Aoi *et al.*, 2009), which allow diffusion of important growth factors from a natural environment to the culture via a special membrane (Siqueria *et al.*, 2013).

1.4.2 Culture-independent techniques

Molecular techniques depend on identification of bacterial DNA and RNA. During the last three decades, a wide spectrum of new techniques have been developed aimed at identifying species DNA or RNA molecular ''finger-prints'' (Mullis *et al.*, 1987).

The polymerase chain reaction (PCR) was developed by Kary Mullis in1993 (Mullis *et al.*, 1987) and is a technique that amplifies a given piece of DNA to a huge number of copies using a thermostable DNA polymerase enzyme. This can be used to amplify specific genes or parts of genes that can, when sequenced, be used to identify the bacterial species they originated from.

Molecular biology techniques have led to new approaches for bacterial identification when trying to analyse the complex picture of different habitats, such as the oral micriobiome. One of the introduced culture-independent approaches is based on amplification and analysis of the 16S rRNA genes in a microbiome sample (Spratt, 2004). 16S rRNA has proven to be the most useful phylogenetic marker to identify bacteria and to determine their evolutionary relationships. Ribosomal RNA gene is essential for life and present in all prokaryotes. It contains nucleic acid sequences with highly conserved and variable regions which allow organism identification at an acceptable specific level (Spratt, 2004; Nossa *et al.*, 2010). In addition, the 16S rRNA gene is large enough (about 1500 bases) to provide sufficient sequence variability among bacteria, thereby making comparisons possible at different taxonomic levels.

This method is truly culture-independent inthat bacteria can be identified within a sample without the need for culture. 16S rRNA gene PCR amplicons

generated from DNA extracted from a mixed sample can be singularised by cloning into *Escherichia coli* and then comparatively sequenced. Sequence data are compared with the existing information in databases, followed by phylogenetic analysis techniques. The rapidly growing Ribosomal Database (RDP) (http://rdp.cme.msu.edu) contains 351796 16S rRNA gene sequences (Spratt, 2004; Nossa *et al.*, 2010).

This method of PCR and sequence analysis of 16S rRNA gene cloning and sequencing have been used in detecting the important species at different specific oral sites; such as subgingival (Paster *et al.*, 2001; Kumar 2006) and supragingival dental surfaces (Becker *et al.*, 2002), toungue surface (Kazor *et al.*, 2003). However, routine cloning and sequencing methods are time consuming (Becker *et al.*, 2002). Molecular methods primarily using 16S rRNA gene-based cloning studies have massively enhanced the field of microbial identification, since they can provide a rapid and reliable way to identify a large number of currently uncultivated microorganisms in the complex samples (Spratt, 2004).

1.4.2.1 Polymerase chain reaction

PCR is an *in vitro* technology for the enzymatic production of specific DNA sequences, via two oligonucleotide forward and reverse primers which hybridise to both strands of the target DNA (Mullis *et al.*, 1987; Henry, 1989). PCR can directly determine the target sequence without molecular cloning and template preparation with host growth and vector purification (Wong *et al.*, 1987; Wrischnik *et al.*, 1987).

PCR relies on repeated thermal cycling process to produce an exponential amplification of target sequences. Thermal cycling can be conveniently and precisely conducted in an automated thermal cycler. The basic PCR process is governed by the available reagents in the reaction volume (Mullis *et al.*, 1987; Henry, 1989):

- DNA template which contain the target DNA region to be amplified.

- Two primers that complementary to the 3' ends of the sense and anti sense DNA strands.

- Thermostable DNA polymerase enzyme.

- Deoxynucleoside triphosphates (dNTPs; nucleotides containing triphosphate groups), the building-blocks from which the DNA polymerase synthesizes a new DNA strand.

- Buffer solution providing a suitable chemical environment for optimum activity and stability of the DNA polymerase.

20 to 40 cycles of PCR amplification produce about million fold amplification of each targe sequence. Each cycle is composed of (i) denaturation stage to denature the DNA and separate the two DNA strands (94°C); (ii) Primer annealing stage in which the primers bind to the complementary sequences of the target DNA at a lower temperature (50-60°C) and initiate the synthesis of the new DNA strand; (iii) An extension stage (72°C) in which the DNA polymerase enzyme performs the DNA building process using the available free deoxynucleoside triphosphates to the 3' end of the primer according to the order of nucleotides in the template. The products of one cycle serve as templates for the next cycles, so the number of target DNA copies double at every cycle. Thus a repetitive series of cycles of template denaturation, primer annealing, and extension by DNA polymerase results in the exponential accumulation of a specific target sequence (Mullis *et al.*, 1987; Henry, 1989).

All PCR applications employ a heat-stable DNA polymerase, such as Taq polymerase, which is an enzyme that can synthesize a new strand of DNA from deoxyribonucleotide triphosphates (dNTPs) using template DNA strand and the primer to initiate the copying process. Taq polymerase is an enzyme that is isolated from the thermophilic bacterium *Thermus aquaticus* (Chien *et al.*, 1976; Saiki *et al.*, 1988), and it has optimum activity temperature at around 75-80°C.

PCR is a technique to that is highly sensitive to contamination (Mullis *et al*., 1987; Henry, 1989) which can impact on both research and diagnostic value of the method. Because of this a number of procedures have been developed in order to minimise contamination. In general, careful handling and strict laboratory precautions are essential to minimize the contamination (Mullis *et al*., 1987; Saiki *et al*., 1988; Henry, 1989).   The PCR procedure is summarised in Figure 1-1.

Figure 1-1 PCR (http://schoolworkhelper.net/2011/06/pcr-uses-steps-purpose/)
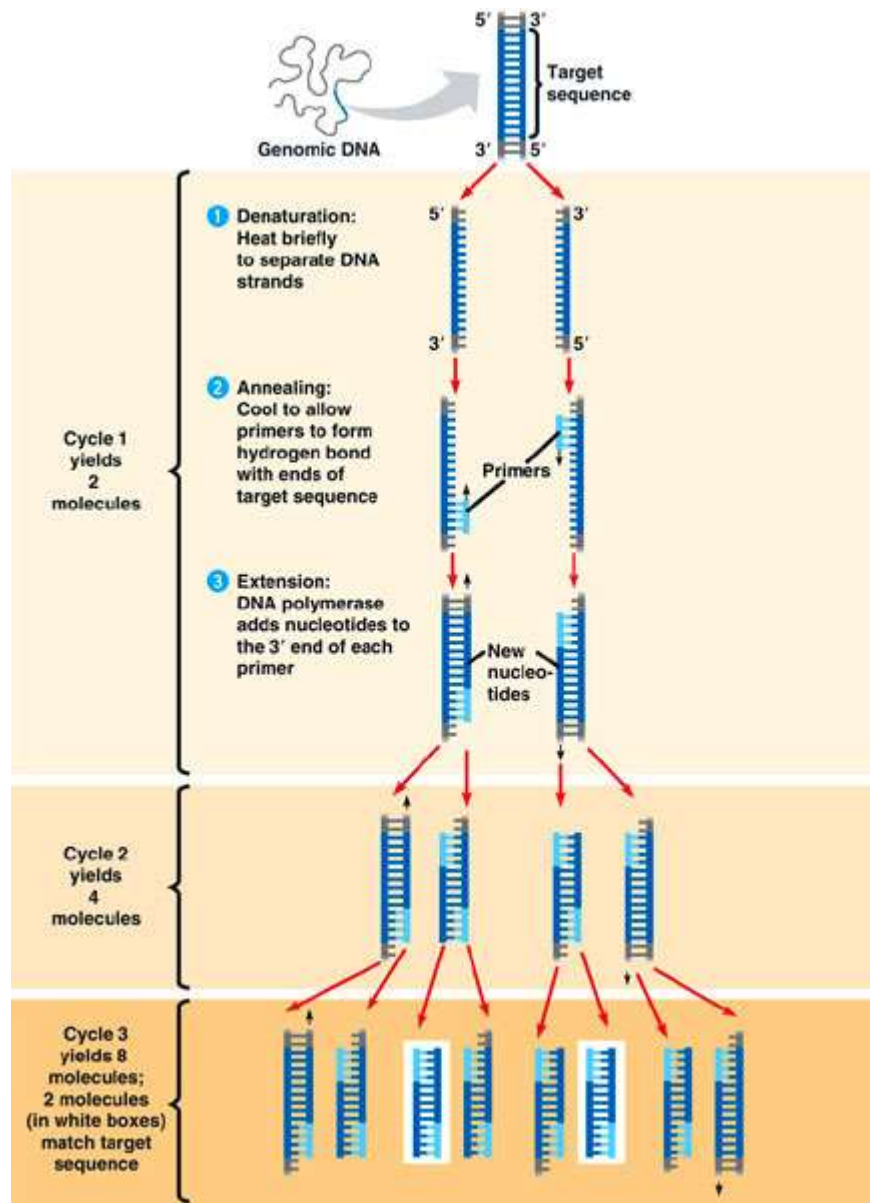
Because of its simplicity, sensitivity and cost effectiveness PCR since its introduction has been the most commonly used tool in research and diagnostic purposes. It has helped in detection of infectious and genetic diseases as a first step in producing enough amount of the target sequence so that efficient methods of identification can be employed (Mullis *et al.*, 1987; Saiki *et al.*, 1988; Henry, 1989). The first application of diagnostic PCR was in the prenatal diagnosis of sickle-cell anaemia (Saiki *et al.*, 1985). Since then, PCR has been used for diagnosis of many conditions such as B-thalassemia

mutations (Wong *et al.*, 1987; Saiki *et al.*, 1988) and phenylketonuria (DiLella *et al.*, 1987). In addition to the enormous applications of pathological diagnosis, PCR has proved extremely useful in bacterial (Sameer Barghouthi, 2011), fungal (Julie Verrier *et al.*, 2011) and viral (Amy *et al.*, 2007) identification.

1.4.2.2 Real -time polymerase chain reaction

Real-time polymerase chain reaction (also known as quantitative real time polymerase chain reaction (qPCR)) is a method based on PCR which is used to amplify and simultaneously quantify targeted DNA molecules for one or more specific sequences in a DNA sample (Chiang *et al.*, 1996; Gibson *et al.*, 1996; Heid *et al.*, 1996; Hellemans *et al.*, 2007). Since its introduction more than 10 years ago (Chiang *et al.*, 1996; Gibson *et al.*, 1996; Heid *et al.*, 1996) qPCR has become the standard method for quantification of nucleic acid sequences. Its ease of use and high sensitivity, specificity and accuracy has resulted in a rapidly expanding number of applications with increasing throughput of samples to be analyzed.

The procedure relies on the same basic principles of PCR; the additional feature is that the amplified DNA is detected and quantified simultaneously as the reaction progresses in real-time. During each cycle the quantitative PCR technology allows measurement of PCR products accumulated during the course of the reaction. This is most commonly achieved through the use of fluorescence-based technologies (Chiang *et al.*, 1996; Gibson *et al.*, 1996; Heid *et al.*, 1996; Hellemans *et al.*, 2007).

Two methods for detection of products in real-time PCR are:

 (1) Commonly used, non-specific fluorescent dyes such as SYBR Green that intercalate with any double-stranded DNA. Because of the ease in designing the assays and its relatively low setup and running costs, this technique is employed by half of all real-time PCR users (Knudtson *et al.*, 2007; Arikawa *et al.*, 2008). The major drawback of SYBR Green assays, however, is that

the dye is non-specific and can generate false positive signals if non-specific products or primer-dimers are present in the assay which can potentially interfere with, or prevent, accurate quantification of the intended target sequence.

(2) Sequence-specific DNA probes that fluoresce upon hydrolysis such as TaqMan assays (Heid *et al.,* 1996). These consist of oligonucleotides labelled with a fluorescent reporter that only permits detection only after hybridization of the probe with its complementary DNA target This method significantly increases the specificity, and enables quantification even in the presence of non-specific DNA amplification (Canales *et al.*, 2006; Arikawa *et al.*,2008). The specificity of fluorescent reporter probes prevents interference of measurements caused by primer dimers, which are undesirable potential by-products in PCR. However, fluorescent reporter probes do not prevent the inhibitory effect of the primer dimers, which may depress accumulation of the desired products in the reaction (Heid *et al.*, 1996).

TaqMan chemistry uses the 5′-3′ exonuclease activity of Taq DNA polymerase, which degrades a nonexpendable fluorescent DNA probe following hybridization and extension in the PCR. The method relies on a DNA-based probe with a fluorescent reporter at one end and a quencher of fluorescence at the opposite end of the probe (Heid *et al.*, 1996; Reynisson *et al.*, 2006, Heather *et al.*, 2008). The close proximity of the reporter to the quencher prevents detection of its fluorescence; breakdown of the probe by the 5' to 3' exonuclease activity of the Taq polymerase breaks the reporter-quencher proximity and thus allows unquenched emission of fluorescence, which can be detected after excitation with a laser. An increase in the product targeted by the reporter probe at each PCR cycle therefore causes a proportional increase in fluorescence due to the breakdown of the probe and release of the reporter (Heid *et al.*, 1996; Reynisson *et al.*, 2006; Heather *et al.*, 2008).

There are numerous diagnostic and basic research applications for real-time polymerase chain reaction. Fluorescent probes can be used in multiplex

assays for detection of several genes in the same reaction. In research settings, real-time PCR is mainly used to provide quantitative measurements of gene transcription. The technology may be used in determining how the genetic expression of a particular gene changes over time, such as in the response of tissue and cell cultures to an administration of a pharmacological agent, progression of cell differentiation, or in response to changes in the environmental conditions. It is also used for the determination of zygosity of transgenic animals used in research (Livak and Schmittgen. 2001; Thiel *et al.*, 2003; Hindiyeh *et al.*, 2003).

1.4.3   Revolutionary molecular technology

The rapid growth of next-generation DNA sequencing methods has enabled novel applications and enormous changes in genetic and ecological research. They have substantially widened the scope of metagenomic analysis of different enviroments (Mardis 2008; Wheeler *et al.*, 2008; Pearson *et al.*, 2007; Margulies *et al.*, 2005). Although these instruments only began to become commercially available in 2004, they already are having a major positive impact on our input to explore and answer genome-wide biological questions (Mardis, 2008). Next-generation platforms are allowing profiling of the microbiomes and metagenomes at sufficient depth which will lead to a better understanding of ecological diversity and the identification of unknown etiologic agents in different atmospheres (Margulies *et al.*, 2005; Pearson et al., 2007; Wheeler et al., 2008; Mardis, 2008).

1.4.4   Next-generation sequencing techniques

The rational of DNA sequencing to identify the precise order of nucleotides within a DNA molecule was proposed about four decades ago. Fred Sanger and co-workers introduced the first independent sequencing method which was based on selective electrophoretic separation of chain-termination products in individual sequencing reactions (Sanger *et al.*, 1975). Unlike the Sanger method, next-generation sequencing is performed by repeated cycles

of polymerase-mediated nucleotide extensions or by machinery automated cyclical ligation of oligonucleotides (Mardis 2008; Voelkerding et al., 2009). As millions of reactions occur in a massively parallel process, these methods produce an extensive amount of nucleotide sequence output in a single machine run, depending on the platform. The three most common widespread platforms for massively parallel DNA sequencing at present are the Roche/454 FLX (Margulies *et al.*, 2005) and Illumina/ Solexa Genome Analyzer (Bentley DR, 2006, Korbel *et al.*, 2007) (Mardis 2008, Voelkerding et al .,2009). Moreover, the most recent powerful NGS platforms with a significant reduction of the run time and remarkable data output, include HiSeq and the Ion Torrent Personal Genome Machine (PGM) (Rothberg *et al.,* 2011).

1.4.4.1 Ion Torrent Personal Genome Machine (PGM)

The Ion Torrent is the first commercial sequencing machine that does not require fluorescence and camera scanning, resulting in higher speed, lower cost, and smaller instrument size. Currently, it enables 200 bp reads in 2 hours and the sample preparation time is less than 6 hours for 8 samples in parallel (Rothberg *et al.,* 2011).

Ion Tottent performs by detecting the protons released as nucleotides are incorporated during synthesis (Rothberg *et al.,* 2011). DNA fragments with specific adapter sequences are linked to and then clonally amplified by emulsion PCR on the surface of 3 micron diameter beads, known as Ion Sphere Particles. The templated beads are loaded into proton-sensing wells that are fabricated on a silicon wafer and sequencing is primed from a specific location in the adapter sequence. As sequencing proceeds, each of the four bases is introduced sequentially. If bases of that type are incorporated, protons are released and a signal is detected proportional to the number of bases incorporated (Rothberg *et al.,* 2011).

1.4.4.2 Illumina Genome Analyzer and the recent HiSeq method

Acquired by Illumina (http://www.Illumina.com) in 2006, was one of the highly targeted NGS approach. It provides more sequence reads per run, than the previous sequencing methods, allowing for more in depth coverage than other NGS technologies (Bentley, 2006; Korbel *et al.*, 2007; Bentley *et al.*, 2008). The Genome Analyzer uses series of steps for a specific number of cycles, utilizing fluorescently labeled reversible-terminator nucleotides, on clonally amplified DNA templates immobilized to an acrylamide coating on the surface of a glass flowcell (Bentley, 2006, Korbel *et al.*, 2007; Bentley *et al.*, 2008),. Figure 1- 2 shows the process of Illumine sequencing. The main drawback of that method was the short read length (200 bp) which limit the depth of the sequencing (Bentley *et al.*, 2008).

In early 2010, Illumina launched HiSeq 2000, which adopts the same sequencing strategy with the Illumina genome analyzer and improved advantages in terms of read length, accuracy, applications (Liu *et al.*, 2012).Compared with 454, and other NGS platforms HiSeq 2000 is the cheapest in sequencing per single read. Using the multiplexing incorporated primers and adapters, it could handle thousands of samples simultaneously and give up to 15 Gb of output with 25 million sequencing reads and 2x300 bp read lengths(Liu *et al.*, 2012).
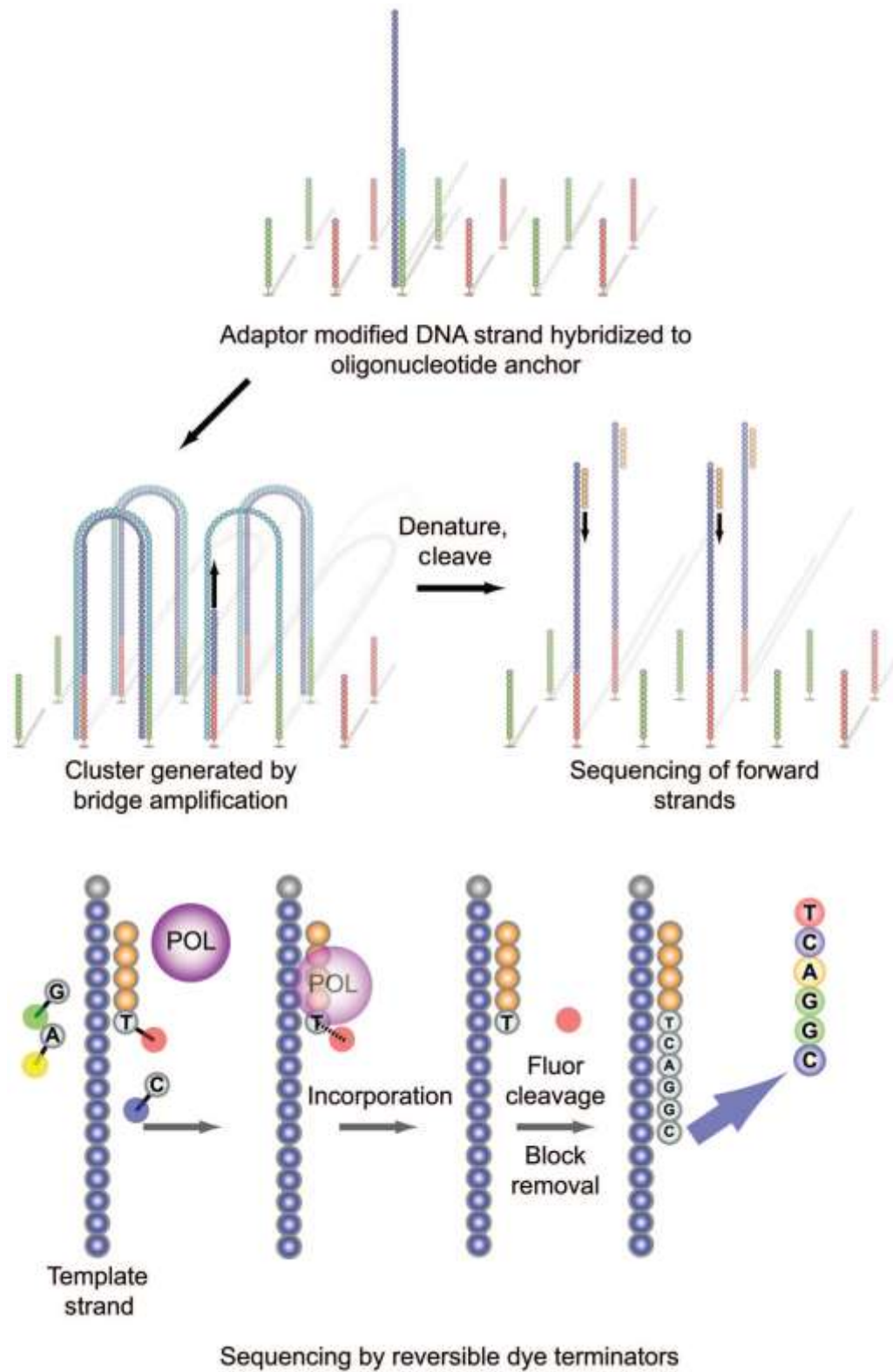
Figure 1-2 Illumina sequencing

Adapted from Voelkerding *et al.*, (2009)

1.4.4.3 454 Pyrosequencing

This next-generation sequencer was the first to achieve commercial introduction (in 2004) and uses an alternative sequencing technology known as pyrosequencing. In pyrosequencing, each incorporation of a nucleotide by DNA polymerase results in the release of pyrophosphate, which initiates a series of downstream reactions that ultimately produce light by the firefly enzyme luciferase. The amount of light produced is proportional to the number of nucleotides incorporated (up to the point of detector saturation). The original Genome Sequencer (GS) 20 machine (Keijser *et al.,* 2008) was able to produce read lengths of approximately 100 bases per sequence. This short read length prohibited detailed analysis of 16S rRNA gene sequencing and very limited phylogenetic data could be inferred. At the end of 2008 a more powerful pyrosequencing machine called GS FLX Titanium was introduced; which had the ability to generate five fold more sequencing reads and an extended read length (~450 bp) compared to the original GS system (Dowd *et al .,* 2008). This system now allowed a much more detailed analysis and phylogenetic inference.

For sequencing, library template DNA is prepared by fragmentation to form fragments of 300 bases. These sequences are then prepared by ligation to adapter neucleotides. The library is then diluted, denatured and hybridized to individual beads containing sequences complementary to the adapter oligonucleotides. Then clonal expansion of the single DNA molecules - bound to the bead- will take place during emulsion PCR. The beads are then separated by dilution deposited in individual picotiter plate wells with the sequencing enzymes. All 4 dNTPs are then added which will subsequently release pyrophosphate and localised luminescence for each incorporation reaction. A special device camera will then record all images. All the data will be then filtered according to quality criteria of signal-to noise ratio and algorithmically translated to a linear sequence output.

Ligation of adaptors to
DNA fragments

Emulsion PCR

Break emulsions and deposit
beads into picotiter plate

Ordered flow of dNTPs

Beads with clonally amplified template DNAs and sequencing enzymes

Polymerase

Template

dNTP

dNTP

Primer

PP_i and APS

ATP-sulfurylase

ATP

Luciferase

LIGHT
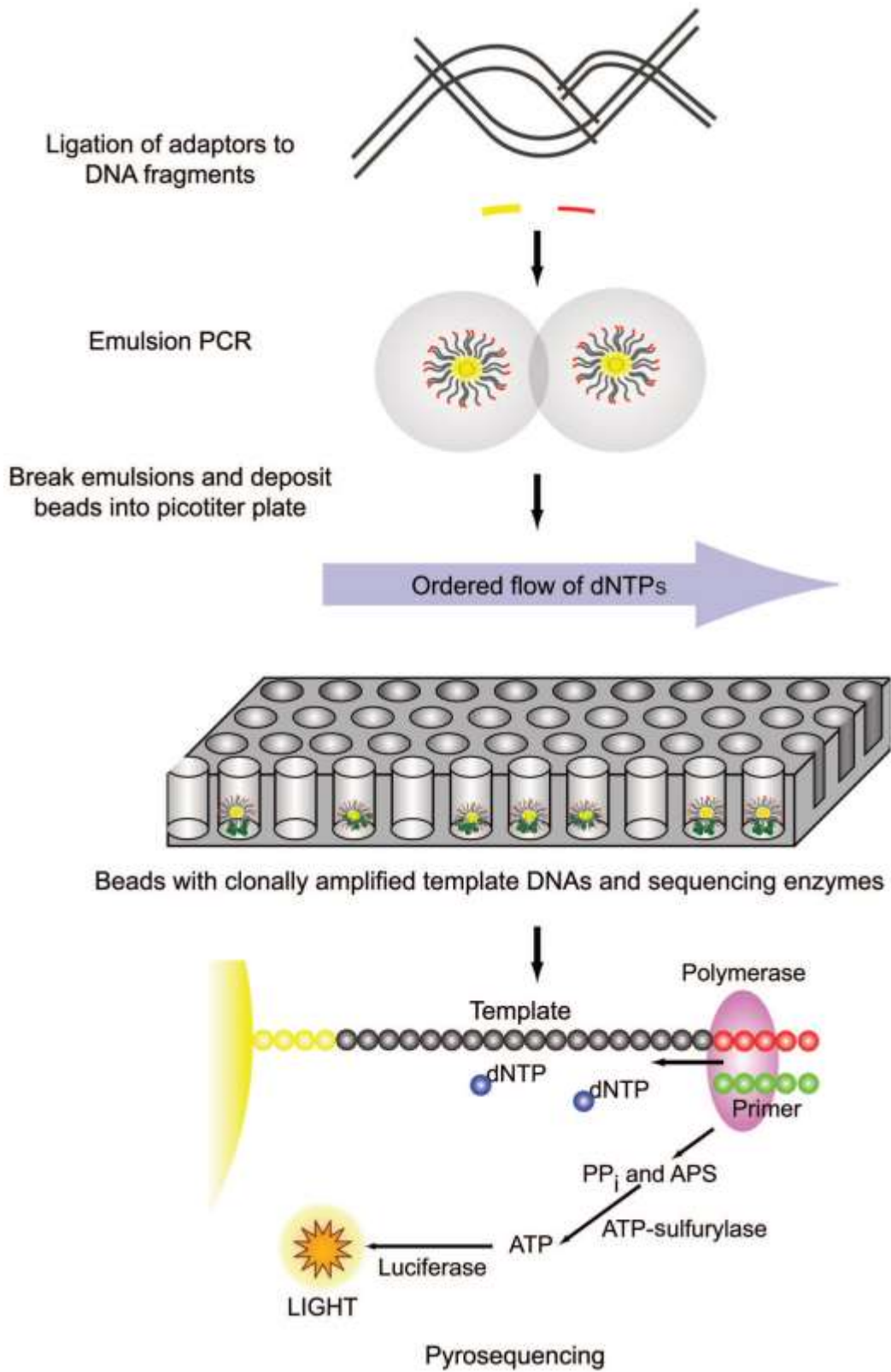
Pyrosequencing

Figure 1-3 454 Pyrosequencing

Adapted from Voelkerding *et al*., (2009)

In order to evaluate the genuine microbiome which previously reported 700 oral microbial phylotypes as identified by cultivation or traditional cloning and sequencing (Paster *et al.*, 2006), a recent study by Zaura et al. (2009) aimed in exploring the nature of the core microbiome and the degree of overlapping of three healthy individual oral cavities at various intraoral niches (different dental surfaces, cheek, hard palate, tongue and saliva) by employing novel pyrosequencing of the 16S rRNA V5-V6 hypervariable. In contrast to the previous culture-dependent methods, the novel 454 pyrosequencing method has revealed an unexpectedly broader scope of microbial diversity found in the healthy microbiome, with over 500 different OTUs. The predominant taxa belonged to *Firmicutes, Proteobacteria, Actinbacteria, Bacteroidetes and Fusobacteria.*

Table 1-3 provides examples of some recent studies that employed the 454 pyrosequencing method in order to investigate different habitats of human microbiome.

Massively parallel pyrosequencing is a new powerful tool that allows for extensive sequencing of microbial populations in a high-throughput, cost-effective manner (Keijser *et al.*, 2008; Rothberg and Leamon, 2008; Zaura *et al.*, 2009). This technique has been successfully employed within several environmental settings such as hydrothermal vents of a deep marine biosphere (Sogin *et al.*, 2006) and soil (Roesch *et al.*, 2007). Microbial communities in the human body have been assessed by this approach including vaginal microflora (Sundquist *et al.*, 2007), bacteria of chronic wounds (Dowd *et al.*, 2008) and oral microflora (Keijser *et al.*, 2008; Zaura *et al.*, 2009). The introduction of high-throughput pyrosequencing has provided a powerful novel approach to identify an entire microbiome at affordable cost without the limitations of cloning/Sanger sequencing (Nossa *et al.*, 2010). This has dramatically increased the resolution at which microbial communities can be analyzed (Keijser *et al.*, 2008).

One 454 sequencing run takes about 8 to 10 hours and can produce up to 1.2 million sequences (Rothberg and Leamon 2008). As the sequences are obtained in one run, the unit cost per sequence read is a very small fraction

compared to the Sanger sequencing (Nossa *et al.*, 2010). In addition, this method limits the cloning bias of directly sequencing the 16S rRNA genes generated by polymerase chain reaction (PCR) (Keijser *et al.*, 2008; Nossa *et al.*, 2010).

The main drawbacks of 454 sequencing is read length. High throughput sequencing reads are about 400 bases per sequence, which are significantly shorter than those from Sanger sequencing which routinely gives up to 900 bases per sequence (Voelkerding *et al.*, 2009; Nossa *et al.*, 2010). Other concerns are the accurate identification of the homopolymers (3-4 base length sequences). With the newest version, termed '' GS FLX Titanium" 454 has reported that the metal coating of picotiter wells aim to improve the accuracy of the homopolymers determination and read length.

Table 1-3 Recent studies using 454 pyrosequencing methodology

Reference	year	Aim of the study	Conclusion
Liu *et al.*	2012	Pilot study used the high throughput pyrosequencing of the 16S rRNA gene and of whole community DNA provide a basic line in understanding, metabolic, and ecological changes associated with periodontitis in 15 subgingival plaque samples, four from each of two periodontitis patients, and the remaining samples from three healthy individuals.	Reveal the disease microbiome to be enriched in virulence factors, and adapted to a parasitic lifestyle that takes advantage of the disrupted host homeostasis. Furthermore, there was a significant difference between diseased samples common structure and the completely healthy samples, suggesting that the disease state may occupy a narrow region within the space of possible configurations of the oral microbiome
Ahn *et al.*	2011	Correlation assessment of the two highly validated pyrosequencing methods: 16S rRNA gene survey by 454 pyrosequencing and Human Oral Microbiome Identification Microarray (HOMIM) among 20 individuals.	The community profiles assessed by 16S rRNA pyrosequencing and HOMIM were highly correlated at the phylum level. Moreover, high correlation at the genus level was detected when comparing the more commonly detected taxa.
Belda-Ferre *et al.*	2011	Oral metagenome analysis in sound and carious teeth surfaces, by 454 DNA pyrosequencing of supragingival, subgingival and active caries cavities plaque samples.	The broad scope of diversity between healthy and diseased individuals; Bacilli and Gamma Proteobacteria are more common in healthy individuals while anaerobic taxa such as Clostridia and Bacteroidetes are more frequent in diseased individuals.

Crielaard *et al.*	2011	Assessing the impact of the physiological transition from deciduous to the permanent dentition on the oral microbiome through 454 pyrosequencing of tagged 16S amplicons of pooled saliva samples from 74 children. qPCR method used in order to quantify the microorganisms.	The deciduous dentition showed high proportion of Proteobacteria compared to Bacteroidetes. In comparison to adults child saliva show high proportion of Firmicutes and Actinobacteria and lower proportion of Bacteroidetes, Fusobacteria and spirochaetes.
Koren *et al.*	2011	Exploring the direct contribution of oral and gut microbiome with development of atherosclerosis through 454 pyrosequencing of 16S rRNA genes of the bacterial diversity of atherosclerotic plaque.	Veillonella, Streptococcus and Chyryseomonas were identified in atherosclerotic plaque bacteria, which suggests possible correlation of oral and gut micriobiome with atherosclerosis markers.
Cephas *et al.*	2011	use pyrosequencing to phylogenetically characterize the salivary bacterial microbiome of edentulous infants and to make comparisons against their mothers. Saliva samples were collected from 5 edentulous infants (mean age = 4.661.2 months) and their mothers or primary care givers	Less richness noted in infants saliva with only 3% divergence Firmicutes, Proteobacteria, Actinobacteria, and Fusobacteria were predominant bacterial phyla present in all samples. The exception was *Streptococcus*, which was the predominant genera in infant saliva.
Lazarevic *et al.*	2010	Comparison of salivary bacterial communities of five individuals at three different intervals using 16S rDNA pyrosequencing in order to evaluate short term stability and inter-individual differences.	Salivary microbial community tends to be stable for at least five days. A novel finding of genus Gemella was identified in all samples at all time points, as it was not defined as a core microbiome member in previous studies.

Ling *et al.*	2010	To explore the diversity of children's oral microbiome and the nature of cariogenic bacterial community. 60 children with and without caries, saliva samples and supragingival plaque analysed first by PCR-DGGE finger-printing and broad-range primers corresponding to bacterial 16S rRNA gene. Characterization of the oral microbiome then conducted by massively parallel pyrosequencing.	The phylotypes in saliva and supragingival plaque were significantly different. The genera of Streptococcus, Veillonella, Actinomyces, Granulicatella, Leptotrichia, and Thiomonas in plaque were significantly associated with caries lesions.
Dominguez-Bello *et al.*	2010	Study the initial bacterial acquisition in infants and its relation to delivery mode. Multiplexed 16S rRNA gene pyrosequencing analysis of different body habitats conducted as follow: mother's skin, vaginal and oral flora taken one hour before delivery and infant's oral, rectal and oral samples obtained immediately after delivery.	Direct correlation was detected between the route of delivery and the nature of bacterial flora. Vaginally delivered infants acquire communities resembling their own mother's vagina such as Lactobacillus, Prevotella, and Sneathia spp. and Caesarean section infants acquire community dominated by skin bacteria such as Staphylococcus.
Lazarevic *et al.*	2009	Evaluate the potential of Illumina high-throughput sequencing to study the human oral microbiota diversity. Saliva and oropharyngeal swab samples collected from three individuals.	Greater depth of coverage of the diversity of oral microbiome than previous studies with predominant phyla: Firmiutes, Proteobacteria, Actinobacteria, and Fusobacteria. On the other hand Bacteroids were less predominant.

| Zaura *et al* | 2009 | One of the first studies that explored the diversity of healthy oral microbiome using the 454 Pyrosequencing technology among three healthy individuals. | In contrast to the previous culture-dependent methods, the novel 454 pyrosequencing method revealed an unexpectedly broader scope of microbial diversity found in healthy microbiome, with over 500 different OTUs. The predominant taxa belonged to Firmicutes, Proteobacteria, Actinbacteria, Bacteroidetes and Fusobacteria. |

1.4.5 Microbial communities analysis tools

The exponential development of these sequencing methods during the last twenty-five years has generated over 109 16S rRNA gene sequence hypervariable regions. This massive amount of data is continuously deposited in public repositories such as GenBank and the number of sequences continues to double every 15 to 18 months (http://www.arb-silva.de/news/view/2009/03/27/editorial/). These massive datasets have been analyzed through the generation of phylogenetic trees (Costello *et al.*, 2009), assignment of sequences to operational taxonomic units (OTUs) (Roesch *et al.*, 2007) and classification of sequences to phylogenentic bins based on similarity to reference sequences (Huber *et al.*, 2007).

Next-generation sequencing methodologies generate hundreds of megabits to gigabits of sequencing reads after each run, which requires processing through a bioinformatics pipeline. This pipeline should ensure that low-quality sequences are discarded and high quality reads or clusters of sequences-operational taxonomic units (OTUs) are obtained.

Several computational tools have been developed to address microbial ecology and interpret all these findings such as DOTUR (Schloss *et al.*, 2005), SONS (Schloss *et al.*, 2006) and the Ribosomal Database Project (RDP). Although these tools have been widely successful, a number of limitations will affect their use as sequencing capacity increases and studies become more complex. Firstly, most of these methods had developed with limited capacity of generic analysis to 102 to 104 sequences which does not match the expanded number of sequences (Schloss *et al.*, 2009). Secondly, most of these programs rely on online data transferring for computational analysis which is not suitable for the new expanded sequences.

To overcome these limitations Schloss et al.(2009) have developed a single software platform, called mothur. Mothur is a C++-based software package used for clustering 16S sequences into operational taxonomic units (OTUs). Mothur creates OTUs using a matrix that describes phylogenetic distances between representative sequences and subsequently estimates within-sample diversity.

This improved tool implements the algorithms implemented in previous tools including DOTUR, SONS (Schloss *et al.*, 2005, 2006) with additional superior features including (i) over 25 calculators for quantifying key ecological parameters for measuring and diversity determination; (ii) visualization tools including Venn diagrams, heat maps and dendrograms; (iii) functions for screening sequence collections based on quality; (iv) a NAST-based sequence aligner (5); (v) a pairwise sequence distance calculator; (vi) the ability to call individual commands either from within mothur, using files with lists of commands (i.e., batch files), or directly from the command line, providing for greater flexibility in setting up analysis pipelines (Schloss *et al.*, 2009).

Chapter Two: Aims

2   Aim of the study

The depth of oral the microbiome has been intensively investigated by both culture and newer DNA based culture-independent methods. Old concepts in the literature of oral microbiome ecology and its role in health and disease are based on microbiological knowledge obtained mainly from bacterial population by using culturing methods that are biased towards bacteria which survive the sampling and transportation procedure from oral environment, and can grow under laboratory conditions. Therefore, there is a possibility that these bacteria detected by culturing techniques are neither dominant nor functionally significant (Aas *et al.*, 2005; Paster *et al.*, 2006; Keijser *et al.*, 2008, Zaura *et al.*, 2009). It was recently demonstrated that count and richness of the oral microbiome is significantly greater than previously thought, with studies reporting that the bacterial diversity in the oral cavity was over 700 species, half of which had never been cultivated (Aas *et al.*, 2005; Paster *et al.*, 2006).

Using the open-ended, high-throughput next generation sequencing approach, Keijser *et al.* (2008) and Zaura *et al.* (2009) confirmed the previous findings of unexpected broad oral bacterial diversity.

The aim of this study was to investigate the acquisition process and development of healthy oral human core microbiome in early life course around two important ecological events; tooth eruption and hormonal changes associated with puberty. In addition, secondary aims include analysing the effect of feeding type on infant group and the impact of oral health condition (DMFT, dmft) on prepuberty and postpuberty oral microbiome groups.Tagged 16S rRNA gene amplicons were sequenced using 454 pyrosequencing and the diversity of phylotypes analysed in supragingival plaque samples in four child cohorts:

- Edentulous infants

- Deciduous dentition group

- Pre-puberty group

- Post-puberty group

Chapter Three : Materials and Methods

3   Materials and Methods

3.1   Ethics statements

Ethical approval was obtained from the Joint UCL/UCLH Committees on the Ethics of Human Research (Committee A), REC reference number (09/H0714/57) (Appendix 1). Written informed consent was obtained by all participants or their parents in this study (Appendix 2). Information sheet about the study aims and objectives was given to patients or their parents according to age (Appendix 3).

3.2   Study population and clinical screening

In this study, samples were collected from different centres according to accessibility. Twenty edentulous infants (1 to 6 months) and twenty deciduous dentition subjects (2 to 3 years old) were recruited at Surestart Daisy Hill Children's Centre located in Kempston. Surestart Daisy Hill Children's Centre is a part of a national initiative to support families with children under five years old, it also offers specialist advice from qualified staff, such as midwives and health visitors.

For older age groups (20 prepubertal and 20 postpubertal) adolescents and their siblings attending the Paediatric clinic at UCL Eastman Dental Institute were recruited. Information on basic demographic data, ethnicity and breastfeeding time were obtained (Appendix 3).

3.3   Inclusion criteria

No antibiotics for previous three months.

No periodontal disease or related systemic illness.

No orthodontic appliance or dentures.

No professional scaling or polishing for the last three months.

The children were clinically examined in the dental clinic by one calibrated dentist on the appointment day. The oral examination included a visual inspection of the oral mucosa, caries experience and plaque assessment (Appendix 2). Caries experience was recorded by dmft/ DMFT index. This index was calculated by adding up the total number of the decayed missed and filled teeth. The plaque amount was assessed via Greene and Vermillion index. Six dental surfaces buccal surfaces of both upper permanent or deciduous molars, labial surfaces of upper right central incisor and lower left central incisor (permanent dentition) or 51 and 71 (deciduous dentition), and lingual surfaces of 36 and 46 (permanent dentition) or 75 and 85 (deciduous dentition) were examined. If the selected tooth was missing the contralateral tooth was inspected instead. Plaque was scored as follows:

0   no visible plaque.

1   plaque only on the cervical third of the tooth.

2   plaque covered the cervical half of the tooth.

3   plaque reached the occlusal surface.

The average plaque index was calculated per child.

Next generation sequencing work flow

Sample collection

DNA extraction

Library creation by fragmenting the DNA and attaching sequences that enable amplification and downstream processing of the targeted 16S rDNA.

Amplification: increasing the amount of DNA available for sequencing by emulsion PCR.

Parallel pyrosequencing using Roche/454 FLX Titanium, Genome Sequencer.

Data filtering by removing low quality sequences, clustering into OTUs and finally taxonomy assignment.

Figure 3-1 Methodology flow chart

3.4   Sample collection

In the infants group, mucosal biofilm swabs were collected from the edentulous ridges using sterile dry cotton swabs (Copan; 154C Rayon, Regular Tip, Brescia, Italy). Supragingival plaque samples were collected from the second group (2 to 3 years) by brushing the sterile dry cotton swab at the gingival margin of deciduous dentition dental surfaces. In older groups samples were collected at the appointment time before dental treatment. Supragingival plaque samples were obtained from the gingival margins of all erupted teeth by brushing for 1 minute with a sterile dry cotton swab. To sample the buccal and lingual dental surface the swab was moved over the enamel from mesial to distal curvature of the tooth crown along the gingival margin and tooth-surface border. After the sample was taken, the tip of the cotton swab was directly placed into a screw capped 1.5 ml tube with 2 ml reduced transport fluid RTF (Syed and Loesche, 1972, Appendix 7) solution, plus three sterile glass beads (3 mm diameter, 1.04015.0500, batch K34740415521, Merck, Germany) and clipped off. All samples were labelled and stored at -70 °C until further processing for DNA extraction.

3.5   DNA extraction

A 1 ml aliquot of sample was transferred to a sterile screw-cap Eppendorf tube and the pellet was resuspended in 0.2 ml of sterile TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Then 0.25 ml of lyses buffer (AGOWA mag Mini DNA isolation kit, AGOWA, Berlin, Germany), 0.2 g zirconium beads (diameter, 0.1 mm; Biospec products, Bartlesville, OK, USA) and 0.2 ml phenol were added to each sample. The samples were homogenized with a bead-beater for 2 minutes at 4 m/sec speed and immediately placed on ice after removal of the beatbeater. DNA was extracted with the AGOWA mag Mini DNA isolation Kit. Briefly, after centrifuging the samples for 15 minutes at 3000 rpm 100 µl of the aqueous supernatant was transferred to a sterile 1.5 ml tubes with 10 µl magnetic beads and 160 µl AGOWA binding buffer, then mixed by gentle pipetting up and down for several times. Samples were incubated for 10 minutes at room temperature to allow sufficient binding and

then placed in the magnetic separator (Dynal MPC; Invitrogen magnetic beads separator) for 1 minute to allow the sedimentation of the pellet (Figure 3-2, Figure 3-3). After removal of the supernatant 200 µl of wash AGOWA Buffer BL1 was added and mixed thoroughly with the pellet. Samples then incubated for 5 minutes at room temperature then placed again in the magnetic separator for 1 minute. 100 µl AGOWA wash buffer LB2 and 1 µl glycogen were added to the pellet and mixed by pipetting up and down for several times. The supernatant was removed after placing the samples in the magnetic separator for 1 minute. Pellets were dried for 10 minutes at 55°C, then 64 µl elution buffer was added, mixed and incubated at 55°C Thermo block for 10 minutes. A vortex mixer was used for sample mixing periodically during the incubation. Samples were stood in the magnetic separator for 1 minute and 50 µl of the supernatant was transferred to a sterile 1.5 ml tube. DNA from every sample was quantified with using a Nanodrop (ND-1000; NanoDrop Technologies, Montchanin, DE, USA). Figure 3-4 demonstrate the main steps of this protocol.
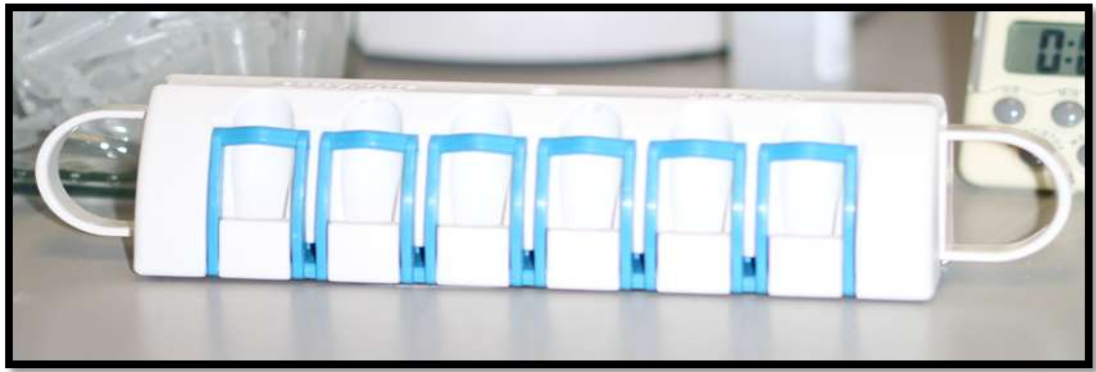
Figure 3-2 Dynal MPC magnetic separator

A=Pellet

B=Magnet

Tube #1   Tube #2   Tube #3   Tube #4
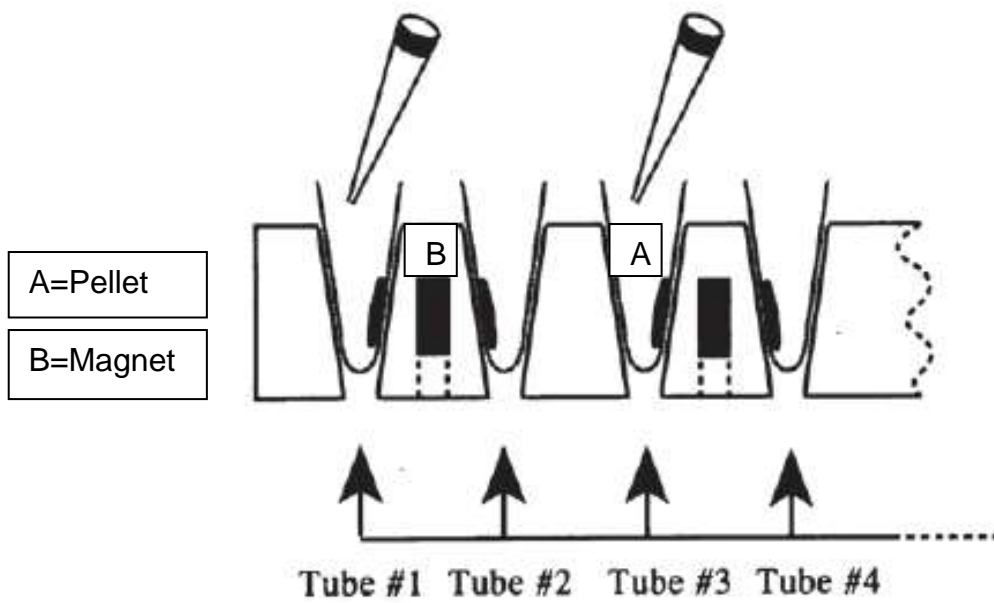
Figure 3-3 Magnetic separator mechanism

Enrich → Lyse → Bind → Wash 1 → Wash 2 → Dry → Elute

Figure 3-4 DNA extraction protocol

3.6   PCR optimization

PCR amplicon libraries of the small subunit ribosomal RNA gene V5-V6 hypervariable region were generated for the individual samples. PCR was performed using the forward primer 785F (GGATTAGATACCCBRGTAGTC) and the reverse primer 1175R (ACGTCRTCCCCDCCTTCCTC) (Appendix 6). The fusion primers included the 454 Life Sciences directional GS FLX Titanium adaptors: A (forward primer) and B (reverse primer) fused to the 5' end of the 16S rRNA gene bacterial primer sequence and a unique trinucleotide sample identification key. The amplification mix contained 1.5 units of Moltaq 16S bacterial DNA-free thermostable DNA polymerase and 1x Molzym reaction buffer (Molzym, Moltaq Bacterial DNA-free thermostable DNA polymerase Kit; Berlin, Germany), 200 µMol L dNTP PurePeak DNA polymerase Mix (Pierce Nucleic Acid Technologies, Milwaukee, WI, USA) and 0.2 µMol L1 of each primer. After denaturation (94°C; 3 min), 30 cycles were performed that consisted of denaturation (94°C; 30 s), annealing (55°C; 40 s) and extension (72°C; 90 s).

Table 3-1 PCR cycling conditions

PCR step	Temperature °C	Time	No. of cycles
	94°C	3 minutes	1
Denaturation	94°C	30 seconds	
Annealing	55°C	40 seconds	32
Extension	72°C	90 seconds	
	72°C	10 minutes	1

3.7   Agarose gel electrophoresis and PCR product purification

All the PCR amplicons were assessed by loading 5 µl of the PCR product in to wells of a 1% (w/v) agarose gel containing gel red (4 µl/100 ml gel). 5 µl of HyperLadder I marker was loaded in to the first well. Five µl of the PCR reaction mixture from each sample was mixed with 2 µl of loading buffer and

inserted into the appropriate wells of the gel. The gel was then immersed in Tris-Acetate-EDTA (TAE) buffer and electrophoresed at 100 V for separation of the fragments. The gel was visualised after excitation under UV transillumination, and the resulting image was captured by a computer software programme (AlphaEase TM, AlphaInnotech). At this stage if the image did not reflect any positive PCR products for an individual sample, the procedure was repeated and re evaluated again under the UV transillumination.

For all the positive PCR amplicons on the agarose gel electrophoresis images, the amplicons were purified by means of the MinElute kit (QIAGEN). Briefly, one volume of the PCR reaction product was added to five volumes of Buffer PB in a QIAquick spin column placed in a 2 ml collection tube, which then was centrifuged at 14,000 rpm for 1 minute to bind the DNA. The flow-through was discarded and 750 µl of PE buffer (containing ethanol) was added to the column to wash the bound DNA. Following centrifuge at 14,000 rpm for 2 minutes, the flow-through was discarded. The tube was centrifuged for an additional minute to remove any residual ethanol. The QIAquick spin column was placed in a sterile 1.5 ml tube and 30 µl of sterilie water was applied to the centre of the column. Each tube was kept at room temperature for 1 minute. The column was centrifuged at 14,000 rpm for 1 minute to give 30 µl of eluted DNA product, which was quantified using the Nanodrop ND-1000 spectrophotometer.

3.8   Bioanalyzer samples assessment

The quality and the size of the amplicons were then analysed for each sample individually on the Agilent 2100 Bioanalyzer with the DNA 1000 Chip kit (Agilent Technologies, Santa Clara, CA, USA) at UCL Genomic Centre/ Institute of Child Health. First the gel-dye mix was prepared by adding 25 µl of DNA dye concentrate (blue) to a DNA gel matrix vial (red) and then the solution was mixed by vortex for 10 seconds and centrifuged at 1400 rpm for 30 seconds. The solution was then transferred to a spin filter and centrifuged at 2,240 rpm for 15 minutes. Secondly, the DNA chip loading procedure was

started by loading 9.0 µl of the gel-dye mix to the specific well marked with the letter G. The chip then was placed to the priming station where it was pressed for 60 seconds by means of the plunger (positioned it at 1ml). The chip then was released and another 9.0 ml of the gel-dye mix was added to the second well marked G. At this stage 5 µl of the DNA marker were added to all 12 wells. The final step was to load 1 µl of the ladder in the well marked with the yellow dot and 1µl of samples were loaded to individually to each well on the chip. The chip then was placed on centrifuge at 2,400 rpm for 1 minute and run in the Agilent 2100 Bioanalyzer within 20 minutes.

3.9   454 pyrosequencing

Sequencing of the PCR products was performed at the UCL Genomic Centre/ Institute of Child Health/ London. The amplicon libraries were pooled in equimolar amounts and sequenced unidirectionally in the reverse direction (B-adaptor) by means of 454 Genome Sequencer using FLX titanium reagents (Roche Applied Science). After sequencing was completed, all reads were scored for quality and any poor quality reads and primer dimers were removed.

3.10 Ribosomal Database Project analysis

After the sequencing run, pre-filtered sequences were searched in Ribosomal Database Project (RDP) using the online program Seqmatch (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp) in order to compare the findings with the higher level analysis using Mothur processing.

3.11 Sequence processing and analysis with mothur

3.11.1 Clustering and OTU assignment

After sequence pre-processing and alignment in mothur, sequence reads were assigned to OTUs based on uncorrected pairwise distances between all aligned sequences. With the "dist.seqs" command a column-formatted

distance matrix was generated, using the "cutoff=0.10" option, which limits the distance matrix to keep only sequence reads with a distance smaller than 0.10 (at least 90% similar).

3.12 Statistical analysis

In order to assess microbial components and diversity at each developmental stage, data were analysed by comparing the mean and standard deviation at the phylum and genus level.

The effect of the feeding pattern in infants and the oral health condition in the prepuberty and postpuberty groups were assessed by a simple t test. Moreover, prevalence of abundant genera were demonstrated by the percentage in relation to these two parameters.

Chapter Four: Results

4   Results

4.1   Sample

Nineteen samples were excluded from the study after the DNA extraction step as there was insufficient yield DNA to allow progression. These were distributed as follows: 4 samples were from each of the infants, deciduous and pre-puberty groups, while 7 samples were from the post-puberty group. The final 61 samples were sequenced using the parallel 454 pyrosequencing GS FLX Genome Sequencer, were distributed as following:

-Edentulous infants (16)

-Deciduous dentition (16)

-Pre-puberty group (16)

-Post-puberty group (13)

4.2   Clinical findings

Data relating to the population ethnicity and the clinical findings are summarised in (Table 4-1) and Figure 4-1.

The plaque amount showed an increase with age with the highest mean value of 0.46 in the prepuberty group. In our population, the dental condition was recorded only in prepuberty and postpuberty groups with the dmft mean 0.75 and DMFT mean 1.38 in prepuberty group; DMFT mean increased with age to reach 2.5 in postpuberty group.

Infants were classified according to feeding pattern in to two groups: breast fed and exclusive formula fed. As (Table 4-2) demonstrates; 31% of the infant group were exclusively formula fed, the remaining 69% had variable breast-feeding history with the highest of 6 months in 18.75% of the group.

Table 4-1 Sample distribution according to their demographic data and clinical findings

Group	Nr of children	Gender	Age in years, mean	dmft mean (sd)	DMFT mean (sd)	Plaque index, mean (sd)
Infants	16	Female= 7 Male= 9	0.5	0	0	0
Deciduous	16	Female= 8 Male= 8	2.3	0	0	0.51 (0.43)
Pre puberty	16	Female= 6 Male= 10	9.5	0.75 (1.12)	1.38 (1.40)	0.8 (0.31)
Post puberty	13	Female= 7 Male= 6	15.6	0	2.5 (1.98)	0.6 (0.22)
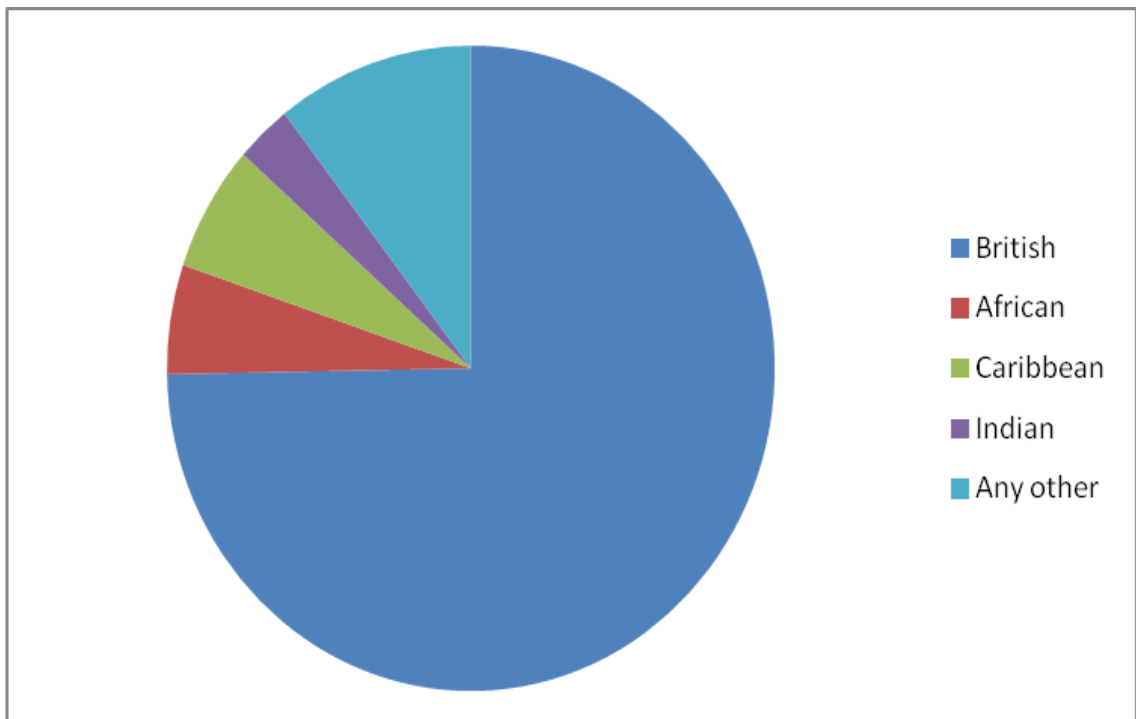
Figure 4-1 Ethnicity distribution: 88.3% British, 6.5% African, 7.5 % Caribbean, 3.5 % Indian, and 12.4 any other mixed.

Table 4-2 Breast-feeding frequency in infants group

Breastfeeding in months	No. of children	%
0.5	1	6.3
1.0	1	6.3
2.0	2	12.5
3.0	1	6.3
4.0	2	12.5
6.0	3	18.75
0	5	31

4.3   Bioanalyzer results

Figures 4-2 and 4-3 show an example of the Bioanalyzer summary and findings. DNA should be between 400 to 500 bp with no detectable irregularities of primadimers.
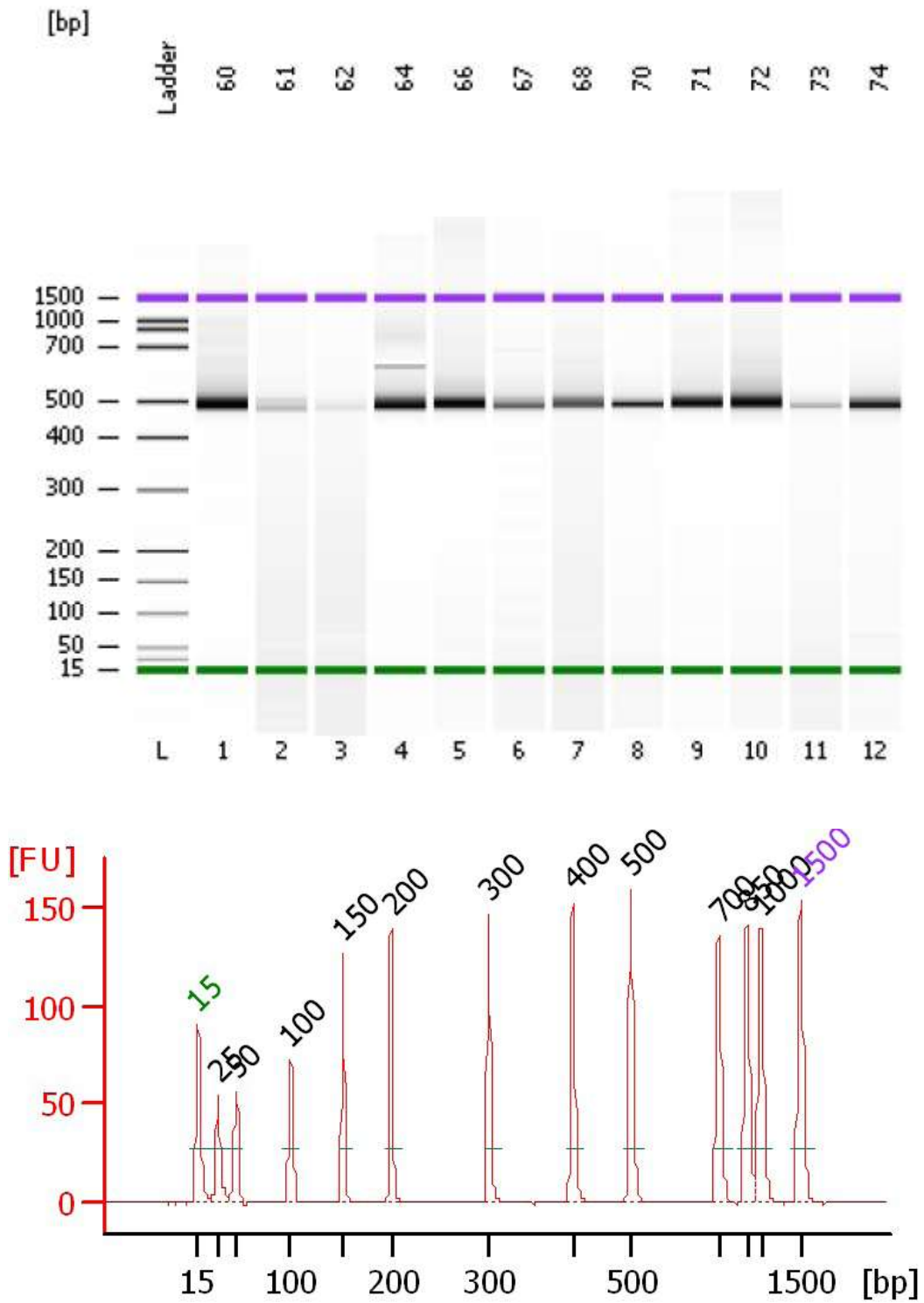
Figure 4-2 Ladder interpretation by Agilent Bioanalyzer
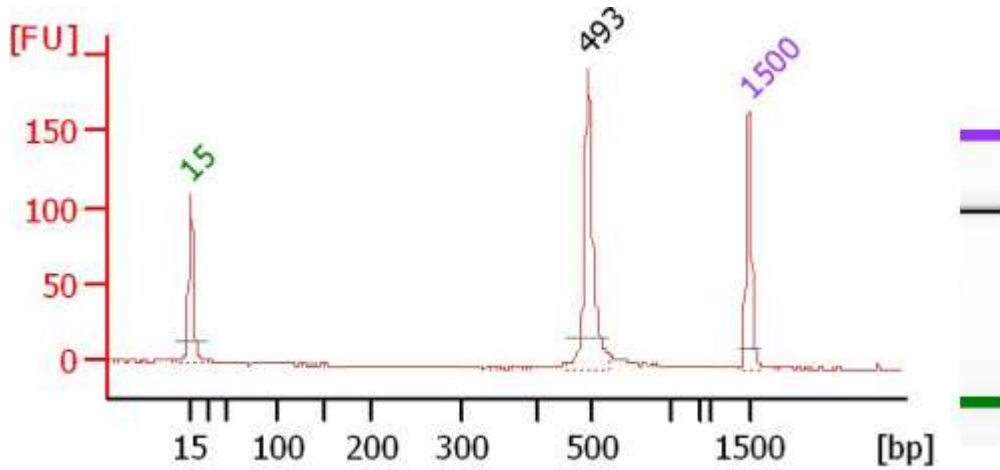
Figure 4-3 Sample interpretation image by Agilent Bioanalyzer

Table 4-3 Sample interpretation image by Agilent Bioanalyzer

Peak	Size [bp]	Conc. [ng/µl]	Molarity [nmol/l]	Observations
1	15	4.20	424.2	Lower marker
2	493	4.76	14.6	
6	1500	0.74	2.1	Upper marker

4.4 RDP sequence analysis

The pre- filtered sequences had been searched initially in the Ribosomal Database Project (RDP) using the online program Seqmatch (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp). The major RDP analysis was at phyla level for the purpose of the comparison with the mothur data after filtering and denoising to assess any inconsistency in findings.

On average there were approximately 15000 reads per sample. Four major phyla predominated in all groups; Firmicutes (68%), Actinobacteria (12%),

Proteobacteria (10%), Bacteroidetes (5.7%), while three phyla (Fusobacteria, TM7 and Spirochaetes) were found in relatively lower proportions (Figure 4-4).
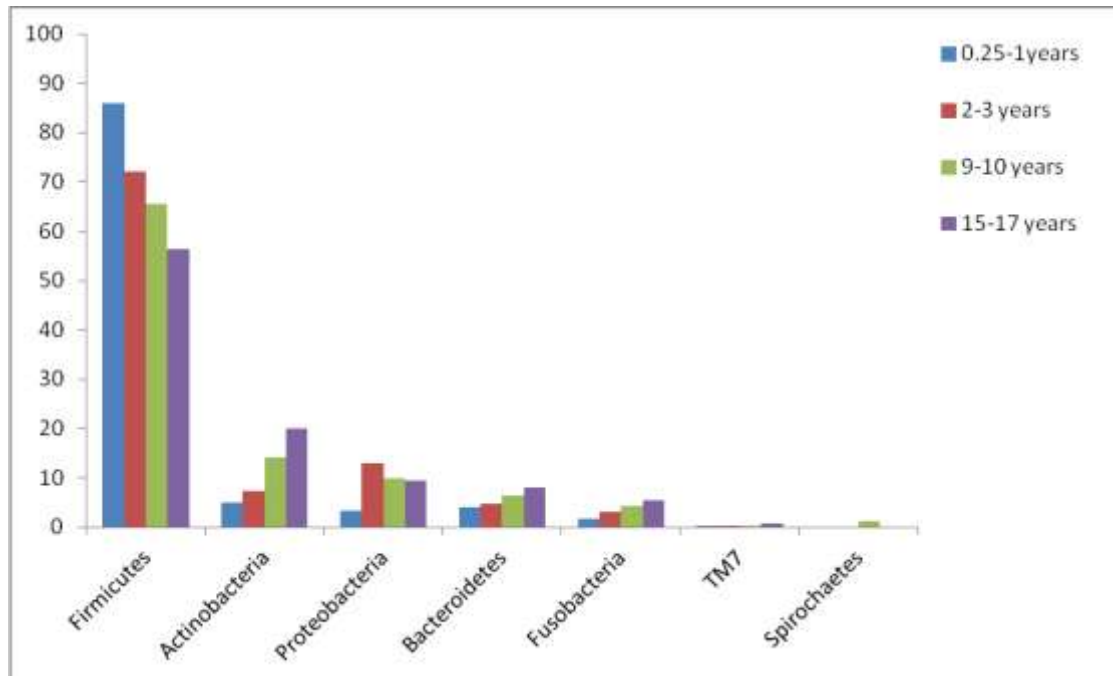
Figure 4-4 (RDP) Relative abundance of the main bacterial phyla, four major phyla predominated in all groups: Firmicutes (68%), Actinobacteria (12%), Proteobacteria (10%) and Bacteroidetes (5.7%).

4.5   Mothur sequence analysis

The analysis of the taxonomic composition and abundance of the oral bacterial community was performed using mothur (Schloss *et al.*, 2009). Sequences were assigned to four distinct pools of samples:

Group 1: edentulous infants (1-6 months)

Group 2: Deciduous group (2-3 years)

Group 3: Pre-puberty (9-10years)

Group 4: Post-puberty (15-17 years)

4.5.1 Relative abundance of the main bacterial phyla

A total of 940,000 sequence reads were obtained (15,409 reads per sample). All sequences that passed the quality filtering were subjected to a 5 read per sample cut-off. After trimming the primer sequences, the average length of approximately 350 bases was obtained first to determine identity or approximate phylogenetic position. Following the processing 837,122 reads were obtained (on average 14,929 reads per sample) for analysis.

The sequences represented eight phyla and 102 higher taxa (genus or more inclusive groups when sequences could not be confidently classified to the genus level) of which 31 were as yet unclassified. Four phyla (Firmicutes, Proteobacteria, Actinobacteria and Bacteriodetes) predominated in all groups (Figure 4-5), while three phyla - Fusobacteria, candidate division TM7 and Spirochaetes - were found in relatively low proportions.

Just as in the RDP initial analysis. Firmicutes was the most dominant phylum among all of the four groups with the highest presentation in infants mouths. The Firmicutes prevalence reduced with age in the next three age groups with a simultaneous relative increase of other dominant phyla (Proteobacteria, Actinobacteria, and Bacteroidetes) (Figure 4-5).
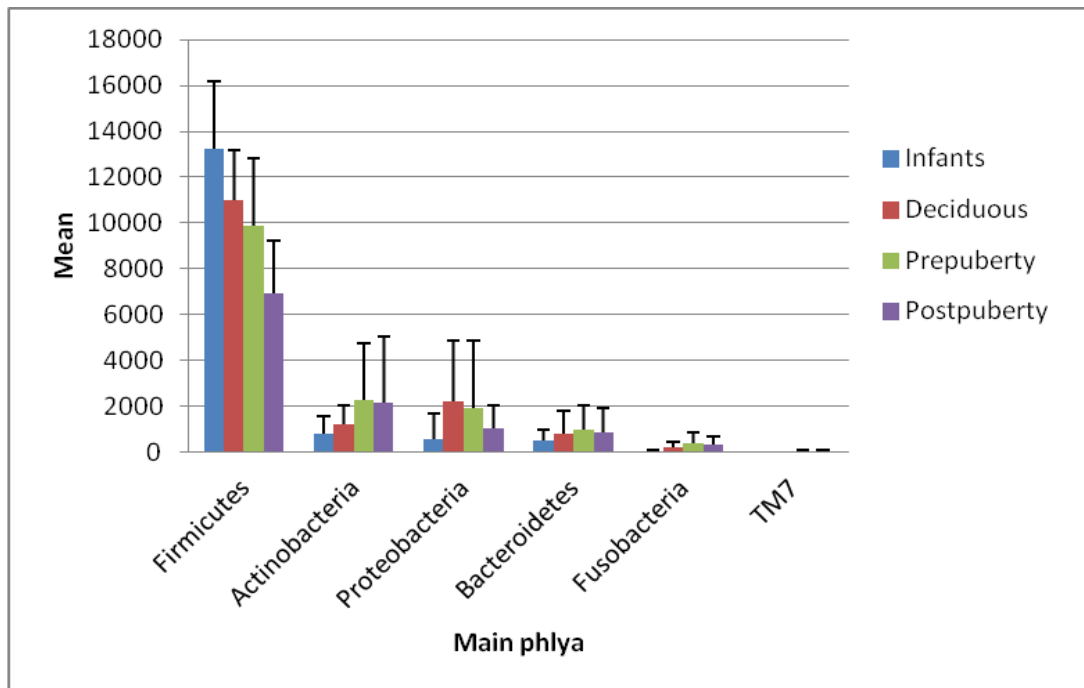
Figure 4-5 Relative abundance of the main bacterial phyla (99% of the reads) identified in supragingival plaque of four age groups. Data displayed by mean and standard deviation for each developmental stage.

Figure 4-6 Relative abundance of the main bacterial genera in Firmicutes identified in four age groups. Data expressed by mean and standard deviation for each developmental stage.

Firmicutes were dominated by the genus *Streptococcus* among all ages with the highest prevelance in edentulous infants (Figure 4-6). However, the richness of *Streptococcus* species increased with increasing age. *Streptococcus* taxa in edentulous babies mouths was dominated mainly by low pH non-*S. mutans*; with a high affinity to mucosal surfaces such as species of *S.Salivaris* group: *S. vestibularis, S. mitis* group: *S. peroris, and Streptococcus viridans* group: *S. Parasanguinis_II.*

After primary teeth eruption the deciduous group *Streptococcus spp* count showed a reduction of *S. vestibularis* and *S. parasanguinis_II* and increase levels of *S. gordonii, S. intermedius,* and *S. mutans,* which are known to have an adhesion property to teeth surfaces.

In the older two groups (prepuberty and postpuberty) similar trends were seen with increased prevalence of *S. constellatus, S. intermedius* and *S. mutans.*

Figure 4-7 Relative abundance of the main bacterial genera in Firmicutes without Streptococcus identified in four age groups. Data displayed by Mean and standard deviation for each developmental stage.

The distribution of other genera apart from streptococcus in the Firmicutes showed a dramatic shift after teeth eruption and around puberty (Figure 4-7). The second highest abundance genus was *Gemella* mainly represented by *G. morbillorum* and unclassified *Gemella.* Representative of the *Abiotrophia* genus was *A. defectiva* which was negligible in infants mouths but showed an abrupt increase with primary teeth eruption but then declined in older groups. In contrast members of the *Veillonellaceae* family (*Veillonella* and *Selenomonas*) showed an increase in the pre-puberty group.
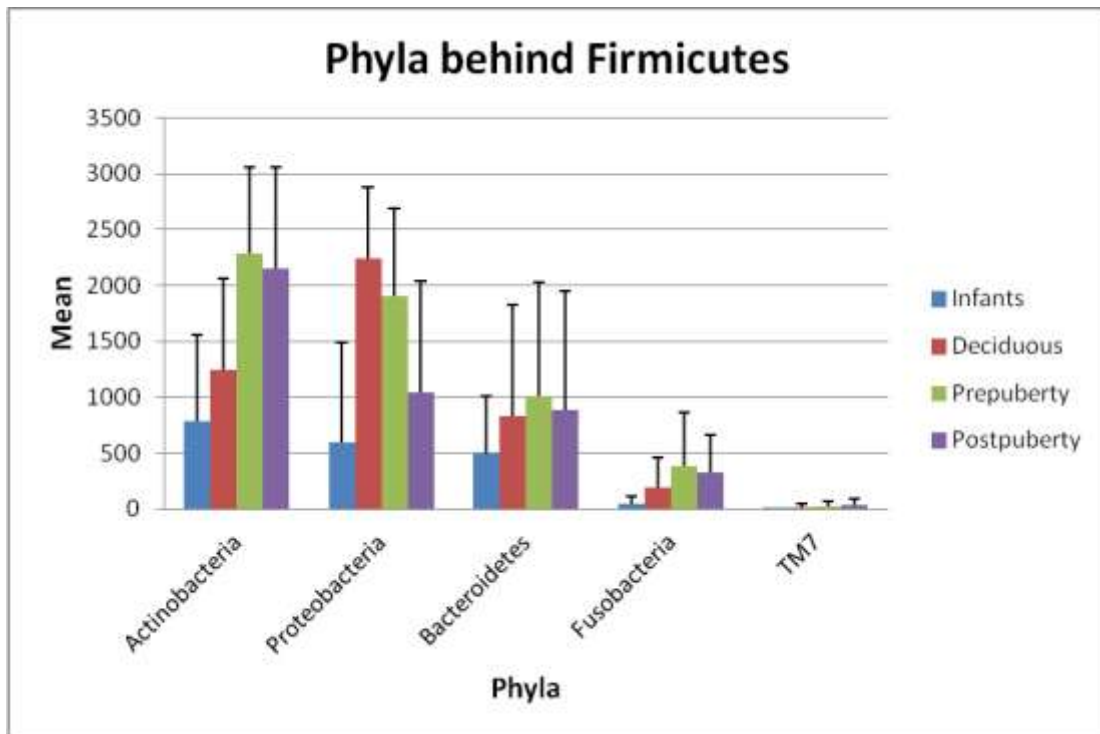
Figure 4-8 Relative abundance of the main phyla behind Firmicutes in four age groups. Data displayed by mean and standard deviation for each developmental stage.

Regarding the prevalence of genera and species in the remaining phyla after Firmicutes (Actinobacteria, Proteobacteria, Bacteriodetes, Fusobacteria and TM7); the pre-puberty group harboured the highest abundance of the phyla Actinobacteria, Bacteroidetes and Fusobacteria. The second most abundant phylum after Firmicutes was Actinobacteria while TM7 represented the least abundant phylum (Figure 4-8). Group two (deciduous dentition) harboured the highest proportion of Proteobacteria compared to other phyla, in all other groups Actinobacteria were found to be more abundant than proteobacteria.
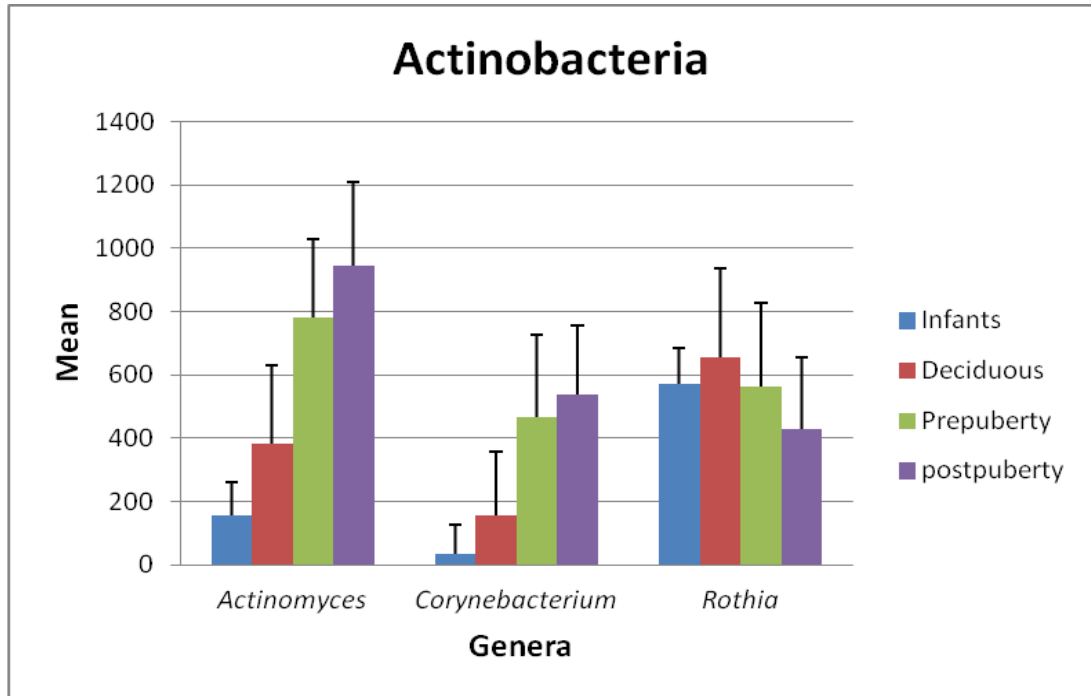
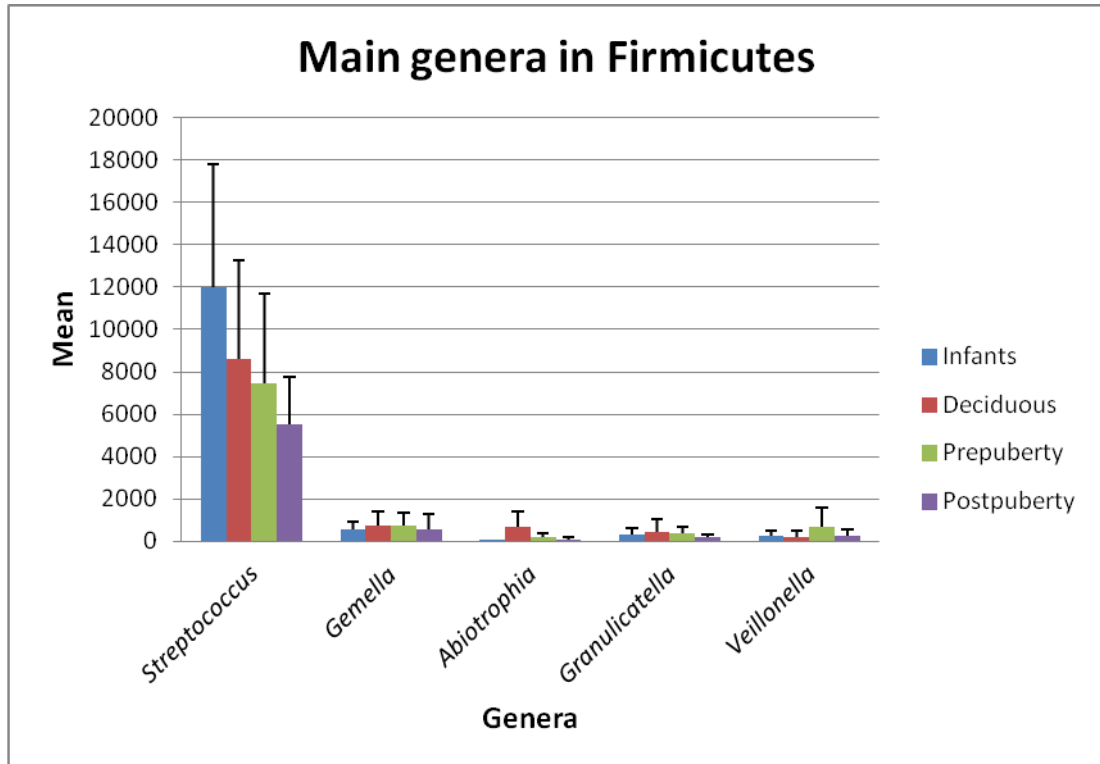**Figure 4-9 Relative abundance of the main bacterial genera in Actinobacteria identified in four age groups. Data displayed by mean and standard deviation for each developmental stage.**

The phylum Actinobacteria (Figure 4-9) was mainly represented by three genera (*Actinomyces, Corynebacterium and Rothia*) in all age groups. *Rothia* showed a similar distribution among all age groups with the lowest presentation in post-puberty group. In contrast to *Rothia*, *Actinomyces* reached the highest prevalence in postpuberty group, represented mainly by unclassified *Actinomyces* and *Actinomyces naeslundii.* In a consistent trend, the *Corynebacterium* genus was positively correlated with age with marked increase in prevalence around puberty; 70% of *Corynebacterium* were represented by *Corynebacterium matruchotii* in the pre-puberty and post-puberty group and the remaining 30% was dominated by *Corynebacterium durum* especially in deciduous group.
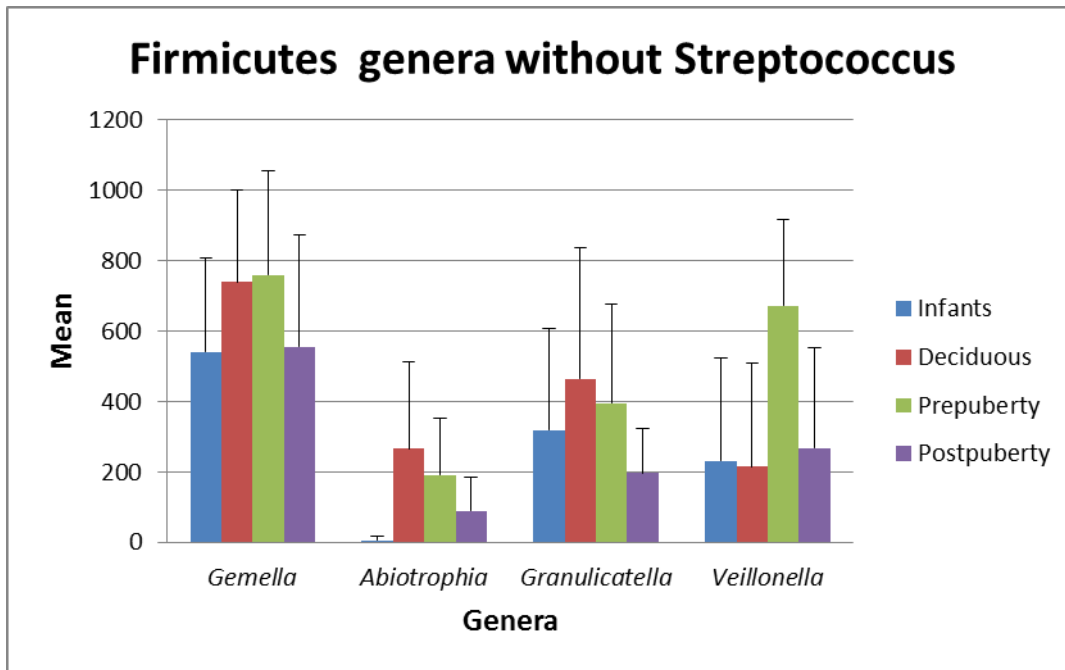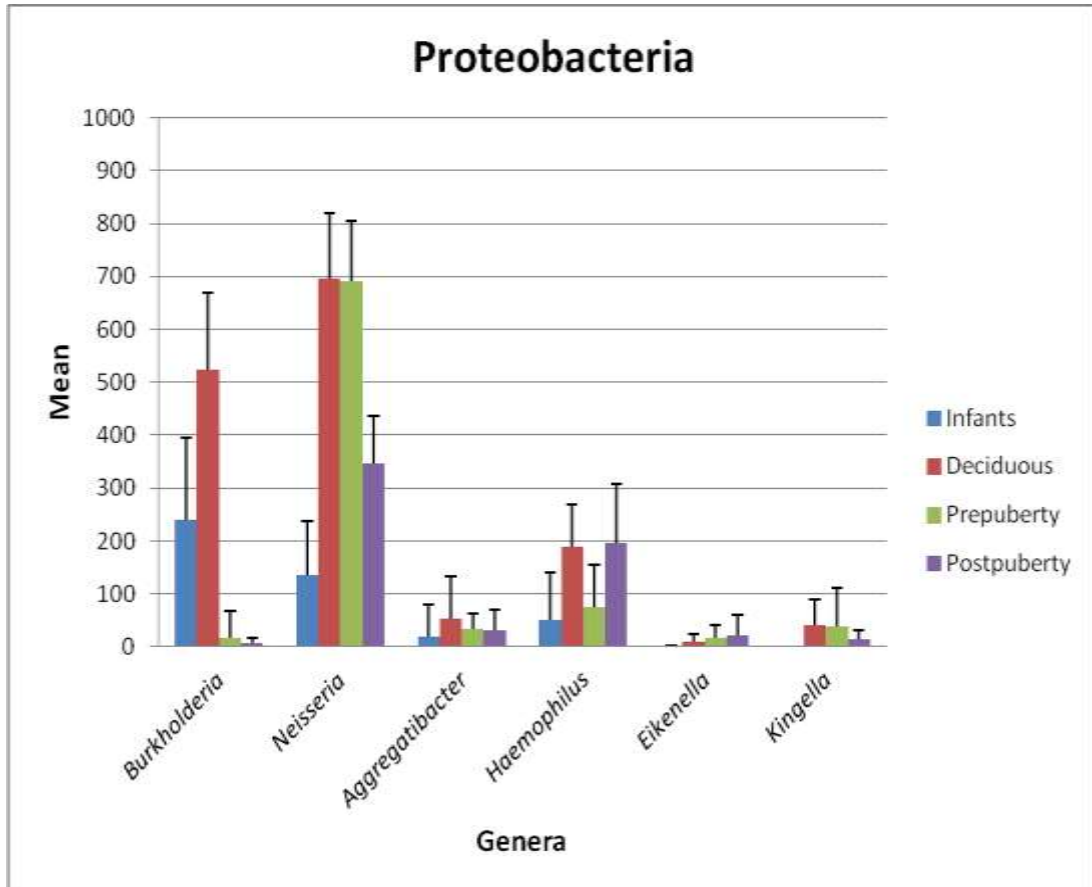
Figure 4-10 Relative abundance of the main bacterial genera in Proteobacteria identified in four age groups. Data displayed by Mean and standard deviation for each developmental stage.

The phylum Proteobacteria (Figure 4-10) showed a marked increase in the deciduous group more than any other stage, which was mainly due to the higher proportion of Betaproteobacteria represented mainly by the *Burkholderiaceae* family (especially unclassified *Burkholderia*) and the *Neisseriaceae* family (represented mainly by *Neisseria* and to a lesser extent by *Eikenella* and *Kingella*). Moreover, representing the Gammaproteobacteria *Aggregatibacter* demonstrated a slight increase after primary teeth eruption then decreased to the same level in pre-puberty and post-puberty groups.
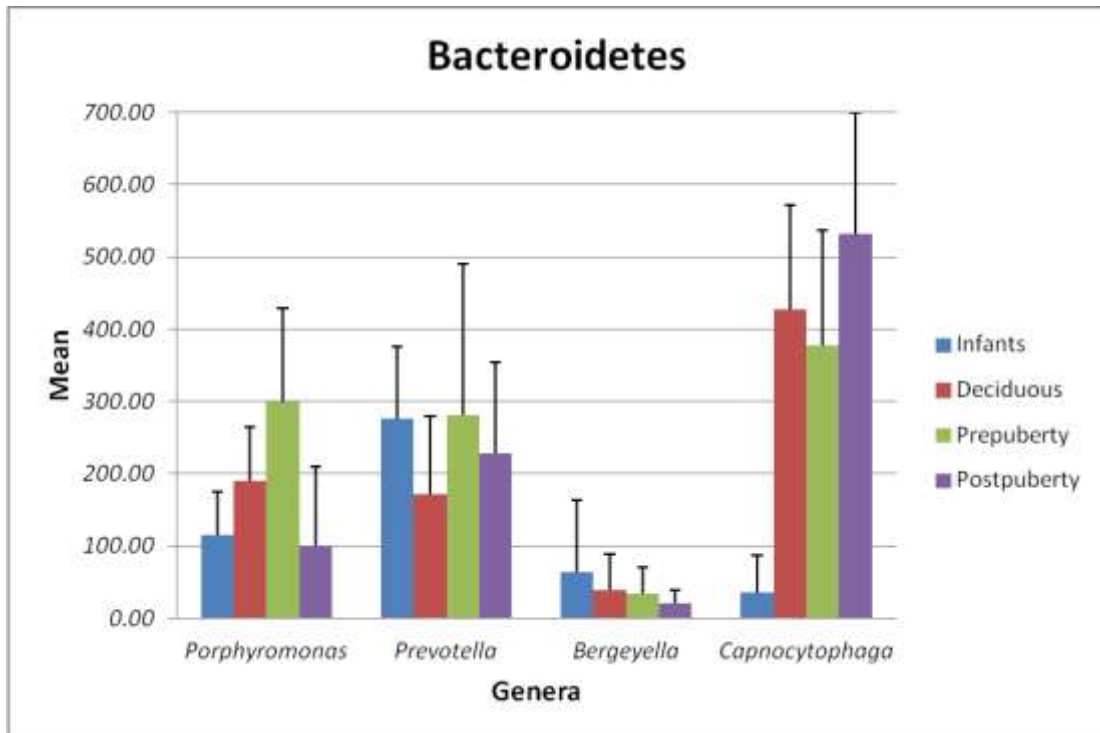
Figure 4-11 Relative abundance of the main bacterial genera in Bacteroidetes identified in four age groups. Data displayed by Mean and standard deviation per each developmental stage.

The distribution of Bacteroidetes (Figure 4-11) showed some striking features. The prevalence of obligatory anaerobic Gram negative rods such as *Porphyromonas* (mainly unclassified *Porphyromonas* and *Porphyromonas catoniae)* was higher in the pre-puberty group than the post-puberty group. *Prevotella* (mainly unclassified *Prevotella*) showed similar prevalence between the infants and prepuberty groups. Carbon dioxide dependent Gram negative rods such as *Capnocytophaga* (represented mainly by *Capnocytophaga granulosa* and *C. gingivalis_A*) increased after primary teeth eruption in the deciduous group and continued to increase to reach the highest prevalence in the post-puberty group.

4.5.2 Relationship between the oral microbiome and breast feeding in the infant group

In order to investigate the effect of breast feeding on the infant oral microbiome, differences between means of two groups were analysed using a two tailed *t*-test. Groups were split into breast-fed and not breast-fed.

The most prevalent species in both group belonged to the phylum Firmicutes (genera *Streptococcus, Gemella* and *Veillonella*). There was no significant difference between the two groups with respect to phyla level.

Although the data did not show significant differences between the two groups, *Prevotella, Neisseria,* and *Granulicatella* were detected more frequently in formula-fed infants. (Figure 4-12)
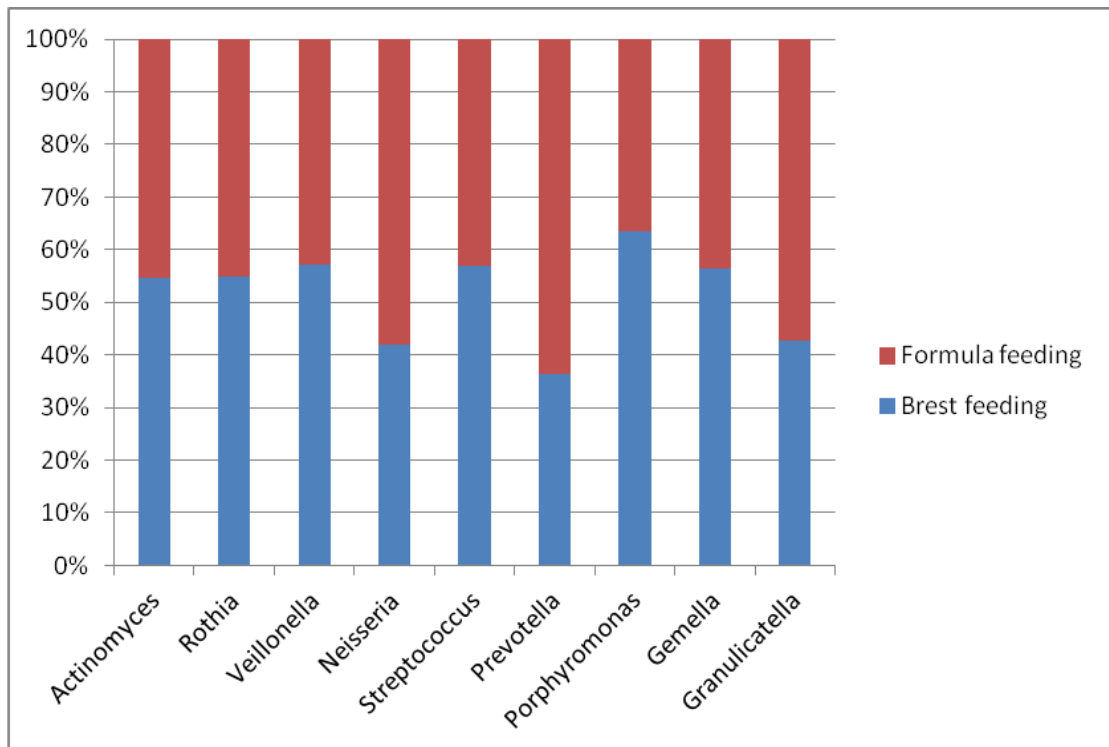
Figure 4-12 Genera prevalence in infants group with respect to feeding pattern

4.5.3 Effect of the oral health condition on the oral microbiome

Oral health was measured by the dmft and DMFT scores (Table 4-1). It was recorded positively in 22 out of 29 subjects in the pre-puberty and post-puberty group (categorised as recorded dmft/DMFT) with seven subjects in these two groups recording 0 dmft/DMFT and thus were categorised as healthy subjects.

Samples were split into two groups: subjects with 0 dmft/DMFT and subjects with any oral disease (dmft+ DMFT >0). Analysis of the plaque microbiome of subjects within these two categories, nine identified phyla of interest. The overall percentage of microbiotic structure for each group is shown in (Figure 4-13). (Firmicutes, Actinobacteria, Proteobacteria, Bacteroidetes, and Fusobacteria) were predominant in both of the groups. Differences between means were tested using two tailed *t*-test. However, there was no statistical significant difference between the two groups (p>0.05).

At genera level *Abiotrophia* (mainly A. *Defectiva*) and *Capnocytophaga* (mainly *C. gingivalis)* were detected more frequently in healthy group. Other major sequences involve (*Streptococcus*, *Neisseria, Lautropia, Fusobacterium, Leptotrichia, Porophyromonas, Prevotella, Actinobaculum, Actinomyces, Corynebacterium, Rothia*) were all recorded to be higher in dmft/DMFT group. *S. mutans* was not detected in healthy subjects, and only 38% of children with recorded dmft/DMFT had detected level of *S. mutans*, with no significant differences between the two groups.
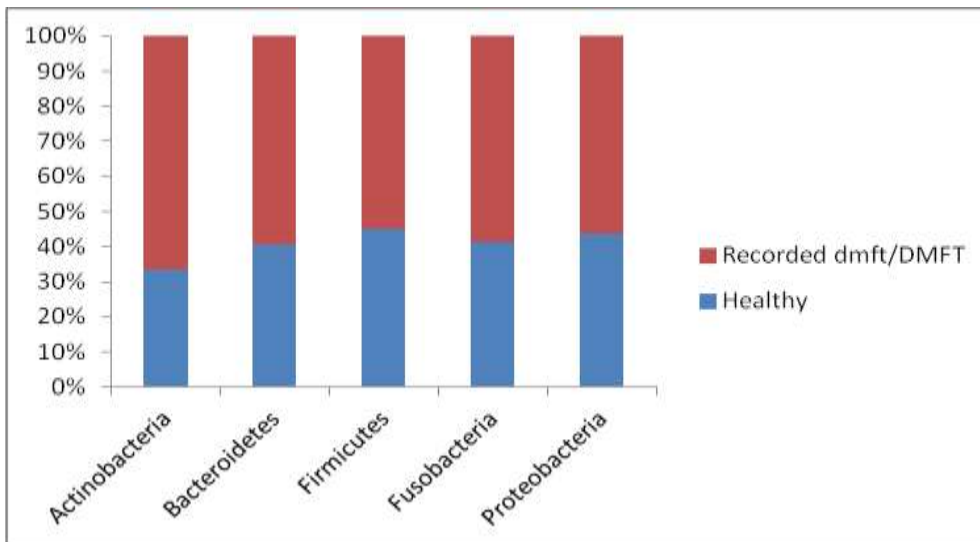
Figure 4-13 Prevalence of bacterial phyla found in dental plaque microbiome according to oral health condition

Chapter Five: Discussion

5   Discussion

Comprehensive investigation of the composition of the oral microbial ecosystem in children is essential for understanding the normal bacterial communities of the oral cavity, disease mechanisms in developing prevention and treatment methods. The oral microbiome in children has been demonstrated to be much more complex than previously thought (Li *et al.*, 2005; Aas *et al.*, 2008; Gezani *et al.*, 2009; Cephas *et al.*, 2011).

To our knowledge this study is unique in that it involves a cohort of edentulous infants (age 1 to 6 months) in addition to the other 3 groups of children as an essential part of analysing oral microbiome ecology. Other recent studies analysing the oral microbiome of edentulous infants using high-throughput sequencing compared newborn vs. mother oral, fecal, vaginal, and skin bacterial populations either immediately after birth (Dominguez-Bello *et al.*, 2010) or at age 2 to 6 months (Cephas *et al.*, 2011).

Dominguez-Bello et al. (2010) found that the neonatal body microbiome in the first 24 hours after birth was considered essentially undifferentiated, and stressed the importance of spotting the timeline over which the distinctive microbiome found in adult body habitats start to establish in different body sites.

Cephas et al. (2011) compared the neonatal oral microbiome with that of the mother or carers and demonstrated that before teeth eruption, the infant oral microbiome already contained most of bacterial species necessary for dental biofilm development and disease. Moreover, they did not find a strong direct relation between the oral bacteria of infants and their mothers.

A recently published study used the Human Oral Microbiome Identification Microarray (HOMIM) and 16S rDNA sequencing to investigate the oral microbiome of 207 infants at age 3 months in order to assess the effect of different types of diet; breast feeding, formula feeding and combination feeding on the oral microbial composition. Results showed that the

prevalence of certain *Lactobacillus* species is significantly different among each feeding group (Holgerson *et al*., 2013).

The goal of the present investigation was to determine the early oral colonization patterns and components of supragingival plaque microbiome in children four chronological stages: edentulous infants, deciduous dentition, pre-puberty and post-puberty.

High-throughput pyrosequencing provides sensitive detection and discrimination for wide spectrum phylogenies and the relative abundances of total bacterial components (Keijser *et al*., 2010; Lazarevic *et al*., 2010). It also overcomes some of the traditional Sanger sequencing disadvantages and intensifies the depth of coverage that can be achieved for each individual sample (Wade, 2013). With our knowledge of the current discussions on the importance of accurate determination of microbial diversity from 454 pyrosequencing data (Huse *et al*., 2007; Quince *et al*., 2009; Kunin *et al*., 2010; Balzer *et al*., 2011) we applied a strict protocol in each step to minimize the artefacts resulting from sequencing of homopolymers (3-4 base length sequences).

5.1 Methodology

The sample size calculation was not conducted as this is a pilot study. In addition, the single 454 run capacity is 80 samples per run in order to give a conclusive data out put. Therefore 20 samples were obtained from each of the four groups to give a total of 80.

The sample groups had representative and comparable data with previous cross sectional studies (Li *et al*., 2005; Aas *et al*., 2008; Gezani *et al*., 2009; Crielaard *et al*., 2011) and was suitable with respect to oral microbiome ecology and evolution from birth to adolescence. However, it did not fully reveal the pathogenicity aspects of the oral microbiome with regard to dmft/DMFT indices or the effect of formula feeding in infants. The main reason for this is the sample size and population; as subjects in the pre-puberty and post-puberty groups were allocated at the Paediatric Department

at Eastman Dental Hospital, which is a main centre for child treatment after referral from the Community Dental Service where most of them had received some treatment. In addition, all subjects in the deciduous dentition group were caries-free.

In order to standardise the sample collection method for all age groups including edentulous infants, a sterile cotton swab was chosen over other techniques such as tooth pick or metal loop. Sterile cotton swabs have been used successfully for sampling by other workers (Lazarevic *et al.*, 2009), and they provide a quick, easy and effective method for collecting both small and large volumes of supragingival plaque (Lazarevic *et al.*, 2009). RTF (Syed and Loesche, 1972) was used as a preservative and transport medium for samples after collection. RTF has proven to be a satisfactory medium for the transportation of bacteria in clinical samples (Syed and Loesche, 1972).

The DNA extraction protocol used in this study was previously optimized by Academic Centre for Dentistry Amsterdam (ACTA) and proven to have high efficiency to acquire sufficient yield DNA from different types of oral bacteria (Personal communication, Egija Zaura). This technology of binding the DNA to magnetic beads and using the magnetic separation for DNA extraction allows removal of contaminations by salt-and alcohol-based wash buffers and a final eluation step improves the purity of the DNA in the samples before the PCR step (Kenk et al., 2012).

Because only relatively short sequences can be obtained via pyrosequencing, the Ribosomal Database Project Classifier (RDP) (Wang *et al.*, 2007) is considered as a limited tool for analysing highly diverse oral microbiome and only allows identification of sequences to genus level. For that reason the mothur analysis pipeline (Schloss *et al.*, 2009) was chosen as it is equipped with satisfactory filtering and quantifying ecological parameters for measuring diversity assessment and phylogenetic analysis. However, an initial analysis using RDP was performed in order to compare findings with mothur data at phyla level.

Recently, researchers have examined the oral microbiome in children populations (3 to 12 years) (Aas *et al.*, 2008; Gizani *et al.*, 2009; Ling *et al.*, 2010; Tanner *et al.*, 2011). The main focus of these studies was to understand the pathological shift in the oral microbiome by analysing caries lesions at different stages and correlate that with specific changes in the oral microbiome community. Results from these studies have suggested that the entire population of oral bacteria, rather than the presence of a small number of specific pathogens, have the greatest impact on disease risk. Aas *et al.* (2008) found that 10% of children with rampant caries have no detectable level of *S. mutans.* In addition, a strong correlation was suggested between dental caries and species such as *Veillonella, Lactobacillus, Bifidobacterium, Propionibacterium and* low-pH non-*S. mutans. Actinomyces, Granulicatella, and Bifidobacteriaceae* have also been identified as contributors to early childhood caries by others as well (Tanner *et al.*, 2011). Ling *et al* used PCR-DGGE and high-throughput sequencing to examine salivary and supragingival plaque in 60 Chinese children with and without caries. A recent similar study by Jiang *et al.* (2013) employed high-throughput sequencing to examine the salivary and supragingival plaque microbiome in forty subjects (3 to 6 years old), twenty children with severe early childhood caries and twenty caries-free children. Results of both studies demonstrated a significant correlation between severe early childhood caries and genera such as *Streptococcus*, *Granulicatella* and *Actinomyces.*

A study by Crielaard *et al.* (2011) provided a descriptive analysis of the childoral microbiome (aged 3 to 18 years) in their natural transition from deciduous to permanent dentition (considering the caries experience) through combination of pyrosequencing method band taxonomic microarray analysis. It was shown that the plaque microbiome of children is already complex by the age of three. However, at the age of puberty it still in the maturation process and differs from the adult microbiome (Crielaard *et al.*, 2011).

5.2   Relative abundance of the main bacterial phyla

At phylum level the sequence distribution on both RDP and mothur were similar with a minimum difference in Actinobacteria and Proteobacteria which were found to be 12% and 10% in RDP and 10.7% and 10.06% in mothur respectively. This therefore suggests that the denoising, trimming does so in an unbiased mannerr.

Phylum level observations of the prevalence between age groups showed some inconsistency with previous studies (Lazarevic *et al*., 2009; Crielaard *et al*., 2011). Proteobacteria were reported to be present at higher levels than Actinobacteria in all age groups in these studies while in our dataset Actinobacteria was observed to be the second most abundant phylum behind Firmicutes followed by Proteobacteria. In addition, our results showd that Proteobacteria was found to be more abundant than Bacteroidetes in all age groups which is comparable to the findings of Lazarevic *et al.* (2009). However, that was shown to be the case only in the deciduous dentition group in the other study (Crielaard *et al*., 2011). In deciduous group in the current study, the phylum Proteobacteria was found to be dominated by Betaproteobacteria mainly represented by Burkholderiaceae family (unclassified *Burkholderia*) and Neisseriaceae family presented first by *Neisseria* and to a lesser extent *Eikenella* and *Kingella*. This was different with the findings of Crielaard *et al.* (2011) where the Proteobacteria component among the same age group was dominated by Gammaproteobacteria.

As in previous studies the oral microbiome was shown to be relatively stable, despite the significant biological changes that occur during teeth eruption. At a genus level 102 distinct genus taxonomic groups were detected, of which 31 were as yet unclassified. Similar to othe recent studies, the principal genera were *Streptococcus, Gemella, Granulicatella, Veillonella, Abiotrophia, Actinomyces, Rothia, Corynebacterium, Burkholderia, Neisseria, Aggregatibacter, Haemophilus, Porphyromonas, Prevotella, Bergeyella, Capnocytophaga,Fusobacterium* and *Leptotrichia*. However, some of the taxa reported to be significant in other studies were not detected in any of our age

groups. For example, Jiang *et al.* (2013) who investigated early childhood caries in children (3-6 years old) observed *Derxia* as one of the predominant genera of healthy plaque microbiome. Crielaard *et al.* (2011) reported positive findings of *Moraxellaceae* family member *Acinobacter*, which is not usually associated with the healthy commensal oral microbiome, in children with primary dentition. Lazarivic *et al.* (2009) who used Illumina GAIIx sequencing reported a less diverse (with respect to genera) population with >70% belonging to *Streptococcus* or *Neisseria*. Discrepancies between studies could be related to primer bias, the hypervariable region selected from sequencing or length of the 16S gene product analyzed, biological variation between subjects and sample preparation.

Despite the differences between studies, many of the 8 predominant genera in infant saliva samples reported by Ling et al. (2010) and Cephas et al. (2011) (*Streptococcus, Prevotella, Neisseria, Haemophilus, Porphyromonas, Rothia, Veillonella*, and *Granulicatella)* were highly prevalent in our samples. The high prevalence of *Streptococcus* in the current study (77%) was in agreement with the findings of Cephas et al. (2011) (62%) but not with Ling et al. (2010) whose study demonstrated an infant streptococcus level of only 25%. Moreover, other studies detected significant levels of *Lactobacillus* (Aas *et al.*, 2008; Gezani *et al.*, 2009) and *Bifidobacterium* (Aas *et al.*, 2008); however, both of these genera were present at low proportion in this study (0.03% and 0.02%, respectively).

In contrast to our findings; the Cephas et al. (2011) demonstrated that edentulous infant saliva has a detectable level of most of the bacterial species that are needed for establishment of a biofilm on the hard dental surfaces prior to teeth eruption. For example, they recorded significantly high levels of early colonisers such as *Fusobacteruim. Periodonticum, Actinomyces. meyeri, Viellonella*) and late colonisers such as *Fusobacterium nucleatum, Tannerella denticola, Prevotella intermedia* and *Porphyromonas gingivalis*. The infants in our study showed less diversity data and all these taxa were not positively recorded. This might be explained by differences in

sampling techniques as other reports collected saliva while only biofilm from the edentulous infants was obtained in the present study.

In addition to *Veillonella, Fusobacterium* and *Actinomyces* which are known to be an early colonisers in infants oral cavity (Kononen *et al.*, 1992; 1994), Firimicutes in the infants oral microbiome in the current study was dominated by the *Streptococcus* genus (90%) especially *S.mitis, S.salivaris* and *S. oralis species* which have high affinity to mucosal surfaces. Moreover, in agreement with the study of Cephas et al. (2011) there were detectable levels of *Gemella* (4%) and *Granulicatella* (2.4%) which are usually associated with early childhood caries in children.

After establishment of the first hard surfaces in childrens mouths *Streptococcus* demonstrated a reduction of *S. Vestibularis* and *S. parasanguinis_II* and increase in the level of *S. gordonii, S. intermedius,* and *S. mutans.* These findings are consistent with those of Crielaard *et al.* (2011).

It has been shown that with increasing age the proportions of periodontal pathogens also increase (Kimura *et al.*, 2002; Papaioannou *et al.*, 2009). We confirmed the maturation of microbial composition by detecting increased proportions of Bacteroidetes (mainly genus *Porphyromonas* and *Prevotella*), *Spirochaetes* and candidate division TM7.

With increasing age, the highest abundance of these anaerobes were found in the prepuberty population. Bacteroidetes was presented mainly by *Capnocytophaga* (41%) followed by *Prevotella* (29%) and *Porphyromonas* (22%). This might be explained by increased amount of plaque and natural enviromental changes in tissues around teeth during eruption and exfoliation which create suitable subgingival habitats for Gram negative anaerobes to grow. Moreover, systemic factors such as developing immune and endocrine changes will also have a role to play. This was not in an agreement with findings of Crielaard *et al.* (2011) who found no detectable level of *Capnocytophaga*, while *Prevotella* and *Porphyromonas* were reported at higher prevalence in the adult dentition group. As none of our subjects

reported positive signs of pathological gingivitis it was not surprising to find a low prevalence of pathogenic taxa such as *Porphyromonas gingivalis.*

Low prevalence of periodontal pathogens such as *Porphyromonas gingivalis* in the permanent dentition in eight year old children have been reported by both culturing methods (Kamma *et al.*,2000) and multiplex PCR (Gafan *et al.*, 2006). Although periodontal disease is rare in healthy children, it is important to investigate the presence of periodontal pathogens as the permanent teeth start to erupt. The detection of periodontal pathogens before puberty may be helpful in identifying which children need to have more effective oral hygiene programs in order to minimize the risk of periodontal disease after puberty.

The maturation of the oral microbiome in our subjects was proven by the richer bacterial diversity in the older population. *Veillonellaceae* family, *Spirochaetes* and candidate division TM7 increased with increasing age, reflecting biological changes with age. The high level of *Corynebacterium* in pre-puberty (7.6%) and post-puberty (8%) groups was dominated by *Corynebacterium matruchotii,* which is well known as a ready calcifier (Moorer *et al.*, 1993) this species was not detected in the infant and deciduous groups.

The highest amount of plaque was recorded in prepuberty group; however, there was no detectedable gingivitis in this cohort. It has been proven that the prevalence of gingivitis and periodontitis is low in healthy children and gradually increases with age (Matsson *et al.*, 1978, 1993).

Although plaque was sampled from a variety of ethnic backgrounds, correlation between ethnicity and pathogen colonisation were beyond the aim of the present study. Further studies specifically addressing ethnicity need to be carried out.

5.3   Correlation with breast feeding in the infant group

In our analysis the most prevalent species in both groups (breast fed and exclusively formula-fed) belonged to phylum Firmicutes/Bacilli (genera

Streptococcus and *Gemella*) and Firmicutes/ Clostridia (genus *Veillonella*). Previous studies using bacterial culture have shown that oral bacterial colonisation in neonates starts with streptococci from the viridans group (Kononen *et al.*, 2002; Pearce *et al.*, 1995). Detectable levels of anaerobes was not recorded before two months of age (Kononen *et al.*, 2002). The present detection of high levels of Firmicutes phylum, especially within the genus *Streptococcus,* is comparable to these studies as is frequent detection of low levels of anaerobes in our infant cohort.

Previously the feeding mode has been found to affect the infants oral microbiome (Holgerson *et al.*, 2013). In that study 207 infants saliva samples were analyzed by a combination of culturing for *Lactobacillus* species, HOMIM analysis and qPCR for *Lactobacillus gasseri*. It was shown that exclusively breast-fed infants demonstrated significantly higher levels of *Lactobacillus Cluster1, Actinomyces gerencseriae* and *Streptococcus australis.*

These taxa were not recorded in our infant samples. This may be due to differences in the methodology as well as the resolution of the 16S r RNA gene identification. However, our exclusively formula fed infants showed a higher prevalence of *Prevotella melaninogenica, Neisseria polysacchare* and *Granulcatella adiacens,* which is consistent with the Holgerson et al. (2013).

5.4   Effect of oral health condition on the oral microbiome

The pre-puberty and post-puberty groups were divided into two categories according to the oral health status, recorded by dmft/DMFT. From our sequencing data, it was not possible to draw any conclusions regarding pathogenicity and correlation with caries experience at this stage due to the type of subjects, and the sample size variation of the two categories. However, *Abiotrophia* (represented by *Abiotrophia defectiva) and Capnocytophaga* (mainly *C. gingivalis*) were detected more frequently in a healthy oral condition. *C. gingivalis* is frequently detected in subgingival plaque and was found in young children's healthy mouths (Corby *et al.*,

2005). *A. defectiva,* a nutritionally variant *Streptococcus,* has been detected in endocarditis and bacteremia and generally requires 16S rRNA gene sequence data for identification (Senn *et al.*, 2006). *A. defectiva* was previously detected at higher levels in caries-free than caries children using 16S r RNA sequencing methods (Corby *et al.*, 2005, 2007; Kanasi *et al.*, 2010). However, the analyses in these studies were conducted on younger age groups (2 to 7 years old) to assess the microbiome change in relation to early childhood caries.

In contrast to this finding, Jiang *et al.* (2013) recorded association of *A. abiotrophia* with early childhood caries. That study analysed the supragingival plaque samples from 40 subjects (20 healthy and 20 with severe early childhood caries) using 16S rRNA gene V1-V3 hypervariable regions.

Despite the direct association between *S. mutans* and caries in the literature, lack *of S. mutans* association by clonal analysis was previously reported in childhood caries, advanced carious lesions and root caries (Aas *et al.*, 2008; Preza *et al.*, 2008). Our findings of low levels of *S. mutans* (38%) in children with caries experience are thus consistent with literature studies. This could be as a result of the quality of subjects recruited, and the method of sequencing to detect *S.mutans.* The other methods such as qPCR have been shown to detect a significant correlation with childhood caries which was negatively found using 16S rRNA gene sequencing on the same samples.

Kanasi *et al.* (2010) investigated plaque microbiome in 80 children (39 severe early childhood caries, 41 caries free) aged 2 to 6 years old. A significant correlation was detected between *S. mutans* and *Bifidobacteriaceae* spp. with childhood caries using specific PCR analysis; on the other hand the clonal analysis of same samples did not reveal any similar detectable relationship. The discrepancy between clonal counts and selective detection (PCR, selective isolation, DNA probes) is likely related to these different approaches used for species detection, with selective detection having the ability to identify species in low proportions of the microbiome.

The data in the present study showed some consistency with the previous studies with respect to the general oral microbiome evolution and ecology early in life (Corby *et al.*, 2005; Kanasi *et al.*, 2010; Lazarevic *et al.*, 2009; Crielaard *et al.*, 2011). However, the most striking feature was demonstrated in the pre-puberty group which harboured the highest level of Gram negative anaerobes. Moreover, our findings in infants samples showed less richness that recorded by Cephas *et al* (2011).

Chapter Six: Conclusion and Further work

6   Conclusion

In this report the overall structure of plaque microbiome ecology and evolution in children from birth to adolescence was comprehensively explored by utilizing 454 pyrosequencing.

Our results demonstrated that the plaque microbiome of children (1 month to 17 years) is still in the process of maturation and that the pre-pubertal stage was characterized by the highest level of Gram negative anaerobes with no visible inflammation while Proteobacteria recorded the highest prevalence in the deciduous group. It is important to investigate the presence of periodontal pathogens as the permanent teeth start to erupt. The detection of periodontal pathogens before puberty may be helpful in identifying which children need more effective oral health programs in order to minimize the risk of periodontal disease after puberty.

In the infant cohort, formula feeding was found to be an important factor in altering the oral microbiome, since exclusively formula-fed infants showed higher prevalence of *Prevotella melaninogenica, Neisseria polysaccharea,* and *Granulicatella adiacens.*

Moreover, no conclusion could be drawn regarding the correlation with dmft/DMFT in the pre-puberty and post-puberty groups due to the discrepancy in the sample size. However, at genera level *Abiotrophia* (mainly A. *Defectiva*) and *Capnocytophaga* (mainly *C. gingivalis)* were detected more frequently in the healthy group. Other major sequences involve (*Streptococcus*, *Neisseria*, *Lautropia*, *Fusobacterium*, *Leptotrichia*, *Porophyromonas*, *Prevotella*, *Actinobaculum*, *Actinomyces*, *Corynebacterium, Rothia*) were all recorded to be higher in dmft/DMFT group. *S. mutans* was not detected in healthy subjects, and only 38% of children with recorded dmft/DMFT had detected level of *S. mutans*, with no significant differences between the two groups.

7   Future work

The microbial richness in the child oral microbiome at different chronological stages provided a novel identification of the potential pathogenic communities and the development of different pathological conditions such as caries and periodontal diseases. This study indicates the essential need for further studies, preferably longitudinal clinical trials at the depth of next generation sequencing, involving a larger population groups to correlate a wide spectrum of different factors with the plaque microbiome composition. Recently developed genetic methods are allowing the functional potential of the oral microbiome to be assessed. Moreover, using the combined metagenomic and 16S rRNA based analyses has allowed correlations between the community structure and metabolic activity to be determined for a variety of sites from the oral cavity.

Fast progress in DNA sequencing technology has made for a substantial reduction in costs and a substantial increase in throughput and accuracy. Employing the most recent powerful sequencing methods such as Hiseq platform will enable up to 15 Gb of output with 25 million sequencing reads and 2x300 bp read lengths in just third the cost of single 454 pyrosequencing run. Species-level identification may not be sufficient however, because of the genetic heterogeneity of bacterial species and the strong influence of the environment on phenotype. A combination of phylogenetic, metagenomic, and metabolomic approaches may be required to fully dissect oral microbiome interactions relevant to health and disease

Characterizing how surrounding conditions such as dietary or other environmental factors early in life may alter the oral microbiome is greatly needed and may impact oral health strategies in the future. Moreover, it would be advantageous to know when children attain their adult microbiome and what factors aid in determination of the microbiome composition to detect individuals at greater risk for diseases and provide essential modifying interventions accordingly.

Chapter Seven: References

8   References

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Chapter Eight: Appendices

9   Appendices

Appendix 1: Ethical approval

The Joint UCL/UCLH Committees on the Ethics of Human Research (Committee A)

National Research Ethics Service

South House

Royal Free Hospital

Pond Street

13 January 20

London

Dr Paul F Ashley

NW3 2QG

Senior Lecturer/Honorary Consultant

UCL Eastman Dental Institute

Paediatric Dentistry, UCL EDI

256 Gray's Inn Road

London

WC1X 8LD

Dear Dr Ashley

Study Title:	**Oral microbiota throughout growth and development: Pilot investigation**
REC reference number:	**09/H0714/57**
Protocol number:	**Version 2**

Thank you for your letter of 08 January 2010, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

<u>Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.</u>

For NHS research sites only, management permission for research ("R&D approval") should be obtained from the relevant care organisation(s) in accordance with NHS research governance arrangements. Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk. *Where the only involvement of the NHS organisation is as a Participant Identification Centre, management permission for research is not required but the R&D office should be notified of the study. Guidance should be sought from the R&D office where necessary.*

Sponsors are not required to notify the Committee of approvals from host organisations.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	Version	Date

Covering Letter	Paul Ashley	09 September 2009
REC application	IRAS Version 2.4 24439/61641/1/698	10 September 2009
Investigator CV	Paul Ashley	
Participant Information Sheet: Patient	Version 2	09 September 2009
Participant Information Sheet: Parent	Version 2	09 September 2009
Letter from Statistician	Aviva Petrie	04 September 2009
Referees or other scientific critique report	Jonathan Pratten	14 July 2009
Investigator CV	Michael Wilson	
Investigator CV	David A Spratt	
Protocol	Version 3	18 December 2009
Data Collection Form	Version 1	18 December 2009
Response to Request for Further Information	Dr Paul Ashley	17 December 2009
Participant Consent Form: Child/Young Person	Version 4	09 January 2010
Participant Consent Form: Assent Form	Version 3	08 January 2010
Response to Request for Further Information	Email - Paul Ashley	08 January 2010

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Service website > After Review

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nres.npsa.nhs.uk.

09/H0714/57	**Please quote this number on all correspondence**

Appendix 2: Consent form

University College London Hospitals **NHS**
NHS Foundation Trust

Patient Identification Number for this trial: ………………………..

CONSENT FORM (V4)

Title of Project: Bugs and bacteria in our mouth - how they change as we grow

Name of Researcher: Paul Ashley

Child /young person to circle all they agree with:

Have you read (or had read to you) about this project?

      Yes/No

Has somebody else explained this project to you?

     Yes/No

Do you understand what this project is about?

     Yes/No

Have you asked all the questions you want?

     Yes/No

Have you had your questions answered in a way you understand?

     Yes/No

Do you understand it's OK to stop taking part at any time?

     Yes/No

Are you happy to take part?

     Yes/No

If you do want to take part, you can write your name below

Your name _____   Date _____

The doctor who explained this project to you needs to sign too:

Print Name _____

Sign _____   Date _____

Thank you for your help.

For further information about this study please contact Paul Ashley

Phone   02079151022   Email   p.ashley@eastman.ucl.ac.uk

UCLH welcomes feedback from their patients who have been involved in research. In the first instance, you should inform the Principal Investigator. If you are not satisfied with the response of the research team then you should address your complaints to the UCLH complaints manager at UCLH postal address or through our website http://www.uclh.nhs.uk/Contact+us/. To help

us identify the research study you have been involved in, please mention the title and the name of the research doctor or principal investigator. You can find this information on the Patient Information Sheet.

Appendix 3: Basic data collection form

University College London Hospitals **NHS**
NHS Foundation Trust

The Eastman Dental Hospital

256 Gray's Inn road

London

Patient Identification Number for this trial: ………………………..

Data collection form (V1)

Title of Project: Oral microbiota throughout growth and development

Name of Researcher: Paul Ashley

Age (years) ………………. Gender M   /   F

Ethnicity (Circle where appropriate)

British

African

Caribbean

Indian

White and Asian

Pakistani

Any other mixed background

Any other ethnic group

Any other Black background

Has the patient had antibiotics in the previous three months   Yes   /   No

Has the patient periodontal disease or related systemic illness   Yes   /   No

Is the patient wearing orthodontic appliances or dentures   Yes / No

Has the patient had a professional scaling or polishing in the last three months   Yes / No

If the answer to any of the above is yes, the patient must be excluded from further participation

DMFT   ……………………………………………

Plaque score ……………………………………………

For subjects in the lower age group - how long were they breastfed for? (months) ……………

How would parents/guardians or participants suggest results from a larger study could be disseminated to participants in future.

……………………………………………………………………………………… ……………………………

……………………………………………………………………………………… ……………………………

Appendix 4: Information sheet page 1

University College London Hospitals **NHS**
NHS Foundation Trust

Information about the research V3

The Eastman Dental Hospital
256 Gray's Inn road
London

Bugs and bacteria in our mouth - how they change as we grow

We are asking if you would take part in a research project to find the answer to the question, *how do the bugs and bacteria in our mouth change as we grow?*

Before you decide if you want to join in it's important to understand why the research is being done and what it will involve for you. So please consider this leaflet carefully. Talk about it with your family, friends, doctor or nurse if you want to.

Why are we doing this research?

There are lots of different bugs that live in all our mouths. The types of bugs we have change as we grow. This is for lots of different reasons. It might be because we get new teeth or it might be because our bodies change as we become grown-ups. Most of the time these bugs don't cause any problems. Sometimes though they might affect our teeth or gums, they might even make us feel unwell. It's difficult to count these bugs, or work out which ones there are. We have a new machine to look for bugs in saliva. What we want to do in this study is use it on a small number of children of different ages. Once we know how well it works we can ask for money to carry out a much bigger study with lots of children and adults. This information might help us better understand how gum or tooth problems start and how the bugs in our mouth could make us unwell.

Before any research goes ahead it has to be checked by a Research Ethics Committee. They make sure that the research is fair. Your project has been checked by the _____ Research Ethics Committee.

Why have I been asked to take part?

Because you are at this clinic and meet our requirements for this study. We want to look at 80 children by the end of the study aged between 6 months an 16 years.

Do I have to take part?

No. It is up to you. If you do, we will ask you to sign a form giving your consent or assent. You will be given a copy of this information sheet and your signed form to keep. You are free to stop taking part at any time during the research without giving a reason. If you decide to stop, this will not affect the care you receive.

Appendix 5: Information sheet page 2

What will happen to me if I take part?

We will ask the adult with you some simple questions to check you meet our requirements. Then we will use a small cotton wool bud on some of your teeth to get our sample. We may ask you to spit in a pot as well. Finally we will have a look at your teeth. These are simple procedures that can be carried out quickly while you wait for your appointment. No more visits will be required. No information that can be used to identify you will be kept (eg name, address etc.)

There are no direct benefits to you from this study, nor are there are any risks in taking part. If we see any signs of dental decay or other problems we can refer you to a dentist if required. Results from this study will be published in a scientific journal. We will also produce a summary sheet that will be circulated on the clinic from which you were recruited.

What will happen if I don't want to carry on with the study?

All your samples and information will be destroyed

Thank you for reading this - please ask any questions if you need to.

For further information about this study please contact the Principal Investigator

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UCL Hospitals is an NHS Foundation Trust comprising: The Eastman Dental Hospital, The Heart Hospital, Hospital for Tropical Diseases, National Hospital for Neurology and Neurosurgery, The Royal London Homoeopathic Hospital and University College Hospital (incorporating the former Middlesex and Elizabeth Garrett Anderson Hospitals).

Appendix 6: Primer sequences

Table 9-1 Sequences for the primers ligated to samples in plate one

Sample	Oligoname	Sequence
REV	TitanB-16S-1175R	CCTATCCCCTGTGTGCCTTGGCAGTCACGTCRTCCCCDCCTTCCTC
2	TCMID002-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAGACGCTCGACAGGATTAGATACCCBRGTAGTC
3	TCMID003-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAGAGACGCACTCGGATTAGATACCCBRGTAGTC
4	TCMID004-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAGAGCACTGTAGGGATTAGATACCCBRGTAGTC
5	TCMID005-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAGATCAGACACGGGATTAGATACCCBRGTAGTC
6	TCMID006-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAGATATCGCGAGGGATTAGATACCCBRGTAGTC
7	TCMID007-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAGCGTGTCTCTAGGATTAGATACCCBRGTAGTC
8	TCMID008-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTCGCGTGTCGGATTAGATACCCBRGTAGTC
9	TCMID009-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAGTATCAGCGGATTAGATACCCBRGTAGTC
10	TCMID010-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTCTATGCGGGATTAGATACCCBRGTAGTC
11	TCMID011-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGATACGTCTGGATTAGATACCCBRGTAGTC
12	TCMID012-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACTGAGCTAGGATTAGATACCCBRGTAGTC
14	TCMID014-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAGCGAGAGATACGGATTAGATACCCBRGTAGTC
16	TCMID016-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCACGTACTAGGATTAGATACCCBRGTAGTC
1	TCMID017-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAGCGTCTAGTACGGATTAGATACCCBRGTAGTC
18	TCMID018-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTACGTAGCGGATTAGATACCCBRGTAGTC
19	TCMID019-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGTACTACTCGGATTAGATACCCBRGTAGTC
20	TCMID020-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAGACGACTACAGGGATTAGATACCCBRGTAGTC
21	TCMID021-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAGCGTAGACTAGGGATTAGATACCCBRGTAGTC
22	TCMID023-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACTCTCGTGGGATTAGATACCCBRGTAGTC
23	TCMID024-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAGAGACGAGGGATTAGATACCCBRGTAGTC
25	TCMID026-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAGACATACGCGTGGATTAGATACCCBRGTAGTC
41	TCMID027-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAGACGCGAGTATGGATTAGATACCCBRGTAGTC
42	TCMID028-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAGACTACTATGTGGATTAGATACCCBRGTAGTC

Sample	Oligoname	Sequence
43	TCMID029-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAG**ACTGTACAGT**GGATTAGATACCCBRGTAGTC
44	TCMID030-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAG**AGACTATACT**GGATTAGATACCCBRGTAGTC
45	TCMID031-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAG**AGCGTCGTCT**GGATTAGATACCCBRGTAGTC
47	TCMID033-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAG**ATAGAGTACT**GGATTAGATACCCBRGTAGTC
48	TCMID034-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAG**CACGCTACGT**GGATTAGATACCCBRGTAGTC
49	TCMID035-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAG**CAGTAGACGT**GGATTAGATACCCBRGTAGTC
50	TCMID037-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAG**TACACACACT**GGATTAGATACCCBRGTAGTC
51	TCMID038-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAG**TACACGTGAT**GGATTAGATACCCBRGTAGTC
52	TCMID039-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAG**TACAGATCGT**GGATTAGATACCCBRGTAGTC
54	TCMID042-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAG**TCGATCACGT**GGATTAGATACCCBRGTAGTC
55	TCMID043-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAG**TCGCACTAGT**GGATTAGATACCCBRGTAGTC

Table 9-2 Sequences for the primers ligated to samples in plate two

Sample	Oligoname	Sequence
REV	TitanB-16S-1175R	CCTATCCCCTGTGTGCCTTGGCAGTCACGTCRTCCCCDCCTTCCTC
57	TCMID002-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAGACGCTCGACAGGATTAGATACCCBRGTAGTC
59	TCMID004-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAGAGCACTGTAGGGATTAGATACCCBRGTAGTC
60	TCMID005-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAGATCAGACACGGGATTAGATACCCBRGTAGTC
62	TCMID007-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAGCGTGTCTCTAGGATTAGATACCCBRGTAGTC
64	TCMID009-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAGTATCAGCGGATTAGATACCCBRGTAGTC
66	TCMID011-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGATACGTCTGGATTAGATACCCBRGTAGTC
67	TCMID012-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACTGAGCTAGGATTAGATACCCBRGTAGTC
68	TCMID013-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAGCATAGTAGTGGGATTAGATACCCBRGTAGTC
70	TCMID015-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAGATACGACGTAGGATTAGATACCCBRGTAGTC
71	TCMID016-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCACGTACTAGGATTAGATACCCBRGTAGTC
72	TCMID017-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAGCGTCTAGTACGGATTAGATACCCBRGTAGTC
73	TCMID018-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTACGTAGCGGATTAGATACCCBRGTAGTC
74	TCMID019-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGTACTACTCGGATTAGATACCCBRGTAGTC
75	TCMID020-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAGACGACTACAGGGATTAGATACCCBRGTAGTC
76	TCMID021-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAGCGTAGACTAGGGATTAGATACCCBRGTAGTC
77	TCMID023-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACTCTCGTGGGATTAGATACCCBRGTAGTC
78	TCMID024-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAGAGACGAGGGATTAGATACCCBRGTAGTC
80	TCMID026-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAGACATACGCGTGGATTAGATACCCBRGTAGTC
S17	TCMID029-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAGACTGTACAGTGGATTAGATACCCBRGTAGTC
S4	TCMID030-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAGAGACTATACTGGATTAGATACCCBRGTAGTC
S7	TCMID033-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAGATAGAGTACTGGATTAGATACCCBRGTAGTC
S10	TCMID037-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACACACACTGGATTAGATACCCBRGTAGTC
S11	TCMID038-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACACGTGATGGATTAGATACCCBRGTAGTC

Sample	Oligoname	Sequence
S12	TCMID039-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACAGATCGTGGATTA GATACCCBRGTAGTC
S14	TCMID042-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCGATCACGTGGATTA GATACCCBRGTAGTC
S15	TCMID043-16S-785F	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCGCACTAGTGGATTA GATACCCBRGTAGTC

CCATCTCATCCCTGCGTGTCTCCGACTCAGTACAGATCGTGGATTA
GATACCCBRGTAGTC

CCATCTCATCCCTGCGTGTCTCCGACTCAGTCGATCACGTGGATTA
GATACCCBRGTAGTC

CCATCTCATCCCTGCGTGTCTCCGACTCAGTCGCACTAGTGGATTA
GATACCCBRGTAGTC

Appendix 7: Reduced Transport Fluid (RTF) prepration

Stock Solution 1

Dibasic potassium phosphate (K_2HPO_4)	0.6 g
Distilled water	100 mL
$MgSO_4$ stock solution	
$MgSO_4$	2.5 g
Distilled water	100 ml

Stock Solution 2

Potassium chloride	1.2 g
Ammonium sulphate	1.2 g
Monobasic potassium phosphate (KH_2PO_4)	0.6 g
$MgSO_4$ stock solution	1 ml
Distilled water	99 ml

Sodium Carbonate Solution

Sodium carbonate	0.8 g
Distelled water	10 ml

Preparation for 100 ml

Stock 1	7.5 ml
Stock 2	7.5 ml
Sodium carbonate	0.5 ml
DW	80 ml

autoclave, then once cool add a filter sterilized solution of DTT (0.02 g in 5 ml DW). This solution is now stable for 1 wee