In-vitro models for the culture of previously uncultured oral bacteria

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Abstract

Around half of oral bacteria have yet to be cultured, and their role in disease is therefore unknown. It is hypothesised that bacteria in biofilms have become dependent on growing in multi-species communities. TM7 phylum has no cultured representatives and some oral TM7 phylotypes have been associated with oral diseases such as periodontitis. The aims of this study were therefore to evaluate the ability of two model culture systems: Cooked Meat Medium and the Calgary Biofilm Device (CBD), to support the growth of mixed oral bacterial communities including uncultured bacteria, and to attempt to culture representatives of the TM7 division.

The Cooked Meat Medium was used to establish a mixed bacterial community from 3 endodontic samples and their composition was analysed by Sanger sequencing and 454 pyrosequencing. A diverse bacterial community closely related to the original endodontic samples was maintained up to 480 days and included some uncultured bacteria present in the original samples.

A mixed oral biofilm was established on the CBD from saliva. The effect of the presence of mucin and glucose in the growth media on community composition was evaluated, but no significant differences were seen. The effect of using propidium monoazide to remove extracellular DNA was assessed and was found to significantly affect the perceived composition of the biofilms. Uncultured taxa detected in culture included representatives of deep branches of *Bacteroidetes* and *Clostridiales*, and TM7 and SR1 phyla.

TM7 members were detected in both models with specific PCR primers, but their proportion never exceeded 1 %. In an attempt to isolate TM7 division representatives a saliva microcosm was grown on agar. TM7 representatives were detected by colony hybridization and specific PCR and subcultured, producing enrichment. Two simple co-cultures of TM7 HOT352 / HOT353 with *Slackia exigua* or *Atopobium parvulum* were obtained, but were not maintained.

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1.1 Oral microbiome

The mouths of animals are heavily colonised by a diverse array of microorganisms. The oral microbiota is characteristic to the host species and specific to individuals within each species, as highlighted by comparison with the canine oral microbiota (Dewhirst, Klein et al. 2012). The factors that determine the specificity of oral microbiomes remain to be fully described but antimicrobial peptides, such as defensins, appear to be important (Linzmeier and Ganz 2005, Gomes Pde and Fernandes 2010). The human oral microbiome is also highly variable between individuals (Ledder, Gilbert et al. 2006, Lazarevic, Whiteson et al. 2010, Hsu, Power et al. 2012).

The infant microbiota may be influenced by the delivery mode. Within the first 24 hours of life, newborns were described as presenting undifferentiated microbial communities across skin, oral, nasopharyngeal, and gut habitats regardless of delivery mode. Vaginally delivered infants would harbour microbiota resembling their mothers' vaginal communities, dominated by *Lactobacillus*, *Prevotella*, or *Sneathia* species, in all body niches, while C-section delivered infants acquire general skin-related bacteria, dominated by *Staphylococcus*, *Corynebacterium*, and *Propionibacterium* species (Dominguez-Bello, Costello et al. 2010). It was also shown that vaginally delivered infants present a significantly higher prevalence of health-related oral bacteria, such as *Streptococcus salivarius*

and *Lactobacillus* species, in the first year of life (Nelun Barfod, Magnusson et al. 2012). The longer-term effects are still to be investigated.

Compared to other human-associated microbiomes, the oral microbiota is habitat and niche specific; for example very few oral bacterial species are found in the colon, and *vice versa* (Rajilic-Stojanovic, Smidt et al. 2007, Maukonen, Matto et al. 2008).

The oral microbiota includes a wide range of microorganisms including bacteria, archaea, fungi, viruses and protozoa. Bacteria are the most numerous, with 10^8 cells per ml of saliva (Richardson and Jones 1958), but other members also play an important role in metabolic pathways and interaction with the host.

1.1.1 Archaea

Several taxa of *Archaea* have been found in the human gastro-intestinal tract (Karlin, Jones et al. 1982), mouth (Belay, Johnson et al. 1988) and vagina (Belay, Mukhopadhyay et al. 1990). All of the *Archaea* detected by culture and culture-independent methods in the human oral cavity belong to the division Euryarchaeota, mostly *Methanobrevibacter*, but also representatives of the genera *Methanobacterium*, *Methanosarcina* and *Thermoplasmatales* (Lepp, Brinig et al. 2004, Li, Liu et al. 2009, Matarazzo, Ribeiro et al. 2011). They are thought to associate with bacteria, in a cross-feeding manner, for the degradation of volatile fatty acids such as acetate, propionate and butyrate, producing methane. The thermo-dynamic constraints render this syntrophic behaviour obligatory, with direct contact between microorganisms required for interspecies hydrogen transfer (Schink 1997, Ishii, Kosaka et al. 2006, Thauer, Kaster et al. 2008). In periodontitis

and endodontic infections, methanogenic *Archaea* could play the role of a terminal degrader, lowering the concentration of H_2 and favouring fermenting bacteria (Matarazzo, Ribeiro et al. 2011, Matarazzo, Ribeiro et al. 2012).

One species in particular, *Methanobrevibacter oralis*, has been shown to be associated with periodontal disease. It was detected in only a subset of periodontitis patients (36 %), but its proportion increased with severity of periodontitis up to 18 % of prokaryotic load (Lepp, Brinig et al. 2004). It had been thought that *M. oralis* was only found in periodontal pockets and was not present at healthy sites (Yamabe, Maeda et al. 2008, Li, Liu et al. 2009), but a more recent study conducted on patients with generalized aggressive periodontitis and healthy volunteers found that *M. oralis* was found in all studied samples, although the numbers of copies of the 16S rRNA gene were significantly different between periodontitis sites ($11.2x10^4$) and healthy gingival crevices ($0.6x10^4$) (Matarazzo, Ribeiro et al. 2011). A positive correlation has also been demonstrated between the numbers of *Archaea* detected in periodontal pockets and those of *P. gingivalis* and *T. forsythia* (Matarazzo, Ribeiro et al. 2012). *Archaea* (*M. oralis*, *Methanococcus maripaludis*, *Methanoplanus endosymbiosus*, and *Methanospirillum hungatei*) were also detected in both symptomatic and asymptomatic endodontic infections (Vianna, Conrads et al. 2006, Vickerman, Brossard et al. 2007, Jiang, Xia et al. 2009).

Further evidence supporting a possible pathogenic role for *Archaea* is that they are recognised by the human immune system. IgG antibodies reacting specifically with *M. oralis* have been found in subjects with periodontitis (Yamabe, Maeda et al. 2008) and archaeosomes, complexes of archaeal membrane lipids,

demonstrate a potent adjuvant activity (Cavicchioli, Curmi et al. 2003, Eckburg, Lepp et al. 2003). It is possible, however, that the anaerobic conditions of the periodontal pocket or root canal environment simply promote the growth of methanotrophic archaea. Whether *Archaea* are truly pathogenic remains to be determined (Cavicchioli, Curmi et al. 2003).

Archaea are also able to transform heavy metals or metalloids into volatile methylated derivatives, which are more toxic than the original compounds, and intestinal *Archaea* have been shown to do this at higher rates than bacteria (Meyer, Michalke et al. 2008, Michalke, Schmidt et al. 2008). Such heavy metals are present in cosmetics and pharmaceutical products. It is unclear if oral *Archaea* exhibit this activity *in vivo*, but it could potentially represent a significant risk with crowns, bridges, amalgams and composites.

The presence of *Archaea* has been historically underestimated because of ineffective DNA extraction methods and the cross-reactivity of archaea-specific PCR primers with human DNA. Enzymatic treatments for the permeabilization of archaeal cell walls have been compared and it has been shown that only recombinant pseudomurein endopeptidase treatment allowed the detection of *Methanobrevibacter* species, and that they were not detected when standard lysozyme or proteinase K protocols were used (Kubota, Imachi et al. 2008). Moreover, in mixed samples where the proportion of archaeal template DNA was low, archaea-specific PCR primers, originally designed for environmental studies, were found to cross-react with human DNA, which is present in high amounts in all oral samples (Horz and Conrads 2011). For this reason, the use of genes specifically

found in archaea, such as mcrA, encoding methyl coenzyme M reductase, a ubiquitous enzyme in the methane generation pathway, may be preferable, for specific detection of members of this domain (Vianna, Conrads et al. 2006).

Archaea are resistant to most antibiotics targeting RNA synthesis such asrifampicin and ofloxacin, and, because they lack peptidoglycan, those inhibiting the synthesis or cross-linkage of the peptide subunit of murein, such as penicillin, cephalosporin, glycopeptides, and aminoglycosides, but are susceptible to antibiotics that inhibit the lipid cycle, such as bacitracin, chloramphenicol, lasalocid, and monesin (Dridi, Fardeau et al. 2011). The inclusion of members of the first group of antimicrobials in appropriate culture media may provide a method for enrichment of archaea.

1.1.2 Protozoa

Entamoeba gingivalis and *Trichomonas tenax* are saprophytic protozoa that have been found in the oral cavity. They appear to be associated with poor oral hygiene (Bergquist 2009) and periodontitis. In a study using real time PCR to detect the presence of *E. gingivalis* in periodontitis patients and periodontally healthy volunteers, 18 out of 26 periodontal pockets were positive for the organism, whilst they were not detected in samples from healthy gingival crevices, even in patients with periodontitis (Trim, Skinner et al. 2011). Bacteria are taken up by protozoa and used for nutrition but some are able to resist digestion (Greub and Raoult 2004). Intact bacterial cells have been observed in the vacuoles of *E. histolytica* and vacuoles and cytoplasma of *E. dispar* (Pimenta, Diamond et al. 2002). This may

enable them to "hide" inside the eukaryote and thus evade the immune system and the effects of antimicrobial treatment (Trim, Skinner et al. 2011).

1.1.3 Oral viruses

1.1.3.1 Pathogenic viruses

Herpes simplex virus (HSV) infections are very common in humans and cause a variety of clinical manifestations, including oral and genital lesions, ocular infections, encephalitis and neonatal disease. One of the main features of the HSV infection is the capacity of the virus to remain dormant for life in host neurones, with no clinical symptoms. In oral infections, HSV can be reactivated following a stimulation of the peripheral nerves, such as dental or oral surgery, chemotherapy, but also the common cold and even stress (Scott, Coulter et al. 1997). The primary HSV infection can lead to gingivostomatitis, affecting the tongue, lips, gingival and buccal mucosa and the hard and soft palate, while reactivation of the virus results in herpes labialis, or cold sores (Arduino and Porter 2008).

Other viruses of the herpes family routinely detected in the oral cavity are Epstein-Barr virus (EBV) and Human Cytomegalovirus (HCMV). Infecting B-lymphocytes and epithelial cells, EBV causes infectious mononucleosis in adults. HCMV infections are usually benign in healthy individuals, but may present a risk in immunocompromised people (for instance HIV-positive patients). Both viruses are often detected in saliva, gingival crevices and periodontal lesions, but do not seem to be associated with periodontitis *per se* or endodontic treatment failures (Dawson, Wang et al. 2009, Sahin, Saygun et al. 2009, Guilherme, Ferreira et al. 2011).

Human papillloma virus (HPV) causes mostly benign infections in the oral cavity, such as papillomas, condylomas and focal epithelial hyperplasia (Kumaraswamy and Vidhya 2011). HPV has, however, also been associated with malignancy, including cervical (Schiffman, Castle et al. 2007), and with head and neck squamous cell carcinomas (HNSCCs) (Chaturvedi 2012). HPV was detected in 35.6 % of oropharyngeal cancers, 23.5 % of oral cavity cancers and 24 % of laryngeal cancers (Kreimer, Clifford et al. 2005). Other studies have shown the integration of HPV into the human genome in HNSCC cancerous cells, high viral copy numbers and strong expression of HPV oncogenes in tumours (Chaturvedi 2012), supporting a possible causal association between HPV and HNSCC. This association was confirmed by several case studies, even after correction for other HNSCC risk factors. Molecular studies need to be undertaken to clarify the role of HPV in other HNSCCs, such as oral cavity and laryngeal cancers (Chaturvedi 2012).

The hepatitis viruses are a heterogeneous family of viruses (HAV to HEV and HGV), causing hepatitis or inflammation of the liver. HBV, a double stranded DNA virus, and HCV, a positive single strand RNA virus, are the most common and endemic worldwide. Both viruses are present in most body fluids of the infected patients, including blood and serum and saliva, which may constitute an infection risk during dental procedures, even in symptom-free patients (Ferreiro, Dios et al. 2005, Heiberg, Hoegh et al. 2010). Viral-induced liver disease can also cause oral bleeding, petechiae, and ecchymoses, and high levels of bilirubin can cause a jaundice of the oral mucosa (Miller 2002). Extrahepatic manifestations of HBV include skin rash, arthritis, arthralgia, glomerulonephritis, polyarteritis nodosa, and

papular acrodermatitis (Pyrsopoulos and Reddy 2001), while oral diseases associated with HCV include sialadenitis, salivary glands lymphoma and lichen planus (Carrozzo 2008, Carrozzo 2008).

Human immunodeficiency virus (HIV) is a lentivirus that causes acquired immunodeficiency syndrome (AIDS). More than 34 million people worldwide live with HIV infection. Even if it proves possible to reduce the number of new infections, there will remain a substantial number of infected individuals in the community as highly active antiretroviral therapy (HAART) increases life expectancy (UNAIDS, available at http://www.unaids.org/en/dataanalysis/datatools/aidsinfo).

The main oral manifestations of HIV are related to the immunocompromised state of the infected individuals, resulting from opportunistic infections due to other viruses discussed in this chapter and *Candida albicans* and other fungal infections (Johnson 2010, Mendes-Correa and Nunez 2010, Thompson, Patel et al. 2010, Vitali 2011). Other oral manifestations include linear gingival erythema, which is a marginal gingivitis with increased numbers of *Candida* species, and acute necrotizing ulcerative gingivitis (ANUG), which can progress to necrotizing stomatitis (Johnson 2010). The effect of HIV infection on periodontitis remains controversial, as studies on different cohorts give contradictory results (Barr, Lopez et al. 1992, Robinson, Boulter et al. 2000, Ranganathan, Magesh et al. 2007, Vernon, Demko et al. 2009).

1.1.3.2 Bacteriophages

There are only a few studies investigating the human residential virome, which is probably dominated by phages, with the majority of viral diversity

uncharacterized. There have been few studies of the range of non-pathogenic viruses found in the oral cavity (Pride, Salzman et al. 2012). Pride *et al*. analysed the viromes (double stranded DNA viruses only) of 5 periodontally healthy volunteers at three time points over 60 or 90 days periods by 454 pyrosequencing. After filtering the samples, they estimated the viral concentration by epifluorescence microscopy after SYBR-gold staining and found it to be 10^8 particles per mL of saliva. Considering that some viral particles adherent to cells or forming aggregates did not pass the filtering, the numbers could be significantly higher. The vast majority (99 to 100 %) of contigs with homology to viral sequences were identified as belonging to bacteriophages (Pride, Salzman et al. 2012).

1.1.4 Fungi

Candida is the most frequently isolated genus of fungi in the human mouth (Aas, Barbuto et al. 2007, Urzua, Hermosilla et al. 2008), as a large proportion of healthy adults are carriers. The prevalence of *Candida* carriage was found to range from 2 to 71.3 % in 32 analysed studies, with a weighted mean of 25.5 % (Scully, el-Kabir et al. 1994). *Candida albicans* is the species found most frequently in humans, both in health and disease, but the importance of other *Candida* species is being increasingly recognised, as non-albicans *Candida* (NAC) species, such as *C. parapsilosis*, C*. tropicalis*, *C. krusei* and *C. glabrata*, cause 35-65% of all candidaemias (Krcmery and Barnes 2002). *Candida* species are usually considered commensal in adults, and a candidosis in an otherwise healthy individual may be a marker of undiagonosed systemic disease. Oral *Candida* infections can be classified in four main categories. Pseudomembranous candidosis, or thrush, takes the form of creamy-white plaques on the surface of the labial and buccal mucosa, which can

be easily rubbed off. Predisposing factors in adults include diabetes mellitus, AIDS, leukaemia or other conditions impairing immune response, and the use of steroid aerosol inhalers and broad range antibiotics (Akpan and Morgan 2002). Acute erythematous candidosis is often related to antibiotic-driven reduction in bacterial competition, while chronic erythematous candidosis is mostly associated with denture wearing. The latest is probably the most common form of oral candidosis, as up to 75 % of denture wearers suffer from the condition (Barbeau, Seguin et al. 2003). Chronic hyperplastic candidosis is characterised by white plaques on the buccal mucosa, tongue surfaces and the angles of the mouth which, unlike in pseudomembranous candidosis, cannot be rubbed off. Speckled red-white areas sometimes seen in this condition present a higher risk of malignant transformation (Renstrup 1970). *Candida* is also a cause of angular cheilitis, as is *Staphylococcus aureus*, and has been associated with median rhomboid glossitis, although the extent of the role *Candida* plays in this condition remains unclear (van der Wal and van der Waal 1986, Smith, Robertson et al. 2003).

In a study analysing oral fungal populations in 20 healthy volunteers using broad range primers targeting universal internal transcribed spacer, 74 culturable and 11 unculturable genera were identified, with 101 species found in total, ranging from 9 to 23 in each individual (Ghannoum, Jurevic et al. 2010). While some low-abundance genera may represent environmental contamination, the most frequently detected genera: *Candida*, *Cladosporium*, *Aureobasidium*, *Saccharomycetales*, *Aspergillus*, *Fusarium*, and *Cryptococcus*, were detected in at least 20 % of samples and are less likely to be contaminants.

1.1.5 Bacteria

One of the predominant members of the oral microbiome, with viruses, are bacteria. They also are the most implicated in oral health and disease. The healthy bacterial population in oral environment is difficult to define, as it greatly varies between sites and individuals and is often defined by the absence of certain species associated with disease, such as *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola* (Aas, Paster et al. 2005). A study analysing the microbiota of 10 healthy volunteers attempted to define a core oral microbiome, by including bacterial taxa found in all 10 individuals: *Streptococcus*, *Granulicatella* and *Veillonella* (*Firmicutes*); *Prevotella*, *Capnocytophaga* and *Bergeyella* (*Bacteroidetes*); *Rothia*, *Actinomyces*, *Corynebacterium* and *Atopobium* (*Actinobacteria*); *Neisseria*, *Cardiobacterium*, *Haemophilus* and *Campylobacter* (*Proteobacteria*); *Fusobacterium* (*Fusobacteria*) and TM7 (Bik, Long et al. 2010). The implications of bacteria in oral diseases are discussed in the next section.

1.2 Oral bacterial infections

The normal oral microbiota is necessary for oral health, primarily because it confers colonisation resistance, preventing exogenous pathogens from causing infections. But a disturbance of homeostasis by environmental factors can lead to a shift in bacterial populations and disease (Marsh 2003).

Dental-plaque associated diseases, which include gingivitis, periodontitis, caries and endodontic infections, are polymicrobial in nature and no single bacterial

species can be described as a causative agent. It is therefore not possible to apply Koch's postulates to these heterogeneous, polymicrobial infections. It might be possible to modify the postulates to include the community as the aetiological agent (Kolenbrander, Palmer et al. 2010). Similar observations have been made for other human polymicrobial infections such as bacterial vaginosis and the infected lung in cystic fibrosis (Nelson, De Soyza et al. 2012).

1.2.1 Periodontal disease

Plaque-induced irritation of the gingiva without loss of connective tissue attachment is referred to as gingivitis (Armitage 1995). This condition is reversible. It was experimentally induced in healthy volunteers abstaining from oral hygiene (Loe, Theilade et al. 1965) and the resumption of a normal oral routine led to the disappearance of symptoms. The inflammation increases the flow of gingival crevicular fluid, providing new nutrient sources, which accelerate bacterial growth in the crevice. If left untreated in susceptible subjects, gingivitis can progress to periodontitis, an irreversible condition characterised by loss of connective tissue attachment and resorption of alveolar bone due to inflammation. Periodontal disease is a polymicrobial inflammatory disorder, resulting from an interaction between a polymicrobial biofilm and the host immune system. Periodontitis is classified into two main categories, chronic and aggressive, primarily related to the speed of progress of the condition (Armitage 1999, Armitage 2010). Approximately 5 to 20 % of any population is affected by severe generalized periodontitis (Burt 2005) which may lead to an increased risk of systemic conditions, including cardiovascular disease, diabetes, respiratory diseases and adverse outcome in pregnancy (Azarpazhooh and Tenenbaum 2012). However not every gingivitis lesion

progresses to periodontitis, as some subjects can harbour an abundant plaque without periodontal destructive symptoms (Loe, Anerud et al. 1986). The tissue damage in periodontitis is partly due to bacterial activity, such as production of endotoxins, proteases, lipases, and sialidases (Smalley 1994), and partly to the host immune response, such as the production of reactive oxygen species and proteolytic enzymes by polymorphonuclear leukocytes (Van Dyke and Serhan 2003, Barnes, Teles et al. 2009).

Differences in periodontal susceptibility have been observed at different sites in the same subject, and the presence of certain bacterial species has been associated with it. Several studies have shown that some bacterial species were found in higher quantity and / or proportion in periodontitis than in health (Listgarten and Hellden 1978, Tanner, Haffer et al. 1979, Slots 1986, Dahlen, Wikstrom et al. 1996). Following several decades of culture and molecular studies, bacteria that are regarded as putative periodontal pathogens include *Porphyromonas gingivalis*, *Treponema denticola*, *Prevotella intermedia*, *Tannerella forsythia*, *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum*, *Capnocytophaga* species, and *Campylobacter rectus* (Paster, Olsen et al. 2006, Kesić, Milasin et al. 2008). Three of these species, *Treponema denticola*, *Tannerella forsythia* and *Porphyromonas gingivalis*, are members of the so-called red complex, associated with chronic periodontitis (Socransky, Haffajee et al. 1998). A more recent study using 454 pyrosequencing of two regions of 16S rRNA to compare bacterial populations in health and chronic periodontitis found several taxa strongly associated with periodontitis, including members of the red complex, but also

Filifactor alocis and *Lachnospiraceae* and *Synergistetes* species (Griffen, Beall et al. 2012). In addition, members of the uncultured TM7 Candidate Division, Human Oral Taxon (HOT) 356, have been associated with periodontitis (Brinig, Lepp et al. 2003, Liu, Faller et al. 2012).

1.2.2 Caries

Dental caries is the dissolution of enamel and dentine by acids produced by bacteria as a result of the fermentation of dietary carbohydrate. These acids can demineralise the enamel and cause tooth structure to be lost. As discussed above, no specific pathogenic agent can be identified, but the shift between health and disease implicates an increase of the proportion of acidogenic and acid-tolerating species such as mutans streptococci and lactobacilli. The Ecological Plaque Hypothesis (Marsh 2006) suggests that environmental changes are responsible of this shift. The frequent intake of fermentable sugars causes more frequent drops of the pH which favour the development of acid-tolerating bacteria (Bradshaw, McKee et al. 1989).

The implication of bacteria in the development of carious lesions has been demonstrated in germ-free animals (Orland, Blayney et al. 1954). *Streptococcus mutans* has been historically implicated in caries, as it was found in large numbers in carious lesions (Clarke 1924). It is more abundant in the saliva of subjects with active caries than of those with no, or treated, caries (Kohler, Pettersson et al. 1981). Furthermore, in primates, immunisation with a *S. mutans* surface antigen I/II was found to protect against the development of caries (Lehner, Challacombe et al. 1975, Lehner, Caldwell et al. 1985). Finally, it is highly acidogenic and acidophilic

(Loesche 1996). Other acidogenic and acidophilic bacteria, such as some other streptococci, *Actinomyces,* and *Bifidobacterium*, have been also associated with caries (van Houte, Lopman et al. 1996, Van Ruyven, Lingström et al. 2000, Beighton, Al-Haboubi et al. 2010) and may contribute to the demineralisation of the tooth. Modern culture-independent studies showed that caries-related microbiota is more diverse that previously thought, with around 30 species found in each lesion (Munson, Banerjee et al. 2004). In addition to the species traditionally thought to play a role in caries, other organisms were found to be associated with active sites including *Propionibacterium acidifaciens*, *Rothia dentocariosa*, and *Prevotella* species. Other studies have detected high proportions of *Atopobium* species (Aas, Griffen et al. 2008). This variety of taxa found in carious lesions can be explained from the ecological perspective, as clearly it is function that is of primary importance, and several different taxa may play similar functional roles (Marsh 2006, Peterson, Snesrud et al. 2011).

1.2.3 Endodontic infection

Endodontic infections are infections of the pulp, which can spread to the surrounding tissues and cause the loss of the tooth and, if untreated, resorption of the mandibular bones. The principal cause of endodontic infections is untreated carious lesions. The degradation of enamel by acids allows bacteria access to the dentine and, subsequently, to the pulp. Dentine is a porous tissue, composed largely of tubules of 1 to 4 µm of diameter, large enough to be colonized by bacteria (Bender, Seltzer et al. 1959, Love and Jenkinson 2002). As long as the pulp is viable, growth of bacteria is limited by the immune system, but some substances produced by bacteria, like endotoxin, can provoke an inflammation of the pulp,

leading to its necrosis (Nissan, Segal et al. 1995). Necrotic tissue provides nutrients for further bacterial growth and the loss of blood supply, hindering the movement of leukocytes to the infection site, makes the root canal a favourable place for bacterial proliferation.

During the 1950s and 1960s, microbiological studies of endodontic infections used microscopic observation, aerobic culture and biochemical assays to detect and identify the associated microbiota (Miller 1890, Brown Jr and Rudolph Jr 1957, Hampp 1957). Members of the genus *Streptococcus* were considered to be dominant in endodontic infections, with species such as *S. mitis*, *S. salivarius*, *S. sanguis* and *S. mutans* the most frequently isolated (Morse 1974). In the next decade, with the adoption of anaerobic culture methods, it was established that the majority of endodontic bacteria were anaerobic (Berg and Nord 1973, Bergenholtz 1974). In a comprehensive study using the most scrupulous contemporaneous techniques, and choosing only teeth with intact crowns to prevent any contamination by oral bacteria, Sundqvist (1976) found anaerobes in 90 % of lesions. The bacteria most frequently found in this study were pigmented *Prevotella* (previously named *Bacteroides melaninogenicus* (Shah and Collins 1990)), *Fusobacterium*, *Campylobacter*, *Selenomonas*, *Veillonella* and *Peptostreptococcus* species. These cultivation studies, however, suffered from the limitation of not being able to detect non-cultivable organisms. The 1990s saw the development of new molecular methods based on 16S rRNA gene amplification, cloning and sequencing, which allowed the identification of bacteria without culture (Conrads, Gharbia et al. 1997, Munson, Pitt-Ford et al. 2002).

Endodontic infections can be classified as chronic, or asymptomatic, and acute, symptomatic, infections. While various studies have found associations between particular species and different types of endodontic infection, no consensus seems to have been reached, which may be due to the great variation in the composition of the microbiota in endodontic infections in different individuals. Saito *et al*. (2006) explored the bacterial diversity of asymptomatic endodontic infections from seven patients by 16S rRNA cloning and Sanger sequencing and reported that 71.7 % of taxa were found only in a single subject. This study was limited, however, by the analysis of relatively few sequences per sample and the use of an unmodified 27F primer (see section 1.3.2). Indeed, a total of 46 phylotypes were detected, from two to fourteen per subject, while most studies usually report a greater diversity. Thus, using similar methods, Munson *et al*. (2002) reported the detection of 65 taxa for five patients, ranging from 7 to 29 per sample.

Although the mouth appears to be the source of the bacteria causing endodontic infections, only a small proportion of the oral microbiota has been detected in the infected pulp. Using the classical Sanger sequencing technique, 14 phyla were identified in the oral cavity (Paster, Boches et al. 2001, Paster, Falkler Jr et al. 2002, de Lillo, Ashley et al. 2006), but only 10 of them were found to be present in endodontic infections: *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Fusobacteria* and *Proteobacteria* (Munson, Pitt-Ford et al. 2002), *Spirochaetes* (Baumgartner, Khemaleelakul et al. 2003), TM7 (Siqueira and Rocas 2005), SR1 (Rocas and Siqueira 2008), *Synergistetes* (Munson, Pitt-Ford et al. 2002), mis-classified in *Firmicutes* or *Deferribacteres* phyla (Vartoukian, Palmer et al. 2007),

and *Tenericutes* (Serene and Anderson 1967), misclassified in *Firmicutes* (De Vos 2009). Gram positive anaerobic bacteria predominate and most taxa belong to the *Firmicutes* phylum. The most frequently detected genera and species are *Streptococcus* species, *Fusobacterium nucleatum, Parvimonas micra, Porphyromonas endodontalis, Olsenella uli, Prevotella baroniae, Treponema denticola* and *Dialister* species (Siqueira and Rocas 2009, Zhang, Hou et al. 2012). The use of next generation sequencing techniques have allowed a deeper coverage of the endodontic microbiota, with more phyla detected. In a recent study analysing the coronal and apical microbiota of 23 teeth with endodontic infection by 454 pyrosequencing, 1 archaeal (*Euryarchaeota*) and 24 bacterial phyla or candidate phyla were detected: *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, BRC1, *Chlamydiae*, *Cyanobacteria*, *Deinococcus-Thermus*, *Firmicutes*, *Fusobacteria*, *Nitrospira*, OD1, OP11, OP3, OP9, *Planctomycetes*, *Proteobacteria*, *Spirochaetes*, *Synergistetes*, *Tenericutes*, TG1, *Thermomicrobia*, TM7, *Verrucomicrobia* and WS3 (Ozok, Persoon et al. 2012). As in previous studies, *Firmicutes* was found to be the dominant phylum. Conversely, another study, using a combination of cloning / Sanger sequencing and 454 pyrosequencing, found *Bacteroidetes* to be the dominant phylum in 6 of the 7 endodontic infections analysed, by both methods (Li, Hsiao et al. 2010). While the authors did not offer an explanation for this divergence from previous studies, it may be explained by bias in collection of samples in that paper points were used, which have been shown to preferentially collect superficial organisms (Sathorn, Parashos et al. 2007), and the DNA extraction method used, which was the QIAamp DNA mini kit (Qiagen), shown to perform

poorly in extracting DNA from Gram-positive bacteria in mixed samples (Nadkarni, Martin et al. 2009).

As with other polymicrobial infections, no single specific pathogen has been identified in endodontic infections. Numerical dominance is sometimes assumed to equate to pathogenicity but this may not be a valid assumption. The microbial compositions of oral, root canal and periapical abscess samples were compared using 454 pyrosequencing and it was found that the dominant species were usually found in all three sites, and were not specific to the diseased sites (Hsiao, Li et al. 2012). By ANOVA tests it was found that the oral taxonomic units (OTUs) associated with disease in that study were relatively rare, belonging to the genera *Bacteroides, Granulicatella, Collinsella, Atopobium, Dialister, Diaphorobacter, Lachnospiraceae incertae sedis*, *Moryella, Prevotella, Streptococcus*, *Veillonella*, unclassified *Bacteroidales* and unclassified *Clostridiales*, based on mothur assignments by alignment with SILVA reference database (Schloss, Westcott et al. 2009).

One of the species often thought to be associated with endodontic infections is *Dialister invisus.* It was found with a high prevalence in both chronic and acute endodontic infections (Munson, Pitt-Ford et al. 2002, Rocas and Siqueira 2005). It is possible that *D. invisus* plays a particular role in tubule invasion, due to its relatively small dimensions (0.3–0.4x0.3–0.6µm (Downes, Munson et al. 2003)). *Enterococcus faecalis* appears to be specifically associated with endodontic treatment failure (Sundqvist, Figdor et al. 1998, Hancock, Sigurdsson et al. 2001, Pinheiro, Gomes et al. 2003). Its ability to resist nutritional deprivation and invade dentinal tubules

seems to allow *E. faecalis* to survive endodontic treatment and cause a persistent infection (Stuart, Schwartz et al. 2006).

Finally, an analysis of the viral populations of HPV and herpesviruses types 1 to 8 in endodontic infections found the most frequently detected viruses to be human herpes viruses HHV-8 and HHV-6, human papilloma virus, varicella zoster virus, and Epstein–Barr virus (Ferreira, Rocas et al. 2011). While two thirds of root canals contained viral sequences, it is unclear if they play an active role in the infection or are just a consequence of bacterially induced disease. If the viruses are active they may cause the release of tissue-destructive cytokines, and the resulting impairment of the immune response may favour the development of bacteria, as suggested for periapical infections (Slots, Sabeti et al. 2003).

1.3 Characterisation methods

1.3.1 Culture

After the development of solid culture media, the description of the morphological appearance of colonies, Gram staining and use of differential media and biochemical tests for phenotypic analyses were the main ways used to classify and identify bacteria. These methods require pure cultures of the test bacteria to be available, which is not always possible, as a large proportion of bacteria elude culture (Amann, Ludwig et al. 1995). Furthermore, such techniques do not allow for reliable population analyses as the conditions used in the laboratory will inevitably differ from the natural environment and will lead to changes in the community

structure. Cultural analysis cannot therefore accurately represent complex bacterial communities (Ranjard, Poly et al. 2000).

1.3.2 Culture-independent methods

The twin developments of the invention of the Polymerase Chain Reaction (PCR) (Mullis, Faloona et al. 1986) and the recognition of the value of the 16S rRNA gene as a bacterial phylogenetic marker (Woese 1987), led to the development of methods for the culture-independent characterisation of complex bacterial communities.

The 16S rRNA gene, often described as an "evolutionary clock", is a housekeeping gene. It includes regions that are highly conserved and others which are essentially species-specific, although there are some closely related bacterial taxa, classified as different species by whole genome DNA-DNA hybridisation, which cannot be distinguished by 16S rRNA gene sequence (Fox, Wisotzkey et al. 1992, Hanage, Fraser et al. 2005).

The PCR allows the amplification of targeted DNA segments *in vitro* using thermostable DNA polymerases. It consists of repeated cycles of denaturation of double stranded DNA, annealing of the short oligonucleotide primers and the synthesis of the complementary strand by the polymerase. For bacterial population analysis, the primers need to be specific for the amplification of the 16S rRNA gene but cover all the diversity of these genes in bacteria. The most frequently used primer pair for this purpose was, for many years, 27F and 1492R (Lane 1991). The forward primer 27F was however shown to not be truly universal (Marchesi, Sato et al. 1998), and was modified to include two degenerate nucleotide positions: 5´-

AGAGTTTGATCCTGGCTCAG-3´ to 5´-AGAGTTTGATYMTGGCTCAG-3´ (27F-YM) which were shown to improve coverage (Frank, Reich et al. 2008).

1.3.2.1 PCR/Cloning/Sequencing

The "classical" molecular method for analysis of bacterial composition in various habitats has been the combination of cloning of PCR amplified 16S rRNA genes and subsequent sequencing using the Sanger method. This is based on the introduction of dideoxynucleotides (ddNTPs) which terminate the DNA elongation process when incorporated. All four ddNTPs are labelled with a different fluorescent dye. The resulting products are run through a capillary and separated by size. A laser is then used to identify which ddNTP is incorporated in each position, resulting in a chromatogram, from which the DNA sequence can be deduced.

This technique allows high fidelity sequencing and the full length of the 16S rRNA gene can be sequenced from the amplicons. The major limitation of the method is the need for clone library construction, which is time-consuming and limits the number of sequences which can be analysed per sample to levels that provide only relatively shallow coverage (Sogin, Morrison et al. 2006, Pedros-Alio 2012).

1.3.2.2 High-throughput sequencing

The recently developed next generation sequencing techniques do not require the cloning step, as they use alternative approaches for the separation of 16S rRNA amplicons. The Illumina platform uses the sequencing by synthesis method (Mardis 2008). DNA fragments are attached to a slide and amplified *in situ*.

Reversible-terminator bases labelled with a fluorescent dye are incorporated into the growing DNA strands. As each dNTP is added, fluorescently-labelled terminator is imaged and then cleaved, to allow incorporation of the next base. The presence of all four reversible terminator-bound dNTPs minimises incorporation bias by competition. This method allows for massively parallel sequencing, but the main drawback is the short length of the sequences obtained. Because of this, only identification to genus level is possible for bacteria, and this level of precision is inadequate for the analysis of the oral microbiota, even if such studies were undertaken (Lazarevic, Whiteson et al. 2009). This is because many oral bacterial genera include species with vastly different metabolisms and functions.

Another commonly used technique is pyrosequencing using the 454 range of sequencing instruments (Roche). Samples are initially amplified by PCR and then individual amplicons are separated and further amplified by means of an emulsion PCR, with each reaction performed in an oil / water emulsion sphere. After the PCR, the micelles are broken, each bead is deposited in a well of a picotiter plate and beads containing the sequencing reagents are added. During the pyrosequencing reaction, the incorporation of a nucleotide is accompanied by a release of pyrophosphate in equimolar amounts to that of the incorporated nucleotide, ultimately resulting in the generation of visible light in a downstream chemiluminescent reaction. A charge coupled device records a peak in the raw data output. Following each insertion reaction, an enzyme degrades any remaining unincorporated nucleotides before a new nucleotide is added. The intensity of the light signal, and therefore the height of each recorded peak, is proportional to the

number of incorporated nucleotides, from which the DNA sequence can be deduced (Mardis 2008). In the first studies using this technique to characterise the bacterial communities in saliva and plaque, estimates of the total species richness were orders of magnitude higher than had been previously described (Keijser, Zaura et al. 2008). It was later demonstrated that this increased species richness was due to the erroneous base-calling of homopolymers. The problem was addressed with a denoising algorithm, PyroNoise (Quince, Lanzen et al. 2009). Pyrosequencing currently offers reads of up to 750 bp, sufficient for species-level identification in most cases. Some bacterial groups, such as the mitis / oralis streptococci, elude precise identification, but for these lineages even full length 16S rRNA sequences may be insufficient. Pyrosequencing therefore enables the production of large number of sequences from mixed bacterial communities with sufficient read length for species-identification. It avoids the biases associated with cloning, but will remain subject to PCR-based artefacts.

Finally, the Ion Torrent Personal Genome Machine Platform, while representing a smaller part of the next generation sequencing market, has also been used for microbial population analysis. However, it presents a problem similar to the Illumina platform as the read length is currently restricted to 200 bp (Whiteley, Jenkins et al. 2012). On this platform, sequence composition is determined by measuring pH changes due to hydrogen ion liberation as nucleotides are incorporated during strand synthesis in picolitre wells (Rothberg, Hinz et al. 2011).

1.4 Uncultured bacteria

Following the widespread use of culture-independent methods for the characterisation of complex bacterial communities and the subsequent introduction of high-throughput next generation DNA sequencing techniques, large numbers of 16S rRNA gene sequences in the DNA sequence databases do not correspond to validly described taxa. Those groups of sequences that represent taxa at the phylum level but which do not include cultivated representatives are referred to as Candidate Divisions. When a cultivated member of the Division is isolated in pure culture and validly described, a new phylum can be created (Dunfield, Tamas et al. 2012).

Currently there are 30 phyla listed in the List of Prokaryotic names with Standing in Nomenclature (LPSN) (http://www.bacterio.cict.fr) : *Acidobacteria*, *Actinobacteria*, *Aquificae*, *Armatimonadetes* (formerly Candidate Division OP10), *Bacteroidetes, Caldiserica* (formerly Candidate Division OP5), *Chlamydiae, Chlorobi, Chloroflexi, Chrysiogenetes, Cyanobacteria, Deferribacteres, Deinococcus-Thermus, Dictyoglomi, Elusimicrobia* (formerly Candidate Division Termite Group 1), *Fibrobacteres, Firmicutes, Fusobacteria, Gemmatimonadetes, Lentisphaerae* (formerly clade VadinBE97), *Nitrospira, Planctomycetes, Proteobacteria, Spirochaetes, Synergistetes, Tenericutes* (formerly class *Mollicutes* in phylum *Firmicutes* (Krieg, Ludwig et al. 2010)), *Thermodesulfobacteria, Thermomicrobia*, *Thermotogae* and *Verrucomicrobia*.

The SILVA database (Quast, Pruesse et al. 2013), a curated database of 16S rRNA prokaryotic sequences, lists 59 phyla, the 29 phyla mentioned above except

Thermomicrobia plus 30 Candidate Divisions: BD1-5, BHI80-139, BRC1, CK-1C4-19, GAL08, GOUTA4, HDB-SIOH1705, Hyd24-12, JL-ETNP-Z39, Kazan-3B-28, KB1, LD1-PA38, MVP-21, NPL-UPA2, OC31, OD1, OP3, OP9, OP11, RF3, RsaHF231, S2R-29, SM2F11, SR1, TA06, TM6, TM7, WCHB1-60, WS3 and WS6, while the Ribosomal Database Project 10 (update 29, 11.06.2012) lists 29 phyla and 6 Candidate Divisions (BRC1, OD1, OP11, SR1, TM7, WS3).

1.4.1 Oral Bacteria

The Human Oral Microbiome Database (http://www.homd.org, (Chen, Yu et al. 2010)) is a curated list of bacteria taxa detected in the human oral cavity. It currently includes 687 taxa at species level in 13 phyla: *Actinobacteria, Bacteroidetes, Chlamydiae, Chloroflexi, Firmicutes, Fusobacteria*, GN02, *Proteobacteria, Spirochaetes*, SR1, *Synergistetes, Tenericutes* and TM7. Un-named taxa at species level have been allocated Human Oral Taxon (HOT) numbers. There are 221 taxa without cultured representatives, that fall into three groups: rare taxa with close culturable relatives, taxa forming deep branches with no culturable representatives and taxa belonging to Candidate Divisions, which are, as discussed above, phylum level groups with no culturable representatives.

1.4.1.1 Rarely encountered taxa, closely related to known and culturable species

Some taxa may not have been cultivated not because of any intrinsic unculturability, but simply as a result of their rarity. The total number of oral bacterial isolates that have been grown and identified remains low, compared to the number of 16S rRNA genes sequenced, particularly since the advent of next generation sequencing. If novel taxa are closely related to well known and easily

cultivated taxa, they are unlikely to have been targeted by a directed isolation attempt. This could be the case for the four uncultured *Streptococcus* species (HOT055, 067, 069 and 487), seen as 1 or 2 clones out of the total of 34879, for instance, or *Capnocytophaga* sp. HOT334 (1 clone) (Chen, Yu et al. 2010, Dewhirst, Chen et al. 2010). Alternatively, these sequences may be artifacts, such as chimeras.

1.4.1.2 Deep uncultured branches within cultured phyla

The uncultured taxa belonging to well established phyla may cluster in deep branches, well represented in culture-independent analyses but not isolated in pure culture. A striking example of this is a deep branch formed by *Bacteroidetes* genera G-3, G-4 and G-5, including 9 species, all uncultured. *Bacteroidetes* [G-3] HOT281 is the most commonly detected taxon within this branch, with 54 clones. Other deep uncultured branches within *Bacteroidetes* include *Bacteroidetes* [G-6], *Flavobacteriales* [G-1] and [G-2], *Bergeyella* sp., *Tannerella* sp. HOT286 and 808 and *Prevotella* sp. HOT292, 293, 300 and 305.

Similar branches are found within the *Firmicutes* phylum, the *Clostridiales* F-1 family, made up of two genera, G-1 and G-2, each represented in the oral environment by only one species, HOT093 and 402, respectively, and the *Clostridiales* F-2 family (3 genera, 4 species), which forms another branch together with *Syntrophomonadaceae* [G-1] HOT435, as well as several smaller branches within the families *Peptostreptococcaceae*, *Lachnospiraceae* and *Veillonellaceae*.

The phylum *Actinobacteria* includes a branch formed by the *Actinomyces* HOT414, 449 and 525. *Actinobacteria* is one of the phyla with the lowest proportion of not-yet-cultured taxa (50.4 %), after *Chlamydiae* (0 %) and *Tenericutes* (25 %),

which can probably be explained by the underrepresentation of *Actinobacteria* sequences in all molecular studies (Krogius-Kurikka, Kassinen et al. 2009).

The *Tenericutes* phylum includes one deeply branching uncultured genus, *Mollicutes* [G-1], with only one species, HOT504.

The *Fusobacteria* phylum includes one uncultured branch consisting of HOT210 and 220, *Fusobacteria* [G-1], and one another of *Leptotrichia* sp. HOT212, 215, 217 and 392. Most *Leptotrichia* species are still to be cultured, but other branches contain cultivated members.

The only taxon representing a deep uncultured branch within the phylum *Proteobacteria* is the γ-proteobacterium *Bdellovibrio* sp. HOT039. *Bdellovibrio* are normally aerobic, predatory bacteria, feeding on other Gram-negative bacteria (Dashiff and Kadouri 2011).

The *Spirochaetes* and *Synergistetes* phyla contain a large proportion of not-yet-cultivated species. *Treponema* is the only genus representing the *Spirochaetes* phylum in the oral cavity. Over the 49 species, only 14 are cultured, but the biggest uncultured branch is formed of 10 HOT (HOT250-256, 508, 517 and 518). Oral *Synergistetes* are comprised of 2 clusters, A and B. Until recently, cluster A had no cultivated representatives, but *Fretibacterium fastidiosum* has recently been successfully cultivated using a co-culture technique (Vartoukian, Palmer et al. 2010, Vartoukian, Downes et al. 2012); the remaining 7 species still form an uncultured branch (Chen, Yu et al. 2010, Dewhirst, Chen et al. 2010).

1.4.1.3 Phyla with no cultured representatives

Three phyla with no cultivated representatives (Candidate Divisions) are listed in the HOMD: GN02, TM7 and SR1. The phylum *Chloroflexi* has no cultivated representatives among the oral microbiota, but environmental relatives have been cultivated (Yamada and Sekiguchi 2009).

The Candidate Division GN02, proposed in a study of the Guerrero Negro hypersaline microbial mat (Ley, Harris et al. 2006), is represented in the oral cavity by three taxa, HOT871, 872 and 873, placed in two classes. Four other taxa were identified in the canine oral microbiome (Dewhirst, Klein et al. 2012). This Candidate Division does not appear to have been the target of any directed studies, thus nothing is known about its morphology or metabolism.

Candidate Division SR1 sequences were first detected in the sediments from Sulphur River in Parker's Cave, and were originally classified in the Candidate Division OP11 (Harris, Kelley et al. 2004). They were predominantly detected in anaerobic habitats such as deep-sea sediments, various extreme environmental sites, the cow rumen and human oral cavity, but have yet to be detected in soil (Davis, Youssef et al. 2009). SR1 Candidate Division is phylogenetically separated in two subphylum level lineages, BH1 and BD2-14, with the former found only in geothermal habitats, while the latter is more widespread. The two lineages are not always monophyletic in trees which include sequences from related Candidate Divisions such as OP11 or OD1, suggesting that they may be in fact two independent phyla. The BD22-14 lineage is made up of 9 subgroups, with all the sequences from mammals (human oral cavity and oesophagus, cow rumen,

rhinoceros faeces) and termite samples falling into subgroup III. This subgroup does not include any environmental sequences. Two distinct morphotypes were identified using FISH in environmental samples. One represented filaments of variable length (2.7 to 137.5 μm) but constant width (0.7 to 0.8 μm), sometimes appearing segmented (Davis, Youssef et al. 2009). This morphotype is strikingly similar to the predominant environmental cell type of the TM7 Candidate Division bacteria (Hugenholtz, Tyson et al. 2001). The second morphotype consisted of bacilli with round ends (2.7 to 5.5 μm in length, 1.8 μm in width), mostly present as single cells but sometimes forming chains. More specific probes showed that the filamentous morphotype represents subgroup V, while rods belong to subgroup I (Davis, Youssef et al. 2009). No similar study was conducted on animal-associated SR1, and thus nothing is known about the morphology of subgroup III. It has been suggested that members of the SR1 division may be implicated in sulphur cycling in the environment (Harris, Kelley et al. 2004), but no metabolic study targeting SR1 members has been conducted. Three species belonging to the same genus of SR1 have been detected in the oral cavity (HOT345, 874 and 875). SR1 bacteria are a rare member of the oral microbiome, present in the clone pool at 1/5,000 only, which would render studies targeting these bacteria in the oral environment extremely challenging. Three species-level taxa of SR1 were also identified in the canine oral microbiome, but they were only seen when a specific *Bacteroidetes*-TM7-SR1 primer was used, further evidence that this phylum makes up only a small proportion of the mammalian oral bacterial community (Dewhirst, Klein et al. 2012).

Finally, the TM7 Candidate Division, which will be discussed in more detail in Section 1.5 of this Introduction, is represented in the HOMD by 12 species, falling within 5 genera and 2 families.

1.4.2 Reasons for unculturability

Some bacteria may have no cultivated representatives not because of any intrinsic unculturability, but simply as a result of their rarity and slow growth, which means that they may be overlooked in cultural studies. Other bacteria, on the other hand, present a genuine resistance to *in vitro* culture. One reason for this unculturability can be the lack of substances essential for the growth of these bacteria in the media used to attempt their isolation or unsuitable atmosphere compositions. Better understanding of the environmental conditions can give clues on how to improve these parameters. Thus high scale culture with 350 isolates analysed per sample and the use of simple solid media with limited nutrients, plus an extended incubation period of 3 months, allowed the isolation of 93 new strains of soil oligotrophs, from 20 different not-yet-cultured families (Joseph, Hugenholtz et al. 2003).

While high-volume culture methods such as those described above have enabled the culture of previously uncultured organisms, more needs to be done to mimic the natural conditions in which bacteria grow. Bacteria living in a biofilm, which represents the vast majority of the bacterial ecosystems on Earth, do not all live in the same environment. Indeed, each microcolony, and even each bacterial cell within the biofilm, is surrounded by very specific concentrations of nutrients, signalling molecules and gases, due to the diffusion patterns of these substances

and the metabolic activity of neighbouring bacteria (Kolenbrander 2000, Stewart and Franklin 2008).

Oxygen gradient is the most studied example of these micro-conditions and its concentration *in situ* can be measured with micro electrodes (Ramsing, Kuhl et al. 1993, de Beer, Stoodley et al. 1994). The micro patterns of oxygen concentration, with anaerobic zones, explain the possible growth of strictly anaerobic microorganisms in biofilms in contact with oxygenated environments. The existence of these zones cannot be explained by physical incapacity of oxygen to diffuse through the biofilm, as it should diffuse freely at 60 % of its diffusion rate in water (Stewart 2003). Instead, oxygen concentration is actively reduced by respiring aerobic bacteria, to the point where it is completely depleted at 175 µm of a 220-µm-thick biofilm (Zhang, Fu et al. 1995). In the oral environment, strictly anaerobic bacteria actively coexist with oxygen-consuming aerobes and more oxygen-tolerant facultative anaerobes (Kolenbrander 2000).

Similarly, the concentration of all nutrients consumed by the bacteria will decrease with depth of the biofilm, while metabolic products of bacteria diffuse in an inverse gradient. For most of the substances there is a balance between production and consumption, creating as many microenvironments as there are bacterial cells in the biofilm. In this context, not-yet-cultured bacterial taxa may be in contact with an essential substance provided either by host or other bacteria even if this substance is not detected in the biofilm supernatant. For instance, *Veillonella* species depend mostly on lactate, as a carbon source, produced by streptococci from carbohydrate fermentation (Marsh 2005).

Another important factor of biofilm growth is communication between bacteria, both intra- and inter-species. While Gram-positive bacteria communicate primarily with small diffusible peptides (Sturme, Kleerebezem et al. 2002) and Gram-negative bacteria use acyl homoserine lactones (AHLs) (Whitehead, Barnard et al. 2001), the autoinducer-2 (AI-2), the product of the LuxS gene, appears to play a role in inter-species communication, as its homologues are expressed by many bacterial taxa, leading to it being described as a "Bacterial Esperanto" (Bassler and Losick 2006). This communication, of which quorum sensing is one example, regulates all functions specific to growth in the biofilm, including competence, adhesion, virulence factors secretion and sporulation. It also influences the metabolic state of bacteria, promoting growth and division or dormancy, as in persister cells. For example, bacterial cytokines, such as Rpf and Sps, are required for the resuscitation of *Micrococcus luteus* from dormancy (Mukamolova, Yanopolskaya et al. 1998, Mukamolova, Turapov et al. 2002, Mukamolova, Turapov et al. 2002, Shleeva, Bagramyan et al. 2002). Other growth promoting factors are required for the growth of many other bacteria (Davies, Parsek et al. 1998, Kell and Young 2000, De Kievit, Gillis et al. 2001).

Most of the factors are soluble, but others can be hydrophobic, such as the quinolone signal (2-heptyl-3-hydroxy-4-quinolone) of *Pseudomonas aeruginosa*. To be able to travel to target cells, such molecules are packaged in extracellular membrane vesicles (Bassler and Losick 2006). Furthermore, a contact communication mechanism allowing the passage of small cytoplasmic molecules, proteins, and non-conjugative plasmids, via nanotubes has been described (Dubey

and Ben-Yehuda 2011). It seems to act even between different species, for instance between *Bacillus subtilis* and *Staphylococcus aureus*, and between *B. subtilis* and *Escherichia coli*.

1.4.3 Recent culture techniques

Although the use of culture-independent molecular methods has greatly expanded our knowledge of the diversity of the bacterial world, pure culture remains essential for the description of the metabolic, physiological and pathological properties of individual species. Efforts are currently being directed at closing the gap between the number of bacterial taxa known by molecular analysis and those available in culture, notably by the Human Microbiome Project (HMP, http://www.hmpdacc.org, (Peterson, Garges et al. 2009)), as part of its initiative for Microbial Reference Genomes.

A number of strategies have been used to improve the range of bacteria that can be cultured, with the greatest success coming from combining one or more approaches.

1.4.3.1 Provision of appropriate conditions for bacterial growth

Anaerobic bacteria dominate environmental and animal-associated microbiota. Although anaerobic cultivation methods were developed in the 19th century (Kursteiner 1907), anaerobic bacteria were not routinely isolated and studied until the second half of the 20th century. A major advance in anaerobic microbial culture was made by the roll tube method (Hungate 1950). The inoculated agar medium in a culture tube is rotated in flowing cold water to solidify the medium on the inside surfaces of the tube, creating a space within the tube

which can be filled with a mix of hydrogen and carbon dioxide. Also important was the realisation that rich culture media, typically used to grow pathogenic bacteria, are often not suitable for the isolation of bacteria from the environment. The use of simpler, low-nutrient, media greatly improved the recovery of isolates from environments such as sea, fresh water and soil (Connon and Giovannoni 2002).

Nucleic acid sequence data, obtained from metagenomic or single-cell studies, can be used to help understand the requirements of not-yet-cultured bacteria. For example, high-throughput sequencing of RNA transcripts (RNA-seq) was used to investigate the metabolism of members of the gut microbiota of the leech *Hirudo verbana* and to use that information to design a medium for their culture and isolation (Bomar, Maltz et al. 2011). The most abundant symbiont, an uncultured *Rikenella*-like bacterium, did not grow in a medium commonly used for *Rikenella microfusus* isolation. RNA-seq data predicted that it was using sulfated- and sialated-mucin glycans, and a modified medium, containing mucin instead of glucose as the main carbon source, enabled its isolation.

When it is not known which substances are required for the growth of a particular organism, a filtered supernatant from the original environment can be added. A study successfully cultivating 27 new bacterial genera from the rumen used a filtered rumen supernatant as an additive to an adapted medium, with modified salt content and low levels of energy sources (Kenters, Henderson et al. 2011). Of 1000 tubes inoculated with 0.05 to 0.5 viable cells, growth was seen in 139. Of these, 93 survived subculture and 54 appeared to be a pure culture. Together with 6 isolates from a pilot study, their 16S rRNA genes were sequenced

and they were placed in 23 genus-level groups (at 93 % of sequence identity). *Firmicutes* made up 87 % of the isolates, with the *Clostridiales* the most represented. Of these 60 isolates, 27 belonged to 14 novel candidate genus-level groups, and 3 candidate genera had no sequence identity matches over 93 % with the GenBank database.

The aim of other studies has been to mimic the environmental habitat in the laboratory. A diffusion chamber developed by Kaeberlein *et al*. (2002) enclosed bacteria in a membrane, impermeable for whole cells but allowing the diffusion of nutrients and quorum sensing molecules from the environment, which was sea water in this case. The recovery rate, compared with microscopic counts, was improved from 0.05 % on traditional Petri plates to up to 40 % in the diffusion chamber. Another study from the same group focused isolation efforts on a novel *Psychrobacter*, designated MSC33, which had been grown in diffusion chambers but not on standard solid media (Nichols, Lewis et al. 2008). It was isolated to pure culture by passages in diffusion chambers and then paired with several cultivable isolates from the same environment, to test their ability to support the growth of MSC33 by co-culture. One of the isolates, closely related to *Cellulophaga lytica*, displayed a strong "helper" activity. After several passages in co-culture, a domesticated variant of MSC33, MSC33c, was obtained. It grew well on standard media, formed macro-colonies and could act as a helper for MSC33 growth. To determine the molecular nature of the substance providing the stimulation, fractioned MSC33c supernatant was added to growth media. Two fractions stimulated the growth of MCS33, but one in particular, which gave a single active

peak in HPLC analysis, was identified. After supplemental analysis, a 5-mer peptide LQPEV was demonstrated to be the strongest inducer of MSC33 growth, at the active concentration of 3.5 nM. This peptide is most probably mimicking a growth-inducing signal, and the independent growth of the MSC33c variant could be due to the production of an increased amount of this factor. Another 23 strains from several previous studies were tested for the appearance of domesticated variants and 74 % were positive after just one to four passages, providing an encouraging perspective for the culture of at least a subset of not-yet-cultivated bacteria.

Other studies have shown that the use of a helper strain can support the growth of a not-yet-cultured bacterium before its isolation and allow the identification of the factor promoting its growth. For example D'Onofrio *et al.* (2010) grew sea water sediment bacteria on agar plates in a mixed culture at different dilutions. As disproportionally more bacteria grew on the more heavily inoculated plates, they attempted the isolation not of single colonies, but of random pairs of colonies. Ten percent of plates inoculated in this way showed a pattern of growth induction and dependence of one bacterium on another. Several of these dependent bacteria were also stimulated by *Escherichia coli*, and so a panel of *E. coli* mutants were tested in an attempt to find the origin of the helper activity. It was discovered that the growth stimulation was due to enterobactin, a siderophore. Similar stimulation was obtained with free iron, but the concentration required (40 µM) exceeded the typical concentration of iron in sea water by a million-fold. Thirteen further isolates were obtained by supplementing the initial growth medium with high levels of free iron, three of which represented putative

new genera. As the energetic cost to maintain siderophore synthesis seems relatively low, authors speculate that not being able to autonomously produce siderophores is a strategy used by some bacteria to initiate growth only in suitable conditions, using it as a communication factor.

Another successful technique, using membranes in direct contact with soil slurry for the culture of soil bacteria, reported the culture of several not-yet-cultivated species and a micro-colony belonging to the TM7 Candidate Division (Ferrari, Binnerup et al. 2005). Unfortunately, the TM7 colony was not a pure culture and could not be passaged further, and was lost.

1.4.3.2 Reduction of complexity of the mixed population

One of the early techniques used to target not-yet-cultured bacteria was dilution-to-extinction (Button, Schut et al. 1993). The method has been successfully used to culture an oligotrophic ultramicrobacterium, *Sphingomonas* sp. strain RB2256 (Schut, Gottschal et al. 1997). The method typically selects for organisms that are predominant in samples, and is not useful for the isolation of cells present in low numbers. Conversely, fluorescence activated cell sorting (FACS) has allowed the recovery of viable target cells, at 70 % purity, originally present at a level of less than 1 % in an environmental sample (Porter, Edwards et al. 1993). For this application, specific antibodies and fluorescently labelled secondary antibodies were used, as they allowed for the recovery of viable cells. The development of the specific antibodies would be a limiting step if this technique had to be applied to uncultured species. This step can be circumvented if targeted bacteria present a physiological peculiarity which can be used for cell sorting. For example, flow

cytometery was successfully used to isolate three species of low nucleic acid content planktonic bacteria belonging to the *Polynucleobacter* group, by staining stream water cells with the non-specific nucleic acid binding dye SYBR green and selecting cells with low nucleic content (Wang, Hammes et al. 2009).

Another technique useful in the isolation of pure cultures from mixtures consists of trapping them in semi-permeable gel microspheres (Manome, Zhang et al. 2001). Zengler *et al.* (2002) used gel microdroplets to encapsulate cells from sea water and soil and incubated them in their natural environments or supplemented media. One of the principal advantages of this technique is its extremely high throughput. Where most "high-scale" techniques use 96-well plates, this study screened 10^7 gel microdroplets, and the technique can be upscaled even further. Secondly, in contrast to dilution to extinction techniques, this method does not prevent existing bacterial interactions, as all the capsules are incubated together and the size of pores allows the circulation of macromolecules. The capsules exhibiting the formation of a microcolony, detected by microscopy, were then sorted by FACS. Several of the isolated microcolonies were identified as belonging to not-yet-cultured taxa, related to the *Planctomycetes*, *Cytophaga–Flavobacterium–Bacteroides* (*Bacteroidetes*), and the α-*Proteobacteria*.

1.4.3.3 Isolation of targeted organisms

The availability of DNA sequence data for as yet uncultivated bacteria makes it possible to design specific oligonucleotide probes which can be used in conjunction with fluorescent *in situ* hybridisation (FISH) to detect and visualise uncultured bacteria in mixed communities (Amann, Stromley et al. 1992, Amann,

Ludwig et al. 1995). FACS can further allow the separation of bacteria of interest, but does not lead to their culture, as it requires the fixation of bacteria, which is lethal. It may nevertheless be used to obtain pure DNA for genome sequencing, which may lead to useful information regarding metabolic requirements (Yilmaz and Singh 2011). As described above, fluorescent antibodies can be used to obtain viable cells after sorting, if it is possible to create an antibody with the required specificity (Porter, Edwards et al. 1993).

Single-cell manipulation techniques such as optical tweezers and laser microdissection (Frohlich and Konig 2000), have also been used to isolate bacteria, such as wood-degrading bacteria belonging to the *Cytophaga-Flavobacterium-Bacteroides* (CFB) complex from a mixed community (Nilsson, Björdal et al. 2008). The method can allow the separation of targeted live cells when these can be discriminated by their morphology, as was done for the hyperthermophilic archaea, where their "grape-like" morphology was observed using FISH (Huber, Burggraf et al. 1995).

The Raman technology is a non-destructive and non-invasive way to analyse single cells and can be used to identify bacteria, with the Raman spectrum serving as a "fingerprint" (Huang, Ward et al. 2009). Cells remain alive after undergoing the Raman-directed sorting with a laser trap. Eleven out of 18 yeast cells (*Saccharomyces cerevisiae*) and 7 out of 18 bacterial cells (*Pseudomonas fluorescens*) were viable after sorting (Huang, Ward et al. 2009). The method has been used to identify bacteria within dried and intact biofilms (Beier, Quivey et al. 2012), discriminating between Gram-positive and negative bacteria (Prucek, Ranc et

al. 2012) and identifying bacteria up to species and even strain level (Almarashi, Kapel et al. 2012). Raman microspectroscopy can also be combined with stable isotope probing for detection and separation of cells that can utilise a particular substrate, as ^{13}C consumption generates a shift in peaks corresponding to phenylalanine and protein bands (Huang, Ward et al. 2009). It has even been proposed as a "chair-side" method of identification of microorganisms for rapid diagnosis and treatment, allowing 10 oral bacterial species: *Streptococcus sanguinis*, *Streptococcus gordonii*, *Fusobacterium nucleatum* ss. *polymorphum*, *Propionibacterium acnes* II, *Actinomyces odontolyticus* I, *Prevotella intermedia*, *Prevotella melaninogenica*, *Streptococcus intermedius*, *Parvimonas micra* and methicillin-resistant *Staphylococcus aureus*, to be differentiated in unprocessed samples (Howell, Haffajee et al. 2011).

Colony-hybridisation directed enrichment allows the targeting, enrichment and isolation of not-yet-cultivated bacteria (Datta, Moore et al. 1993). Bacteria are transferred from plates to membranes, on which the RNA or DNA are fixed. The localisation of specific bacteria on plates can be inferred from zones of the membrane hybridising with specific probes, allowing the subculture of targeted bacteria. The target organisms are therefore subcultured with their neighbouring natural helper strains and the culture is progressively simplified and enriched for the target. This technique has been successfully used to isolate *Lactococcus lactis* ss. *cremoris* from environmental samples (Salama, Sandine et al. 1993) and *Fretibacterium fastidiosum* from subgingival plaque in periodontitis (Vartoukian, Palmer et al. 2010, Vartoukian, Downes et al. 2012). This technique, although time-

consuming, is valuable because succeeding passages lead to both an enriched, possibly pure, culture and, at the same time, encourages the emergence of domesticated variants, less dependent on helper strains.

1.5 TM7

TM7 Candidate Division presents a striking example of a phylum level group of bacteria with implications in human health and no cultivated representatives.

1.5.1 Discovery of TM7 in the environment

TM7 is a Candidate Division with no cultivated representatives. It gets its name from Torf, Mittlere Schicht, or peat, middle layer, in a German peat bog (Rheims, Sproer et al. 1996), where the 16S rRNA sequences belonging to this division were first identified. Subsequently, TM7 bacteria have been detected in a wide range of environments: waste water and batch reactor sludges (Bond, Hugenholtz et al. 1995, Hugenholtz, Tyson et al. 2001), fresh and sea water (Connon, Tovanabootr et al. 2005, Newton, Kent et al. 2006, Neulinger, Gartner et al. 2009), soil of different origins (Borneman and Triplett 1997, Costello and Schmidt 2006, Oline 2006), decayed wood (Valaskova, de Boer et al. 2009), city air (Brodie, DeSantis et al. 2007) and composting (Le Goff, Bru-Adan et al. 2010) aerosols.

Three subdivisions were initially identified within the group from an analysis of all the sequences available at that time, which had a maximum intradivision sequence divergence of 17 % (Hugenholtz, Tyson et al. 2001). Subdivision 1 was

composed of 29 sequences of sludge or soil origin, while subdivision 2 consisted of 4 sequences from sludge. The 24 sequences making up the third subdivision were from soil, sludge, seawater, mouse feces and human oral cavity.

Probes specific for all or part of the TM7 division were designed based on comparative analysis of all sequences in the database using the ARB software package (Wolfgang, Strunk et al. 2004), and used with samples obtained from laboratory-scale activated sludge, which had previously been shown to be positive in TM7-specific PCRs (Hugenholtz, Tyson et al. 2001). Probe TM7305, which targets most of TM7 subdivision 1, revealed a filament morphotype. Probe TM7905, targeting nearly the entire division, was used in combination with TM7305 and confirmed that the filament morphotype belonged to the TM7 division. In addition, TM7905 hybridized to cocci that may be representatives of TM7 subdivisions 2 and/or 3.

The filament morphotype was described as having a morphology closely resembling Eikelboom Type 0041 (Eikelboom 1975). Type 0041 is a filamentous Gram variable to positive bacterium, forming straight or bowed, but not branched, filaments. It is non-motile and can occur free in the water as well as attached to flocs. The filament length is variable, with visible septa separating the filament into square cells. It is unclear from the description if the filament is one cell, with septa being internal structures, or if it is a chain of square cells. The filament is embedded within a sheath, which would suggest the former possibility. The diameter of the cells ranges between 0.6 and 1.5 µm. Most of the filaments of this morphotype exhibit attached bacteria of diverse types, which may play a role in collaborative

utilisation of available nutrient sources. The filaments reacting with both specific probes in the laboratory scale sludge did not exhibit attached growth, however (Hugenholtz, Tyson et al. 2001). The interpretation of the FISH staining was that since the majority of the filaments from the sludge gave positive hybridisations with both the TM7305 and TM7905 probes, they could be positively identified as belonging to the TM7 division. However, not all of the filaments were positive for the specific probes and further work has suggested that only a proportion of filamentous cells from this habitat are representatives of TM7 (Thomsen, Kjellerup et al. 2002).

Two studies using stable isotope probing have identified members of the TM7 Candidate Division as main degrader of toluene (Luo, Xie et al. 2009) and as one of the main degraders of benzene (Xie, Sun et al. 2011).

1.5.2 TM7 in animals and humans

Among invertebrates, TM7 members have been found in the microbiota associated with sponges and corals (Ceh, Van Keulen et al. 2011, Webster, Soo et al. 2011, Webster and Taylor 2011), termites (Nakajima, Hongoh et al. 2005, Nakajima, Hongoh et al. 2006, Miyata, Noda et al. 2007) and nematodes (Ladygina, Johansson et al. 2009). They have also been detected in a wide range of mammals, from the mouse (Salzman, de Jong et al. 2002, Ley, Backhed et al. 2005) the bovine digestive tract (Brulc, Antonopoulos et al. 2009, Fernando, Purvis et al. 2010, Kong, Teather et al. 2010), dogs (Xenoulis, Palculict et al. 2008), and pigs (Lowe, Marsh et al. 2012). A study analysing the intestinal microbiota in 60 mammalian species, mostly from San Diego Zoo and the San Diego Zoo's Wild Animal Park animals, detected

the presence of TM7 members in elephants, gazelle, bighorn sheep, takin, buffalo, bonobo and gorilla (Ley, Hamady et al. 2008).

In humans, TM7 bacteria have been identified in several habitats, such as the intestinal tract (Krogius-Kurikka, Kassinen et al. 2009), skin (Gao, Tseng et al. 2007), vaginal fluid (Fredricks, Fiedler et al. 2005) and oral cavity (Paster, Boches et al. 2001, Kazor, Mitchell et al. 2003, Bik, Long et al. 2010).

Although TM7 bacteria are frequently found in healthy individuals (Bik, Long et al. 2010) and were among 25 OTUs found in all sites and subjects in the human digestive tract (Stearns, Lynch et al. 2011), some associations have been found with disease. TM7 sequences have been detected in the airways of individuals with asthma (Hilty, Burke et al. 2010), bronchoalveolar lavage fluid from cystic fibrosis (Harris, De Groote et al. 2007, Guss, Roeselers et al. 2011), bacterial vaginosis (Fredricks, Fiedler et al. 2005), oral inflammation (Kumar, Griffen et al. 2003) and inflammatory bowel disease (IBD) (Frank, St Amand et al. 2007, Kuehbacher, Rehman et al. 2008). In the latter study more phylotypes of TM7 were detected in patients with Crohn's disease than in ulcerative colitis and control subjects, but no quantitative analysis was carried out. Most of these phylotypes were closely related to taxa originally found in the mouth. Surprisingly, some closely related taxa (Human HOT356, corresponding to the clone name I025, and HOT437, genomospecies P1) showed differing disease associations in IBD. Clones closely related to HOT356 were detected only in ulcerative colitis, while those resembling HOT437 were detected exclusively in healthy subjects. This finding raises once again the need to identify bacteria to species level but this is a particular challenge in this

Division, which, due to its lack of cultured representatives, does not have a well-defined taxonomy. This study also analysed the morphology of TM7 using in-situ hybridisation, and revealed a filamentous morphotype. But the choice of the probe appears to have been injudicious. Probe TM7-305, as described by Hugenholtz *et al*. (2001), was designed to target the TM7 subdivision 1, which included no sequences associated with animals. It has at least one mismatch with all human oral sequences except for HOT347, and up to 4 mismatches for HOT355. When its specificity is determined with the RDP Probe Match program, it shows 100 % identity to 1452 bacterial 16S rRNA gene sequences, only 671 of which belong to the TM7 division (of the total of 3307 TM7 sequences). When one mismatch is allowed, it matches 181318 sequences (including 135464 *Proteobacteria* sequences) of which 1512 are TM7.

Members of the TM7 Candidate Division are typically found to constitute only a small proportion of the microbiota of any habitat, usually under 1 %. This may explain the lack of consistency in their detection in related samples, as, until recently, most of the molecular analysis of bacterial populations was performed by the cloning of 16S rRNA genes and Sanger sequencing, with usually no more than 96 clones analysed per sample. The advance that has come with the adoption of next generation sequencing, which allows a far greater depth of analysis, is likely to confirm the ubiquity of TM7 bacteria.

1.5.3 Oral TM7 in health and disease

The presence of members of the TM7 division in human oral samples was first reported by Paster *et al*. (2001). Analysing 2522 clones from the subgingival plaque

of healthy subjects and patients with acute necrotizing ulcerative gingivitis and different forms of periodontitis, they identified 34 sequences belonging to this division, falling into five phylotypes closely related (> 99 % identity) to HOT346, 347, 349, 355 and 356. HOT346 and 356 were predominant, as they were detected in more than 4 subjects, with 20 and 6 clones respectively. HOT346 was the only one found in health, although it was the most frequently detected phylotype overall and relatively few clones in the study came from healthy subjects (268 of 2522 clones, 10.6 %). Nevertheless, HOT356 (represented by clone I025) was described as associated with periodontitis, as it was detected in at least 4 diseased subjects, but not in healthy ones; no statistical test was performed to confirm the association.

Quantitative real-time PCR and FISH has been used to detect TM7 in samples from healthy and diseased sites of 42 subjects with different degrees of chronic periodontitis and from 4 healthy subjects (Brinig, Lepp et al. 2003). Among 85 TM7 clones from 4 healthy and 3 diseased sites, 6 phylotypes were identified: HOT346 and 356, previously detected by Paster *et al*. (2001), and 4 phylotypes defined as new by the study, SBG1, 2, 3 and 6. SBG1 is 99.6 % identical to HOT437 and 99.5 % identical to HOT356, SBG2 is 99.5 % identical to HOT350, SBG3 is 98.8 % identical to HOT348 and SBG6 is 99.1 % identical to HOT346. It is unclear why sequences named SBG1 and SBG6 were not included in the previously defined taxa HOT356 and HOT346, respectively. HOT346 was again found to be the most frequently detected, with 62 clones, found at all seven sites. TM7 bacteria were detected in 96 % of samples, including all healthy sites tested. The proportion of TM7 compared to the total number of bacteria was greater in mild periodontitis (0.54 %) than in healthy

sites (0.21 %, $p<0.01$) or severe periodontitis (0.29 %, $p<0.05$). A difference was also found between subjects from different ethnic origins, with smaller proportions of TM7 being found in Caucasian subjects (0.22 %) compared to Asian (0.49 %, $p<0.008$) and black patients (0.37 %, $p<0.05$), but no differences were found in subjects for gender, smoking status or age. The TM7 cells detected by FISH were described as filaments from 4 to 30 µm long and 1 to 1.5 µm thick. The "long filaments", however, appeared to be composed of multiple cells, each 3 to 4 µm long. The I025-136 probe hybridised exclusively to long filaments. There were no significant differences in morphology or numbers of TM7 between healthy and diseased sites, as detected by FISH. This study confirms the association between the I025 subgroup (HOT356) and periodontitis, as it was detected in only 1 in 18 healthy subjects, compared to 38 of 58 diseased sites.

Specific probes for TM7, TM7-905, targeting the entire TM7 division (Hugenholtz, Tyson et al. 2001), and I025-136 (Brinig, Lepp et al. 2003), directed against the I025 TM7 clone (HOT356) were designed and validated by means of cloned artificial targets for FISH (catFISH) (Ouverney, Armitage et al. 2003). TM7 bacteria were detected in healthy and periodontally diseased sites using the TM7-905 probe (4 out of 9 and 12 out of 12, respectively) and I025-specfic probe (5 and 10 respectively), with higher numbers detected in periodontitis than in health (3 times more for TM7-905 and 2.5 for I025-136). It is not clear why cells that hybridised with the I025-specfic probe did not react with TM7-905 in one of the healthy subjects, as the latter has a broader specificity and covers all oral taxa detected to date. The TM7-905 probe reacted with a variety of morphotypes, from

cocci (1.0 to 0.45 µm) to filaments (3.0-75.0 by 0.6-1.0 µm), with an average of three segments per filament. The length of the filaments was four times greater in disease than in health for I025 subgroup, while it did not vary significantly for the rest of the TM7 bacteria. Intriguingly, not all segments of the filament hybridised with TM7-specific probes, indicating possible metabolic inactivity.

Specific PCR primers were used by Kumar *et al.* (2003) to detect HOT356, among other "novel" bacteria, in health and chronic periodontitis. TM7 HOT356 was detected with a prevalence of 71 % in health and 91 % in disease, and was considered to be associated with periodontitis with a p value of 0.004 (not significant, when the correction for multiple comparisons was applied).

TM7 have also been detected in non-specific surveys of oral samples. For example, a study analysing the microbiota in halitosis, identified 3 phylotypes of TM7 bacteria (Kazor, Mitchell et al. 2003). One of them, HOT352, first identified in this study, was associated with halitosis, as it was detected in 4 out of 6 halitosis subjects and not at all in health. The other two, HOT351 and HOTA56, were detected in only one subject each. Similarly, TM7 HOT346, 356 and 437 were detected in larger proportions in refractory periodontitis than in good responders or healthy controls ($P < 0.05$; χ^2 test) in a study using the Human Oral Microbe Identification Microarray (HOMIM) (Colombo, Boches et al. 2009).

1.5.4 TM7 culture and genome sequencing

Microcolonies of TM7 visible to the naked eye were detected on low-nutrient solid media after 50 days incubation (Hugenholtz 2002). This report, however, did not give specific details of this apparently successful isolation and no subsequent

work has been published to date, suggesting that either the colonies may have not been pure or did not survive subculture.

A soil substrate membrane system was successfully used to grow microcolonies of TM7 from soil (Ferrari, Binnerup et al. 2005). Several morphotypes were detected after 7 days of incubation using FISH (TM7-905 probe), which comprised 6.7 % of the total of growing bacteria. The two dominant types were a fast-growing short rod, forming microcolonies of several hundred cells, and a slow-growing long rod (15 µm) with colonies of less than 50 cells. No pure culture of TM7 was obtained, however. The same method was used in an attempt to grow TM7 bacteria from waste water by Abrams *et al.* (2012), who reported the formation of microcolonies of 15 to 30 µm after 2 weeks of incubation, with the main morphotypes being cocci, diplococci and short rods. All of the colonies appeared to be mixed, as they included cells that were detected by the universal probe, but not with a TM7-specific probe. The co-growing bacteria included *Proteobacteria*, mostly *β-Proteobacteria*, *Actinobacteria*, *Planctomycetes*, *Bacteroidetes* and *Chloroflexi*.

Sequencing of the TM7 genome from single cells has been attempted. Podar *et al.* (2007) used FACS and the TM7-905 probe with soil samples to obtain pools of TM7 cells. Most of the largest pools (containing 100 cells) contained several TM7 lineages and also non-TM7 cells, due to the broad spectrum of the probe. It was determined experimentally that the best conditions for effective genome amplification and best purity were obtained for 5 cells. The genome from one pool was amplified with multiple displacement amplification. The affiliation of isolated cells was analysed by sequencing of the 16S rRNA genes and 61 out of 69

sequenced clones belonged to the TM7 phylum (89 % identity). These sequences presented some polymorphism but were considered as belonging to the same "species", named TM7_GTL1 (90 % identity to any oral TM7 sequences). The other sequences presented 99.5 % identity to the *Pseudomonas* sp. isolates, such as *Pseudomonas rhodesiae*. During the genome analysis, authors detected some contigs showing 90 % identity to known *Pseudomonas* genes. These sequences presented also a relatively high GC content, > 53 %, compared to the majority of sequences (< 50 %). As these sequences were removed from further analysis, it is possible that some genuine TM7 sequences were removed. The coverage depths of this partial genome sequencing varied extensively, exceeding 50-fold for some contigs, but were low for others. The final data included 132 contigs made up of 679,515 nucleotides, and encoded 670 predicted proteins, six tRNAs, a full-length SSU rRNA gene (that allowed a second structure prediction), and a partial large-subunit rRNA gene. The average GC content was of 48.5 %.

A more complete genome was obtained by Marcy *et al.* (2007), who used a microfluidic device, designed for the purpose, to isolate 35 rod-shaped bacteria from the human subgingival crevice. On-chip whole genome amplification from isolated cells yielded 50 ng of DNA. It was followed by a second off-chip amplification to obtain sufficient DNA for sequencing. Of the 35 isolated cells, 4 belonged to the TM7 phylum. Three were closely related to the SBG3 clone from the Brinig *et al.* (2003) study (> 99.6 % identity). The fourth was a more distant relative (97.3 %) of TM7 HOT353. Genomes of three TM7 cells related to the SBG3 were sequenced by 454 pyrosequencing. The coverage, once again, was low and

some regions could not be assembled. The whole assembly of the genome of one of the cells, referred to as TM7a, was achieved with ‘‘metagenomic assumptions’’ but low-coverage regions (error-prone) were excluded from analysis. The total genome size was not estimated due to the bias intrinsic to the single-cell genome amplification technique. Genomes of TM7b and TM7c were less complete and served mostly to confirm the assembly of theTM7a genome. A stringent analysis of the data from TM7a including only genes from large contigs identified 1,474 genes on 288 scaffolds, 43 % of which were assigned a predictive function. The G+C content of the sequenced genome was 34.3 %. Some contaminant DNA from *Leptotrichia* species was found but made up less than 10 % of the sequence.

Most of the TM7 genes sequenced were found to have low homology with related genes from other phyla, as 80 % of the predicted TM7 proteins had less than 60 % sequence identity to proteins from other sequenced organisms, and 33 % had less than 30 % identity. Nevertheless, some genes presented more than 60 % identity to those from *Bacilli, Clostridia*, and *Fusobacteria* by BLAST. It should be borne in mind, however, that most of the closest relatives of TM7 bacteria, such as *Chloroflexi* and SR1, are still largely uncultured, and therefore the sequences of their metabolic genes are hugely underrepresented in databases. It is possible that DNA from other organisms contaminated the TM7 preparations or were bound to the membrane of TM7 cells, but these genes do not cluster together by organism. This finding could also result from horizontal gene transfer. Some evidence for gene transfer was seen by Podar *et al.* (2007) as a second operon of ATPases had high homology to similar genes from *Chlorobi*.

Genes implicated in several metabolic processes were identified, such as glycolysis (3-phosphoglycerate kinase, phosphoglycerate mutase triosephosphate isomerase, and pyruvate kinase), the tricarboxylic acid cycle (succinyl-CoA synthetase), nucleotide biosynthesis, and amino acid biosynthesis. Potential substrates include oligosaccharides, arginine and possibly other amino acids. Genes encoding putative virulence factors were also seen. The presence of genes implicated in type IV pili biosynthesis was reported in both studies. Genetic evidence of a novel sortase, predicted to add an atypical amino acid to the growing peptidoglycan chain was found, that has been implicated in chronic granulomatous inflammation (Marcy, Ouverney et al. 2007). Type II and Type IV secretion systems, as well as a putative autoinducer 2 exporter (AI-2E), implicated in quorum sensing, were also seen (Podar, Abulencia et al. 2007).

One of the primary aims of a genomic analysis of an uncultured bacterium is to obtain clues related to its metabolism that could allow its culture, but unfortunately no such clues were identified in these studies. Furthermore, both studies presented some bias and both were contaminated with non-TM7 DNA. Further work is needed to generate genomic data of value in improving cultural techniques for members of the TM7 division.

1.6 Aims of research

The overall aims of the research described in this thesis were:

1. To evaluate the ability of two model culture systems: Cooked Meat Medium and the Calgary Biofilm Device, to support the growth of mixed oral bacterial communities.
2. To determine if such in-vitro communities can support the growth of previously uncultured oral bacteria.
3. To attempt to culture representatives of phylum-level Division TM7, as a pure culture or as a member of a simple bacterial community.

Chapter 2: Cooked Meat Medium culture of endodontic infections

Chapter 2. Cooked meat medium culture of endodontic infections

2.1 Introduction

There have been numerous attempts at recreating the oral habitat *in vitro*, primarily for the assessment of metabolic activity or antibiotic resistance of mixed biofilms. Such systems would also be useful for the study of as yet uncultured bacteria, if these microorganisms could be established as part of a mixed culture. These culture systems may allow phenotype and metabolic capacities of uncultured taxa to be studied, and provide a source of cells for other analyses. Probably the first model oral microcosm was described by Miller (1890) who incubated extracted human teeth in water and bread mixture and observed lesions identical to naturally observed caries. Artificial mouth systems reproducing the oral environment have been used in many studies mainly focused on plaque development and caries (Pigman, Elliott et al. 1952, Sidaway, Marsland et al. 1964, Russell and Coulter 1975, Sissons, Cutress et al. 1991). Attempts to closely mimic the in-vivo environment, however, frequently result in complex systems which reduce the practicality of repeated sampling and lead to a lack of reproducibility. Liquid continuous cultures have also been used to grow oral microcosms, but they fail to reproduce the bacterial interactions found in a natural biofilm. Plaque bacteria grow naturally in biofilms and it was noticed that biofilms formed on walls of liquid culture vessels (Marsh, Hunter et al. 1983). Keevil *et al.* (1987) developed a continuous culture chemostat where dental plaque biofilm was grown for up to 21 d on acrylic tiles in slightly modified basal medium without glucose (Shah, Williams et al. 1976, Keevil,

Bradshaw et al. 1987). Growth rates were found to be comparable to those observed in the natural environment and a biofilm formed which appeared to be very similar to natural plaque in its appearance when viewed by scanning electron microscopy. Similar models were subsequently used to establish oral microcosms on different types of surface, such as glass, polycarbonate, silicon, hydroxyapatite (HA), nitrocellulose, enamel or dentin. A variety of culture media were used in these models, including modified basal medium with or without mucin, a peptone yeast extract-based medium supplemented with mucin (Sissons, Cutress et al. 1991, Sissons, Wong et al. 1995), defined medium mucin (Wong and Sissons 2001) and a chemically defined saliva analogue with mucin (Sissons, Wong et al. 1995).

A number of models have been specifically designed to mimic the root canal habitat. Such systems have usually been used to test the efficacy of biofilm removal by different techniques (Shen, Qian et al. 2009). Some use entire teeth with naturally or experimentally infected root canals (Villette, Manek et al. 2008, Xie, Johnson et al. 2012), or flow cells (Chavez de Paz, Bergenholtz et al. 2010, Chavez de Paz 2012). A weakness of these models, particularly those using extracted teeth, is that they are complex, can be difficult to sample and are frequently prone to contamination.

Cooked Meat Medium (CMM) was developed for the culture of anaerobic bacteria from wounds (Robertson 1915). Cooked meat was used because sulfhydryl groups, desirable for their reducing properties, are more available in denatured proteins and meat granules and the reducing properties are greatly lessened if the meat particles are removed by filtering. Both anaerobic and aerobic bacteria can be

grown in the medium, if it is incubated in aerobic conditions with the cap loose. This creates a redox gradient providing conditions suitable for organisms with a variety of atmospheric requirements. The primary source of energy in the medium is proteinaceous. The carbohydrate concentration is low at around 1 % and is primarily muscle glycogen converted to glucose and isomaltose. It has been shown that a bacteriotoxic factor is formed when glucose and phosphate are present during heat sterilization (Finkelstein and Lankford 1957), so a low level of glucose should prevent this. The meat particles are also able to buffer medium acidification by acids produced by bacteria. CMM is known to be able to support bacterial growth from a small inoculum and to maintain the viability of cultures over a long period. Mixed cultures of bacteria survive in CMM without the loss of slower-growing organisms. CMM was successfully used to reisolate bacteria after prolonged incubation times of six to ten months with only few exceptions (Holman 1919). The medium also enabled the successful growth of *Actinomyces* species and *Propionibacterium acnes* and a range of other oral bacteria previously uncultivated at that time (Holman 1919). Finally, the presence of solid meat particles provides a substrate for biofilm formation. The maintenance of the culture can be achieved by serial batch culture, transferring an aliquot of developed CMM culture to a new tube containing fresh CMM. Compared to models for biofilm with continuous growth, CMM has the advantages of simplicity of use and reduced risk of contamination of the culture.

It was long assumed that oral bacteria used the host diet for their nutrition. But a simple study by Keene *et al*. (Keene, Coykendall et al. 1966) showed that

sugars were rapidly cleared from saliva following ingestion. Comparing caries-active and caries-resistant volunteers they determined that after an oral rinse with a 20 % corn syrup solution glucose was cleared from saliva in about 12 min, with no regard to the caries status. Another study showed that the recolonisation of teeth by streptococci after scaling was similar in feeding and fasting monkeys (Beighton and Hayday 1986). Therefore, bacteria would need to rely on the substances produced and secreted by the host, primarily saliva and gingival crevicular fluid. The principal protein-containing constituents of saliva are mucins, heavily glycosylated proteins of high molecular weight (millions of Da), representing up to 26 % of salivary proteins. Two cell-bound mucins are found in the oral cavity: MUC1 and MUC4 (Swallow, Gendler et al. 1987, Nollet, Moniaux et al. 1998), and two secreted mucins can be detected in saliva: high-molecular-weight mucin, MUC5B (MG1), and low-molecular-weight mucin, MUC7 (MG2). They are secreted by the submandibular and sublingual glands, as well as minor salivary glands (Derrien, van Passel et al. 2010). Mucins consist of a protein backbone, called apomucin, with a large amount of O-linked glycans, representing up to 90 % of the molecular weight. Oral bacteria can use both the glycans and the protein core as energy and carbon sources (Beighton, Smith et al. 1988, Glenister, Salamon et al. 1988). The degradation of glycans is sequential and requires a number of species to work together. Wickstrom *et al*. (2008) used a range of glycosidase substrates to determine the glycosidic activity of natural dental plaque. Among the enzymes found to be synthesised by oral bacteria to degrade mucins were sialidase (neuraminidase), β-galactosidase, β-N-acetylglucosaminidases and α-L-fucosidase. Apart from their nutritional role, mucins can also play a structural role in adhering

to oral surfaces and promoting attachment of bacteria. Bovine salivary mucin has been primarily used in oral bacterial culture models. But Glenister *et al.* (1988) showed that hog gastric mucin supplemented basal medium performed better in supporting the growth of *Actinomyces*, *Bacteroides* and *Treponema* species, forming a mixed community resembling subgingival plaque. Hog mucin is considered to be chemically more similar to human mucin than the bovine form, as well as being cheaper to produce and Glenister *et al.* developed a crude method of purification which is still used today.

Other supplements incorporated into culture media for oral bacteria include haemin and vitamin K_1. Haemin is an iron-containing porphyrin, a source of the X factor. Vitamin K_1 can be used by bacteria in electron transfer as a part of anaerobic respiration. Both haemin and vitamin K have been reported as necessary for the growth of some *Prevotella* and *Porphyromonas* species (Shah, Williams et al. 1976). Blood, of horse or sheep origin, and horse serum (ter Steeg, Van der Hoeven et al. 1987) are often used as broad range supplements providing a range of vitamins and growth stimulating factors. Nicotinamide adenine dinucleotide plays an essential role in metabolism as a coenzyme in redox reactions and can additionally function as a substrate for bacterial DNA ligases (Wilkinson, Day et al. 2001). Finally, some media also incorporate arginine and urea, to provide a natural buffering effect because some bacteria metabolise these substrates to ammonia, which increases the pH (Wijeyeweera and Kleinberg 1989), and formate and fumarate (Tanner 1987), which are required by some *Campylobacter* species for growth.

2.2 Aim

The aim of the work described in this chapter was to evaluate the ability of Cooked Meat Medium to support the growth of a mixed bacterial community of endodontic origin that would include not-yet-cultured members of the microbiota. The composition of the microbiota of the endodontic samples and the CMM cultures derived from them was determined by culture and molecular methods and compared.

2.3 Methods

2.3.1 Validation of the batch co-culture using CMM

2.3.1.1 Bacterial strains and culture

The bacterial strains used in this study are listed in Table 1. The strains were taken from the departmental collection and cultured on Fastidious Anaerobe Agar (LabM), supplemented with 5 % horse blood (TCS) (FAAB), under anaerobic conditions (Don Whitley MACS MG1000 anaerobic workstation, 80 % N_2, 10 % H_2, 10 % CO_2) at 37 °C. Their identity was verified by partial sequencing of the 16S rRNA gene. For viable count determination, strains were grown in Brain Heart Infusion broth (BHI, LabM), under the same conditions.

Table 1. Bacterial strains used in the study, with the American Type Culture Collection (ATCC) number.

Species	Strain number
Fusobacterium nucleatum ss*. nucleatum*	ATCC 25586
Parvimonas micra	ATCC 33270
Porphyromonas endodontalis	ATCC 35406
Prevotella buccae	ATCC 33574
Propionibacterium acnes	ATCC 6919
Streptococcus intermedius	ATCC 27335

2.3.1.2 Viable count determination

The optical density (OD) of cultures was measured at a wavelength of 600 nm. Doubling dilutions were prepared to assess the OD in the linear range of the spectrophotometer. FAAB plates were inoculated, in triplicate, with 100 µL of serial dilutions of the broth culture and incubated under anaerobic conditions for up to 5 d. After incubation, plates with between 30 and 300 colonies were counted, and the calibration curve constructed by plotting OD_{600nm} against log of CFU/ml (colony forming units per mL).

2.3.1.3 Cooked Meat medium co-culture

1 x 10^5 CFU of each bacterial strain were added to a glass universal bottle containing Cooked Meat medium (CMM, Oxoid). The mixed bacterial culture was incubated in anaerobic conditions at 37 °C for 7 d after which a 500 µl aliquot was transferred to a new vial and the composition of the culture analysed. Similar analyses were performed at one week intervals for 11 weeks. After 6 weeks, the CMM was supplemented with 20 % defibrinated horse serum (TCS) and after 7 weeks, with serum, 5 µg/ml haemin and 0.5 µg/ml vitamin K.

2.3.1.4 Culture analysis of bacterial composition of mixed culture

One hundred µl of supernatant were collected 5 mm from the bottom of the vial, in the mass of meat particles. Ten-fold dilutions of the supernatant in pre-reduced Phosphate Buffered Saline (PBS, Oxoid) were prepared and 100 µl of dilutions 10^{-4} to 10^{-6} were spread on FAAB plates in triplicate and incubated under anaerobic conditions at 37 °C for 5 d. Colonies of each bacterial strain were counted on plates containing between 30 and 300 colonies. Colonies were identified by phenotypic observation and Gram stain appearance. The identification of colonies was facilitated by the fact that the chosen strains had very different colony morphologies. The three plates of the chosen dilution were counted and a mean value was calculated. The data were expressed in CFU/ml of CMM.

2.3.2 Endodontic sample analysis and CMM culture

2.3.2.1 Sample collection

The study protocol was approved by Joint RNOH/IOMS Research Ethics Committee (REC reference number 09/H0724/12) and informed consent was obtained from the patients.

Samples were collected from three patients presenting clinical signs of chronic apical periodontitis with radiological evidence of bone destruction. Two affected teeth were sampled from one patient and one tooth from each of the other two. None of the patients received any systemic antibiotic treatment within the preceding 3 months. The teeth were isolated with a rubber dam and cleaned with sodium hypochlorite. Access cavities were made into the pulp chamber by

means of a sterile bur. A 100-µl quantity of sterile saline was introduced to the root canal with a needle and the canal was gently irrigated to disturb the biofilm.

The saline was then aspirated and added to 250 µl of reduced transport medium (RTM) in a screw-cap 2 ml tube. RTM (1 % w/v tryptone, 0.5 % w/v yeast extract, 0.1 % w/v L-cysteine, 0.1 % w/v D+ glucose, 2 % v/v horse serum in water, pH 7.5) was prepared, filter sterilised and pre-reduced in the anaerobic workstation overnight in preparation of sample collection. Samples in RTM were taken immediately to the laboratory, within 3 min of collection. They were placed inside an anaerobic workstation, with cap unscrewed, for 30 s, to replace the head space with anaerobic gas, taken out to be homogenized by vortexing for 30 s, and finally returned to the anaerobic workstation.

2.3.2.2 Sample processing

A vial containing 20 ml of CMM supplemented with 20 % of horse serum, 5 µg/ml haemin and 0.5 µg/ml vitamin K was inoculated with 100 µL of cell suspension. Another 100 µl of the sample A1 were used to prepare tenfold dilutions up to 10^{-4} in RTM which were spread on FAAB in triplicate. Plates were incubated in an anaerobic workstation at 37 °C for 10 d. In addition, dilutions 10^{-2} and 10^{-3} were spread on Blood Agar (LabM) plates supplemented with 5 % horse blood and incubated aerobically in hermetic jars with 5 % CO_2 (CO_2Gen, Oxoid) for 4 d. DNA was extracted from the remainder of the sample for molecular analysis of the bacterial composition of the endodontic sample.

2.3.2.3 In-vitro culture of endodontic sample

The mixed microbiota present in the sample was cultivated in serial batch culture in CMM supplemented with 20 % defibrinated horse serum, 5 µg/ml haemin and 0.5 µg/ml vitamin K with passages every 10 d. The mixed bacterial culture was incubated under anaerobic conditions at 37 °C. The first batch was inoculated with 100 µl of endodontic sample; all subsequent batches were inoculated with 500 µl of the previous culture, taken 5 mm from the bottom of the vial. At the end of each 10-d period, bacterial composition was analysed by molecular methods. Similar analyses were performed at 10 d intervals up to 480 d for samples A1 and A2 and 140 d for sample C.

2.3.2.4 Analysis of cultivable bacteria

Ten-fold dilutions of sample A1 in RTM were prepared and 100 µl of the dilutions 10^{-4} to 10^{-6} were plated on FAAB in triplicate. After 10 d of anaerobic incubation, plates with between 30 and 300 colonies were counted. Ninety six randomly selected colonies were isolated on FAAB plates with a streak of the feeder strain, *Propionibacterium acnes*, and identified.

2.3.2.5 Analysis of endodontic samples and subsequent CMM culture composition

2.3.2.5.1 DNA extraction

DNA was extracted from 100 µl of the endodontic sample, 1 ml of the CMM culture and pure culture isolates growing on FAAB, with the Genelute bacterial genomic DNA extraction kit (Sigma Aldrich) according to the manufacturer's instructions and following the modification recommended for Gram-positive bacteria. Cells were harvested by centrifuging at 13 000 g for 2 min, the pellet

resuspended in 200 µl 45 mg/ml lysozyme solution and incubated at 37 °C for 30 min. RNase A solution was added (20 µl) and incubated at room temperature for 2 min, after which 20 µl Proteinase K and 200 µl Lysis solution C were added. The suspension was vortexed and incubated at 55 °C for 10 min. Columns were prepared with 500 µl Column Preparation Solution. Following incubation at 55 °C, 200 µl 99.6 % ethanol was added to the lysate and mixed by inversion. The complete lysate was transferred to the binding column and centrifuged at 13 000 g for 1 min. The column was then washed with Wash Solution 1 and Wash Solution concentrate (diluted with the appropriate amount of ethanol). The column was centrifuged to dry and DNA eluted in a fresh collection tube with 200 µl of the Elution Solution after a 5-min incubation at room temperature. An aliquot was stored at 4 °C for short term use, while the remainder was stored at -70 °C. DNA and subsequent PCR products were detected and quantified by visual comparison with known amounts of lambda phage DNA (New England Biolabs) on 0.8 % agarose (Bioline) gel in 0.5x Tris-Borate EDTA (Sigma-Aldrich), containing 0.1 µg/mL of GelRed (Biotium).

2.3.2.5.2 PCR amplification of 16S rRNA genes

16S rRNA genes from pure or mixed cultures were amplified using universal primers 27F-YM and 1492R (Table 2) (Nercessian, Fouquet et al. 2005) (Frank, Reich et al. 2008). The reactions contained 22.5 µl of Thermoprime *Taq* polymerase master mix (Abgene), 1 µl of template, 1 µl each (0.4 µM final concentration) of 27F-YM and 1492R primers. Initial denaturation, 95 °C for 5 min, was followed by 25 cycles of denaturation at 95 °C for 45 s, annealing at 56 °C for 45 s and extension at

72 °C for 90 s, with a final period of extension at 72 °C for 5 min. A touch PCR was used to amplify 16S rRNA genes from isolates, as follows. Isolated colonies were touched with a sterile pipette tip viewed under a dissection microscope and the cells suspended directly in the PCR reaction mix. The amplification cycle was as described above, with the exception of 1 µl of water added to the reaction mix (to compensate for the template volume) and the length of the initial denaturation step was increased to 15 minutes.

Table 2. Primers used for 16S rRNA gene amplification and sequencing ([1](Lane 1991), [2] (Amann, Stromley et al. 1992) [3] TOPO-TA Cloning kit, Invitrogen, [4] (Fierer, Hamady et al. 2008), **IUPAC notation of degenerate bases: M = A or C; Y = C or T; R = A or G; W = A or T, S = G or C, N = any base**)

Primer	Primer sequence (5'-3')
27F-YM	AGAGTTTGATYMTGGCTCAG[1]
342R	CTGCTGCSYCCCGTAG[1]
357F	CTCCTACGGGAGGCAGCAG[1]
519R	GWATTACCGCGGCKGCTG[1]
907R	CCGTCAATTCCTTTRAGTTT[2]
926F	GGTTAAAACTYAAAKGAATTGACGG[1]
1100R	GGGTTGCGCTCGTTG[1]
1114F	GCAACGAGCGCAACCC[1]
1392R	ACGGGCGGTGTGTRC[1]
1492R	TACGGYTACCTTGTTACGACTT[1]
M13F	GTAAAACGACGGCCAG[3]
M13R	CAGGAAACAGCTATGAC[3]
27F-YM-A	CCATCTCATCCCTGCGTGTCTCCGACTCAGNNNNNNNNNNNNAGAGT TTGATYMTGGCTCAG[4]
519R-B	CCTATCCCCTGTGTGCCTTGGCAGTCTCAGGWATTACCGCGGCKGCTG[4]

2.3.2.5.3 Cloning of amplified 16S rRNA genes

For the mixed cultures, 16S rRNA genes products amplified with the *Taq* polymerase were cloned using the TOPO TA Cloning kit (Invitrogene, UK), according to the manufacturer's instructions. Two µl of PCR product were combined with 1 µl salt solution (Invitrogen), 2 µl dH_2O (Sigma-Aldrich) and 1 µl of the TOPO-TA cloning

vector (Invitrogen). The solution was incubated for 30 minutes at room temperature, placed on ice and 2 µl of the cloning solution added to TOP-10 chemically competent cells and after gentle mixing, incubated for 10 min on ice. Cells were heat shocked at 42 °C for 30 sec on a heating block and immediately placed on ice. Aliquots of 250 µl SOC medium (Invitrogen) were added and the cells incubated for 1 h at 37 °C, while shaking at 250 rpm in a Forma scientific orbital shaker. Finally, the cell suspension was spread in 50 µl and 100 µl aliquots on pre-warmed Luria Bertani (LB) agar supplemented with 50 µg/ml kanamycin (Gibco/Invitrogen) and incubated aerobically at 37 °C over night.

Clone colonies were touched with a sterile pipette tip and the cells suspended in 50 µl of sterile water. One µl of the suspension was used as the template in a PCR reaction using Thermoprime *Taq* polymerase, as described above, using the primer set M13F/M13R (Table 2). Initial denaturation step was run at 95 °C for 15 min. This was followed by 30 cycles of denaturation at 95 °C for 45 sec, annealing at 55 °C for 45 sec and extension at 72 °C for 90 sec, with a final period of extension at 72 °C for 5 min.

2.3.2.5.4 Sanger Sequencing

2.3.2.5.4.1 Purification of amplicons

Prior to sequencing, the PCR products were purified using ExoSAP-IT (USB). The method was modified from the manufacturer's instructions in that 1 µl of product, plus 1 µl of water, were added to 5 µl of PCR product, instead of 2 µl of ExoSAP-IT. It was then incubated in a Thermo cycler for 15 min at 37 °C followed by heat inactivation of the enzymes at 80 °C for 15 min.

2.3.2.5.4.2 Sequencing reaction

Clones and isolates were partially sequenced using the universal primer 519R (Table 2). The reaction mixture was composed of 0.5 µl Big Dye, 1.75 µl of 5X sequencing buffer (400 mM Tris Base pH 9.0, 10 mM $MgCl_2$), 5.45 µl sterile UHQ distilled water, 0.3 µl of primer (0.3 µM) and 2 µl of PCR product. The cycle, repeated 30 times, consisted of a denaturation step, 96 °C for 10 s, an annealing step, 50 °C for 5 s, and an elongation step, 60 °C for 2 min.

For some clones and isolates, full length sequencing was performed with 9 additional primers (27F-YM, 342R, 357F, 907R, 926F, 1100R, 1114F, 1392R and 1492R, Table 2).

2.3.2.5.4.3 Sequencing product clean up

Ten µl of water was added to all wells of the 96-well sequencing plate, followed by 50 µl of precipitation mix (sodium acetate 57 mM, EDTA 4 nM, 91 % ethanol). The plate was vortexed and incubated at RT for 20 min. The plate was then centrifuged for 25 min at 4000 rpm, the supernatant was discarded and the plate spun upside-down at 300 rpm for 10 s. One hundred µl of chilled 70 % ethanol were then added to each well and the plate was centrifuged for 10 min at 4000 rpm. The ethanol was discarded and the plate spun inverted at 300 rpm for 15 s. The plate was dried and 10 µl of 0.1 TE buffer (1mM Tris, 0.1 mM EDTA, pH8.0) were added to each well.

2.3.2.5.4.4 Sequencing facility

Sequencing was performed using an automated sequencer ABI 3730x (Applied Biosystems). Data quality was analysed with Sequence Scanner Software (Applied Biosystems).

2.3.2.5.5 454 pyrosequencing

For amplicon library construction, 16S rRNA genes of the extracted DNA from the initial endodontic samples and from selected points of CMM culture were amplified using the broad range 16S rRNA gene specific primers 27F-YM and 519R along with unique 12-mer Golay 'barcode' sequences on the forward primer and the Roche GS-FLX-454 Titanium series adaptor sequences (A + B) for the Lib-L kit emPCR method. These primers were named 27F-YM-A and 519R-B (Table 2), where the Ns in the 27F-YM-A primer represent the barcode sequence, different for each multiplexed sample.

Three replicate amplification reactions were set up for each sample. Reactions were prepared containing 12.5 µl Extensor PCR mastermix (High fidelity Taq polymerase) (Thermo Scientific), 2 µl of template, 0.5 µl of each primer (10 µM) and 9.5 µl sterile water. Initial denaturation was at 95 °C for 5 min, followed by 25 cycles of denaturation at 95 °C for 45 s, annealing at 53 °C for 45 s, extension at 72 °C for 90 s and a final extension at 72 °C for 15 s. A negative no template reaction was set up for every primer set.

PCR amplicons were pooled and purified using the QIAquick PCR purification kit (Qiagen) following the manufacturer's instructions to remove un-used primers and nucleotides. Purified product was eluted in 30 µl 0.1 x TE buffer

The size, amount and purity of purified amplicons were evaluated using the Agilent 2100 Bioanalyzer along with the Agilent DNA 1000 kit (Agilent Technologies, Inc.). DNA samples were accepted for further analysis if they had an OD 260/280 ratio of 1.8 or above and were at a concentration of 5 ng/µl or greater.

Accurate quantitation of the amplicons with the Quant-iT-Picogreen fluorescent nucleic acid stain (Invitrogen), a fluorometric assay, was performed to determine the concentration (ng/µl) of each amplicon. This was converted to molecules per µl and subsequently amplicons for each library were pooled in equimolar concentrations (1 x 10^9 molecules /µl).

The samples were amplified clonally by emulsion-PCR using the GS emPCR Lib-L Kit. The GS PicoTiterPlate Kit was then used to sequence individual clonally amplified molecules on a Roche 454 GS-FLX Titanium sequencer.

2.3.2.5.6 Data analysis

2.3.2.5.6.1 Sanger sequencing

Sequences were handled and aligned using the BioEdit Sequence Alignment Editor (BIOEDIT) software (Hall 1999). They were provisionally identified by means of the BLAST (Basic Local Alignment Search Tool) tool available with the Human Oral Microbiome Database (HOMD, www.homd.org). Sequences showing homology of less than 98.5 % to databases reference sequences were subjected to full length

sequencing of the 16S rRNA gene. Sequences were assembled using the Contig Assembly Program (Huang and Madan 1999), and identified. The closest possible identification was then obtained by BLASTN 2.2.22 interrogation of the GenBank database (http://blast.ncbi.nlm.nih.gov (Zhang, Schwartz et al. 2000)) and by means of the Seqmatch tool available from The Ribosomal Database Project II, (RDP) (http://rdp.cme.msu.edu/index.jsp).

2.3.2.5.6.2 454 pyrosequencing

The sequences were subjected to the mothur (Schloss, Westcott et al. 2009) shhh.flows command to de-noise the data. The trim.flows command was used to remove the primer sequences and barcodes, sequences shorter than 350 bp, and sequences with mismatches in barcode and primersequences.

The data was de-replicated using unique.seqs and aligned to the silva.bacteria 16S rRNA reference file by means of align.seqs (Pruesse, Quast et al. 2007). Sequences that had more than two ambiguous bases, that did not start by position 1044 (97.5 % - tile) or end by position 7000 (2.5 % - tile) were removed using the screen.seqs command. Any columns with a '-' in every sequence were removed using filter.seqs and any further redundant sequences were removed using unique.seqs again. Pre.cluster was used to merge sequences that were within 1 bp per 100 bp of total sequence length of a more abundant sequence with that sequence.

Chimerae were detected using chimera.uchime and removed using remove.seqs. The classify.seqs command was used to classify sequences using the HOMD version 10 reference sequence and taxonomy databases. The dist.seqs

program calculated uncorrected pairwise distances between aligned DNA sequences and the cluster command was used to assign sequences to OTUs. Following this, a table was created indicating the number of times an OTU was present in each sample using the make.shared command. A randomised normalisation to the same sample size was performed using the command sub.sample where appropriate. The classify.otu command was used to obtain a consensus taxonomy for each OTU at a value of 98.5 % (or 0.015) using the HOMD version 10 reference sequence and taxonomy databases.

2.3.2.5.6.3 Statistical analysis

The α and β diversity of samples was estimated in mothur for datasets from both Sanger and pyrosequencing. The collect.single command was used to calculate the Chao1 richness and the inverse Simpson diversity index, while the rarefaction.single command was used to compile rarefaction curve data. A table containing the number of sequences, sample coverage, number of observed OTUs and the Inverse Simpson diversity estimate was compiled using the summary.single command. Uncorrected pairwise distances between aligned DNA sequences were calculated using dist.seqs and were used to compare the community membership and structure by Jaccard index and theta YC metric, respectively. The obtained matrices were visualised by the principal coordinate analysis (PCoA). The corr.axes command listed correlation coefficient for each OTU to the axes displayed in a PCoA file, with corresponding p value. The phylogenetic comparison of communities was also performed by weighted and unweighted UniFrac analysis. Finally, the amova command was used to analyse nonparametric analog of traditional variance (Excoffier, Smouse et al. 1992).

To complement the analyses implemented in mothur, Wilcoxon signed-rank test with cut-off p value of 0.05 was used for statistical comparison of α and β-diversity (Wilcoxon 1946). Differences in relative abundances of individual OTUs were determined with LEfSe (Segata, Izard et al. 2011).

2.4 Results

2.4.1 Validation of the co-culture experiment

A combination of five bacterial species - *Streptococcus intermedius, Parvimonas micra, Fusobacterium nucleatum* ss *nucleatum, Prevotella buccae* and *Porphyromonas endodontalis* - was used to assess the ability of Cooked Meat Medium (CMM) to sustain a mixed community for 11 weeks (Figure 1).

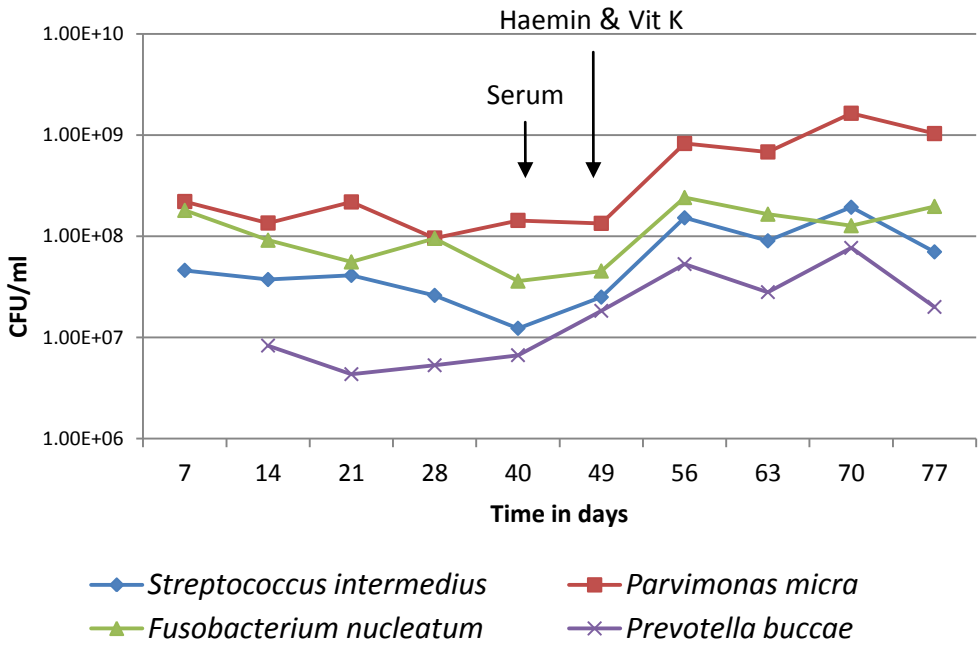


Figure 1. Bacterial counts in a defined member community in Cooked Meat medium.

P. endodontalis was not detected at any sampling time. *P. buccae* was not detected at 7 d of mixed culture, but was detected from 14 d. The other three species were detected at all time points. In the first phase of the experiment (up to

40 d), the number of CFU/mL of these 4 species slightly decreased, by a factor between 1.2 and 5.0 (*P. buccae* and *F. nucleatum*). *P. micra* was the most numerous, with a mean count of $1.6x10^8$ CFU/mL, followed by *F. nucleatum* ($9.2x10^7$), *S. intermedius* ($3.3 x10^7$) and finally *P. buccae* ($6.2 x10^6$, on the period 14 to 40 d). In the second phase, after supplementation with horse serum, haemin and vitamin K, the number of CFU/ml of all four species increased, by factors varying from 3.0 for *P. buccae* to 7.2 for *P. micra*, with no change in their relative proportions. There was no significant difference for any species when cultures before supplementation (7-40 d) were compared to supplemented cultures (49-77 d) (Wilcoxon signed rank test).

The successful establishment of a simple mixed community in this validation experiment suggested that CMM was a suitable medium for in-vitro growth of a complex microbial community and that its supplementation with horse serum, haemin and vitamin K would improve the recovery rate of initial inoculum.

2.4.2 Samples from endodontic infections

Two endodontic samples, A1 and A2, were collected from two infected teeth in a 55 year-old male patient. A third sample, B (male, 64), was heavily contaminated with blood and the DNA extracted from this sample could not be amplified by PCR, even after purification by phenol/chloroform method or on columns. After one week of growth in CMM, the appearance of the culture was unusual in that the supernatant remained clear but hundreds of small white aggregates, 0.5 to 1.5 mm in diameter, were visible on the surface of the meat particles (Figure 2). The composition of the CMM culture derived from sample B

was analysed by cloning and sequencing and 70 % of clones were identified as *Actinomyces israelii*. The other species detected were: *Parvimonas micra* 17 %, *Mogibacterium diversum* 6 %, *Eubacterium infirmum* 2 %, *Olsenella uli* 2 % and *Peptostreptococcus stomatis*, *Pseudoramibacter alactolyticus* and *Streptococcus constellatus* at 1 %. Given the dominance of *A. israelii*, it was considered that this was a case of actinomycosis and not a typical endodontic infection. Analysis of sample B was therefore discontinued. A fourth sample, C, was obtained from a 54 year-old male.

Figure 2. Bacterial growth in CMM. The vial on the right contains a typical multi-microbial community (Sample A1) while the vial in the centre contains white aggregates typical of *Actinomyces* growth (Sample B). An uninoculated vial is shown on the left.

The bacterial composition of samples A1, A2 and C was analysed by PCR / cloning / Sanger sequencing and by direct 454 pyrosequencing. In addition, the composition of sample A1 was also analysed by anaerobic and aerobic culture.

2.4.2.1 Analysis of 16S rRNA profile data

A summary of the α-diversity of the endodontic samples analysed by cloning and Sanger sequencing and pyrosequencing is shown in Table 3. Ninety clones were analysed per sample and 6078 sequences were obtained in total from the pyrosequencing analysis after de-noising and trimming. Two chimeric sequences were detected by uchime among the pyrosequence data, both of which were from sample A2. No chimeric sequences were detected in other samples. Of the remaining 6076 sequences, 1580 belong to sample A1, 3366 to A2 and 1127 to C. The pyrosequence data were normalised by random sub-sampling to 1127 sequences per sample, although phylogenetic analysis was carried out on the complete dataset.

The α-diversity analysis of datasets obtained by Sanger or pyrosequencing is presented in Table 3. The pyrosequencing analysis detected 3 to 4 times more observed OTUs than the clonal analysis, but the Good's coverage values are very similar, with all coverage estimates over 90 %. In contrast, the CatchAll estimator calculated coverage is lower than the Good's coverage value for samples A1 and A2, but not for sample C, which is less OTU-rich than the others and the estimate of OTU richness is the same as the observed value. For samples A1 and A2, the CatchAll estimates are substantially higher for the pyrosequencing data. The Chao1 estimate of richness gives estimates of a similar order to those obtained by CatchAll

except that the value for the pyrosequencing data for sample C is substantially higher at 117 compared to 33 from CatchAll.

Table 3. α-diversity and richness of endodontic sequence data for samples A1, A1 and C, analysed by Sanger sequencing (Sanger) or 454 pyrosequencing (py). Detailing number of sequences for each subject (Nseqs), observed OTUs (S_{obs}), Good's coverage, CatchAll richness estimate, coverage estimate based on CatchAll value, Chao1 richness estimator and the Inverse Simpson diversity index.

Sample	N seqs	S_{obs}	Good's coverage	CatchAll estimate	CatchAll cov. %	Chao1	invsimpson
A1 py	1127	60	0.98	114.3	52.49	88.88	16.49
A1 Sanger	90	16	0.98	19.5	82.05	16.2	9.89
A2 py	1127	62	0.98	109.9	56.41	120.50	12.84
A2 Sanger	90	19	0.91	24.1	78.84	26	9.96
C py	1127	33	0.98	33	100	117.33	2.90
C Sanger	90	9	0.98	9	100	9.5	4.51

Finally, the Inverse Simpson diversity index is higher for the pyrosequencing data compared to clonal analysis for samples A1 and A2, but smaller for sample C. Because of the small number of samples analysed, it was not appropriate to perform statistical analysis on these α-diversity estimates.

Rarefaction curves for the three endodontic samples analysed by cloning and Sanger sequencing are shown in Figure 3. Samples A1 and A2 exhibit similar initial curves but the curve for A1 flattens earlier. The curve for the sample C has a shallower initial slope and lower plateau, reflecting the lower number of OTUs detected.

Rarefaction curves for the pyrosequencing data are shown in Figure 4. The curves for samples A1 and A2 are very similar, while the curve for sample C is again different, reflecting the lower richness of this sample. None of the curves level out completely, suggesting that additional sequencing effort would reveal greater richness.

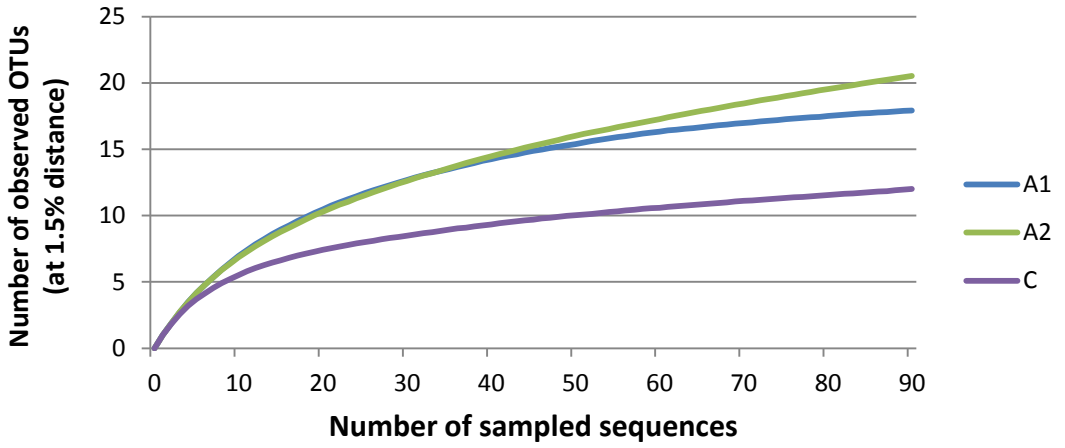


Figure 3. Rarefaction curves for endodontic samples A1, A2 and C analysed by cloning / sequencing.

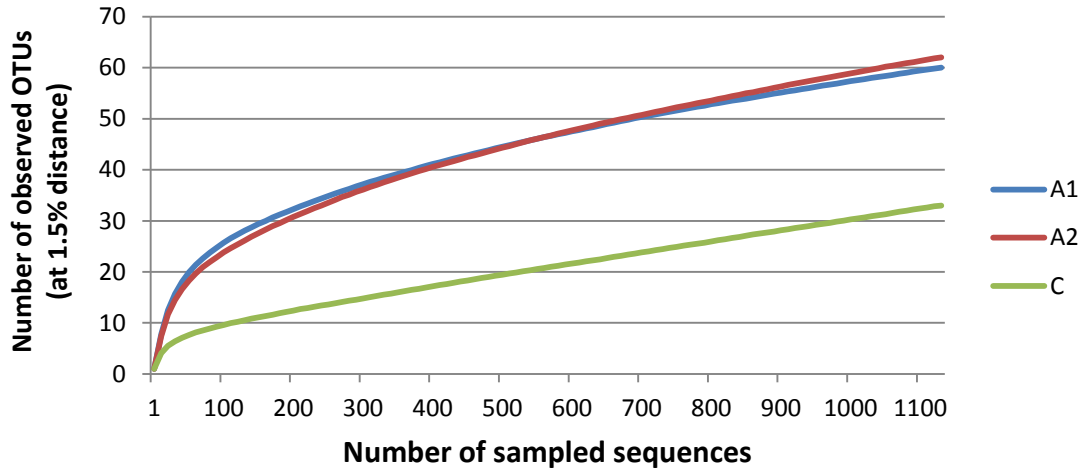


Figure 4. Rarefaction curves for endodontic samples A1, A2 and C analysed by 454 pyrosequencing.

2.4.2.2 Cultural analysis of the sample A1

A total of 90 isolates from anaerobic incubation of FAAB were identified by 16S rRNA gene sequence analysis. The number of observed OTUs was of 18, higher than the corresponding value for the cloning / sequencing method. The estimated Catchall richness was 24.1, with an estimated coverage of 75 %, while the Good's coverage value was of 0.92. The Chao1 value was higher than that of the cloning / sequencing method (23.3 to 16.2), but the diversity index by Inverse Simpson was lower (7.7 to 9.89). No colonies were observed on plates incubated in aerobic conditions.

One of the isolates was identified as not-yet-cultured *Prevotella* sp. HOT300. Its growth was dependent on the presence of helper strain *P. acnes* (Figure 5). The full length sequence of the 16S rRNA gene showed 99.2 % identity to that of the HOMD reference sequence for *Prevotella* sp. HOT300. This isolate was submitted to HOMD for genome sequencing as it was the first cultivated strain for this taxon.

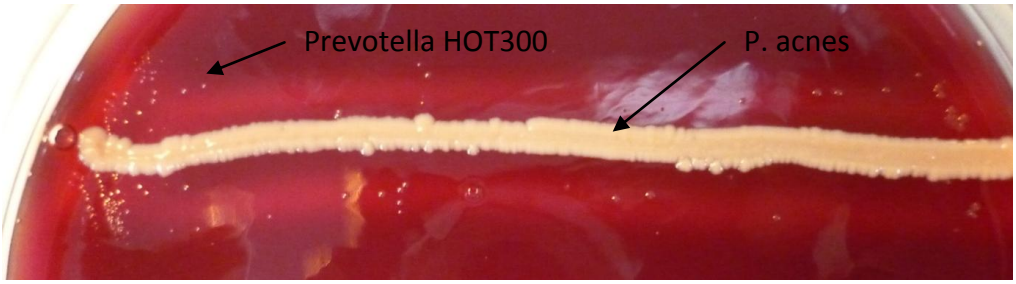


Figure 5. The growth of *Prevotella* sp. HOT300 was dependant on the presence of helper strain.

2.4.2.3 Phylogenetic assignment

The phylogenetic identification of the sequences is shown in Table 4 and Figure 6. All samples were dominated by the phylum *Firmicutes*, which comprised 61 % of the sequences. Representatives of the phyla *Bacteroidetes*, *Fusobacteria*, *Actinobacteria*, *Spirochetes*, *Synergistetes*, *Proteobacteria*, *Tenericutes* and TM7 were also detected. Of these, only *Firmicutes*, *Actinobacteria*, *Bacteroidetes* and *Tenericutes* were detected by culture of sample A1, with *Actinobacteria* overrepresented compared to the molecular methods.

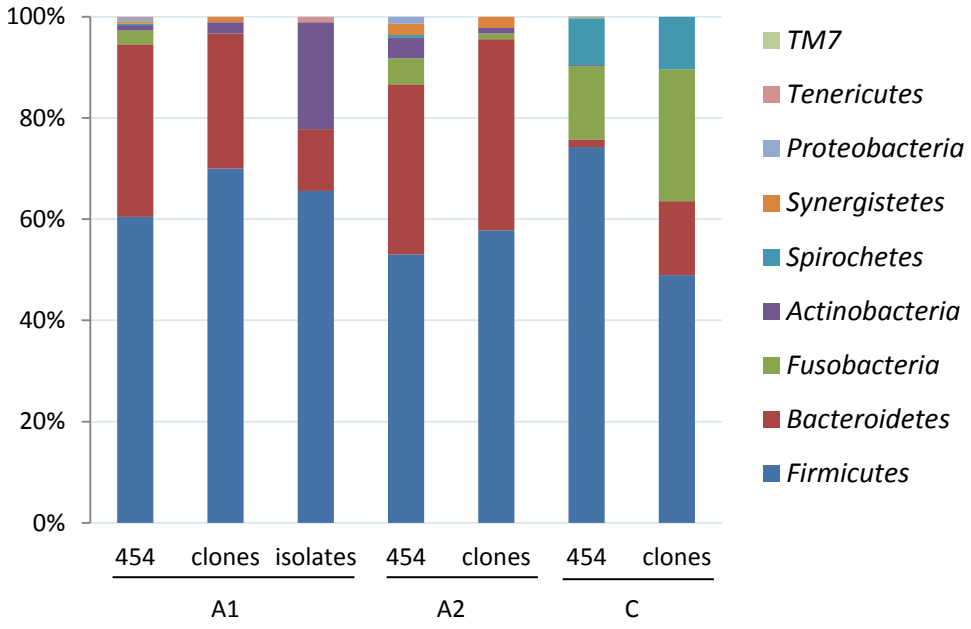


Figure 6. Bacterial composition of endodontic samples at phylum level, analysed by 454 pyrosequencing, cloning and Sanger sequencing, and isolation.

The composition of each sample is shown in the phylogenetic trees in Figure 7 to Figure 11. The cut-off used for species identification was 99 %. Where there were several hits of over 99 %, all are listed, separated by slash symbols.

Table 4. Number of taxa detected and the percentage of the sequences for each phylum in endodontic samples A1, A2 and C, analysed by pyrosequencing (py), cloning and Sanger sequencing (Sanger), and isolation (I).

Samples **Phylum**	A1 py	A1 Sanger	A1 I	A2 py	A2 Sanger	C py	C Sanger	Total/ mean
Firmicutes	20 60.4	9 69.9	10 62.1	22 53.0	9 57.8	10 74.1	4 48.9	33 60.9
Bacteroidetes	18 34.1	4 26.7	4 13.3	12 33.6	5 37.7	6 1.5	1 14.6	22 23.1
Fusobacteria	2 2.6			3 5.1	1 1.1	3 14.5	2 26.0	4 7.0
Actinobacteria	3 1.1	1 2.2	3 24.4	4 4.0	1 1.1	2 0.3		7 4.7
Spirochetes	2 0.3			4 0.6		2 9.2	2 10.4	6 2.9
Synergistetes	2 0.5	1 1.1		2 2.2	1 2.2	1 0.2		2 0.9
Proteobacteria	5 0.8			8 1.5		1 0.2		9 0.4
Tenericutes	1 0.3		1 1.1					1 0.2
TM7						1 0.1		1 0.01
Total	53	15	18	55	17	26	9	

The most frequently detected species in sample A1 was *Dialister invisus* / HOTA97. It made up around 20 % of all identified sequences by both pyrosequencing and Sanger sequencing. The proportion recovered by culture was lower at 4.4 %. *Peptostreptococcus stomatis* / HOTE46 and *Pseudoramibacter alactolyticus* were the next most frequently seen, with approximately 7 % of

pyrosequencing and 15.6 and 13.3 % of Sanger-identified sequences, respectively. *Parvimonas micra* / HOT393 made up 7 % of pyrosequencing sequences and 22.2 % of identified isolates, but only 5.6 % of Sanger sequences. *Prevotella nigrescens*, *oris* and sp. HOT526 were the predominant *Bacteroidetes* species, with more than 20 % of total sequences for both culture-independent methods, but only *P. nigrescens* was detected by culture. *Olsenella uli* made up 20 % of identified isolates, but only 2.2 % of clones and was not detected by pyrosequencing.

Sample A2 was dominated by *Prevotella nigrescens*, with around 20 % of sequences for both Sanger and pyrosequencing methods. Another well represented *Bacteroidetes* taxon was *Bacteroidetes* sp. HOT365 / HOTG44 / HOT281, a member of a deep branch with no cultivated representatives. For the *Firmicutes* phylum, the dominating taxa were *Filifactor alocis, Pseudoramibacter alactolyticus* and *Peptostreptococcus stomatis* / HOTE46.

Peptostreptococcus stomatis / HOTE46 made up a substantial proportion of Sample C with 56.25 % of pyrosequencing and 35.4 % of Sanger sequences. The next commonest taxa were *Fusobacterium nucleatum* ss. *animalis*, with 13.5 and 25 %, and *Eubacterium yurii* / *Peptostreptococcaceae* sp. HOT106 (13.8 and 6.3 %). *Treponema* species (*T. maltophilum* and *T. vincentii*) were also well represented and made up 10 % of sequences obtained by Sanger sequencing and just under that for pyrosequencing.

No representatives of the *Proteobacteria* phylum or *Veillonella* or *Streptococcus* genera were detected in any of the samples by the traditional cloning and sequencing method or culture. They were detected only by pyrosequencing.

Mogibacterium diversum / *vescum* / *neglectum, Peptostreptococcus stomatis* / HOTE46, some of the *Prevotella* species, *Fusobacterium nucleatum* ss. *animalis* and *Fretibacterium fastidiosum* were among species detected in all three samples. *Dialister* species, *Lactobacillus catenaformis, Solobacterium moorei, Eubacterium infirmum* (and closely related *Peptostreptococcaceae* species), *Eubacterium nodatum* / HOTG32, *Pseudoramibacter alactolyticus, Atopobium rimae* and *Olsenella* species, *Fretibacterium* sp. HOT360 / HOT453 and *Campylobacter gracilis* were among species detected in both samples A but not in sample C. On the other hand, *Veillonella dispar* / *parvula* / HOTG30, *Capnocytophaga* species, *Actinomyces* species, *Fusobacterium naviforme*, *Treponema vincentii* and TM7 phylum representatives were detected only in sample C.

Figure 7 (page 100). The phylogenetic tree of taxa belonging to the phylum *Firmicutes* detected in endodontic samples. Numbers represent the percentage of sequences belonging to the corresponding taxon for each detection method.

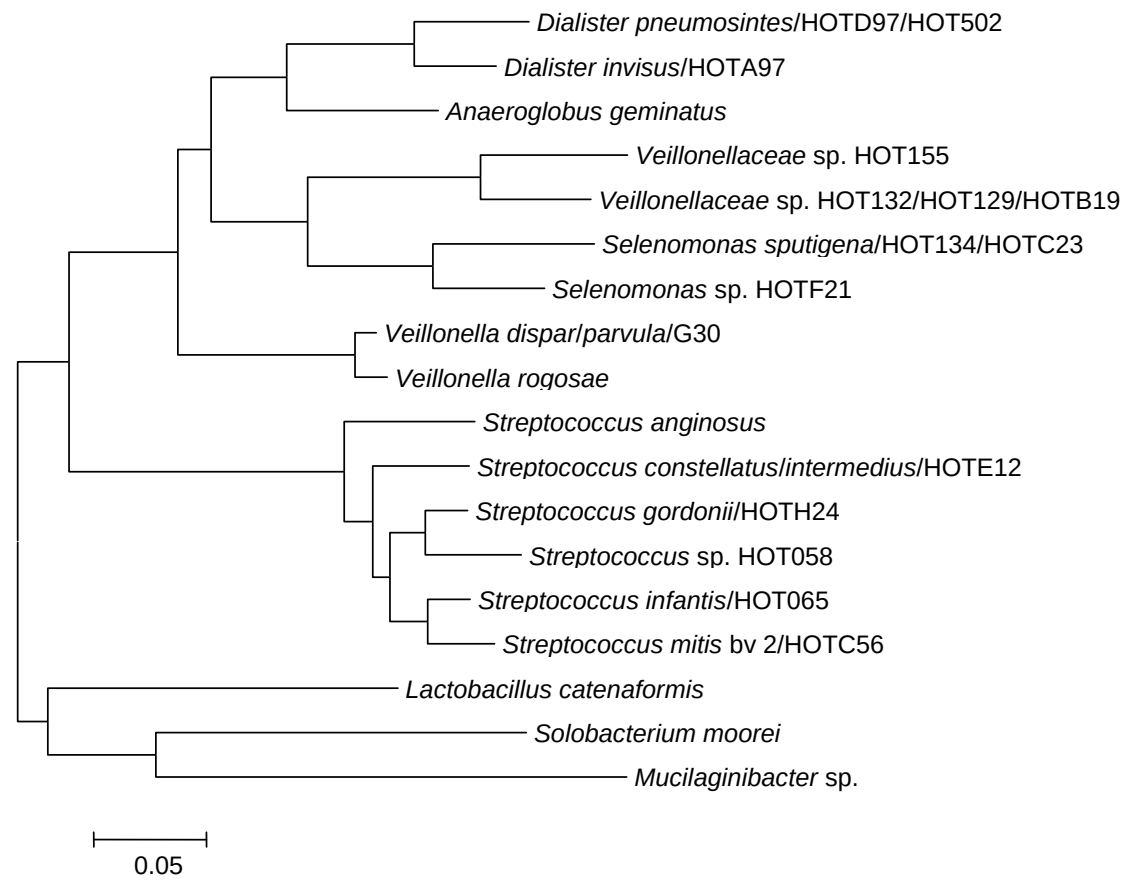
Figure 8 (page 101). The phylogenetic tree of taxa belonging to the phylum *Firmicutes* detected in endodontic samples (continued).

Figure 9 (page 102). The phylogenetic tree of taxa belonging to the phylum *Bacteroidetes* detected in endodontic samples.

Figure 10 (page 103). The phylogenetic tree of taxa belonging to the phylum *Actinobacteria, Fusobacteria, TM7* and *Synergistetes* detected in endodontic samples.

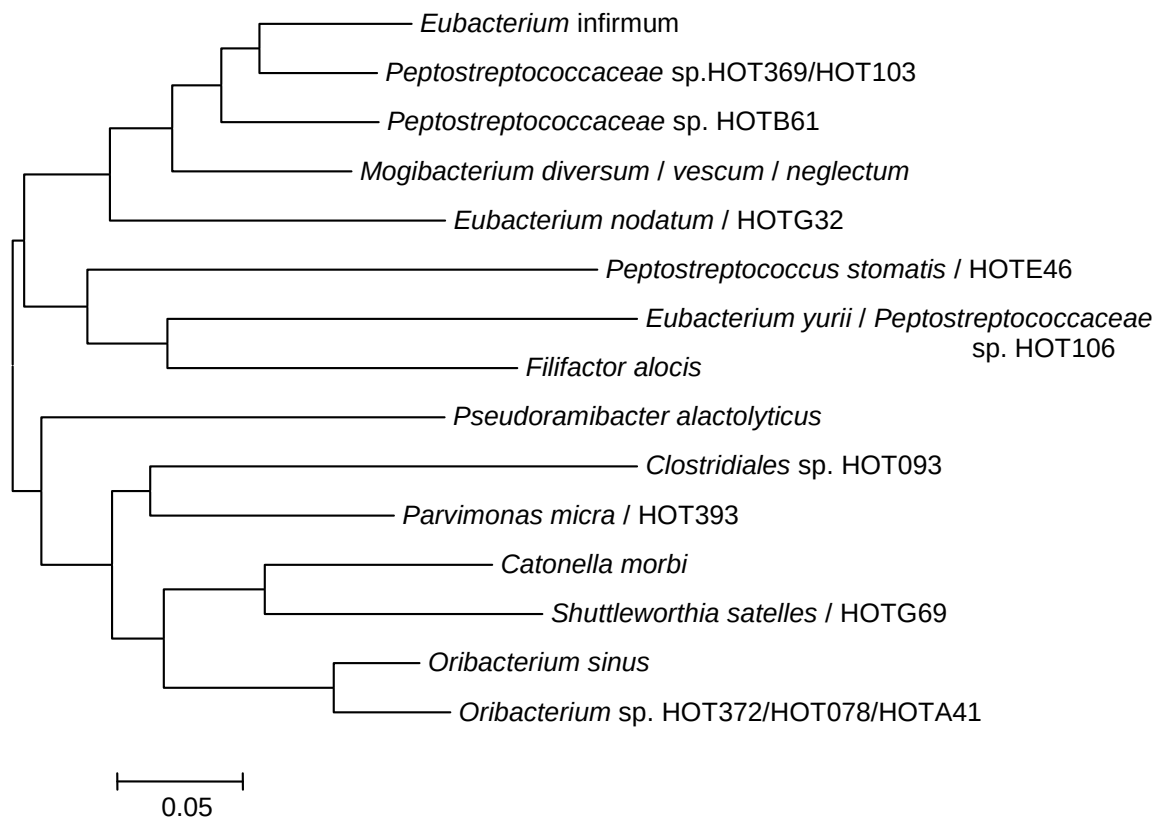
Figure 11 (page 104). The phylogenetic tree of taxa belonging to the phylum *Proteobacteria, Spirochetes* and *Tenericutes* detected in endodontic samples.

***Firmicutes* 1**



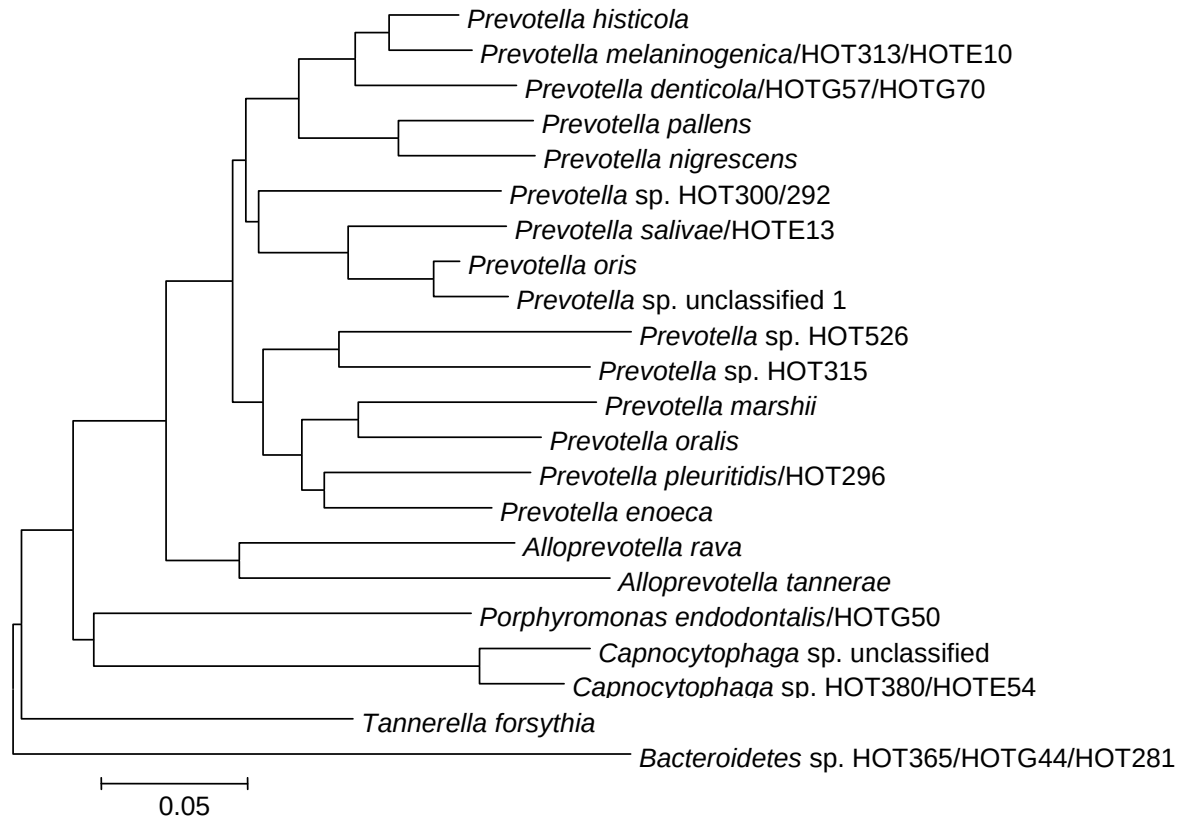
A1 py	A1 Sanger	A1 cult	A2 py	A2 Sanger	C py	C Sanger
2.72	3.3	2.2	4.21	1.1		
19.30	21.1	4.4	8.52	5.6		
					0.09	
0.76						
0.32			0.03			
0.19			0.36			
					0.09	
					0.27	
0.06						
					0.09	
			0.03			
					0.09	
			0.03			
0.06						
0.06						
3.80			0.92			
4.43		3.3	3.68			
			0.03			

Firmicutes 2



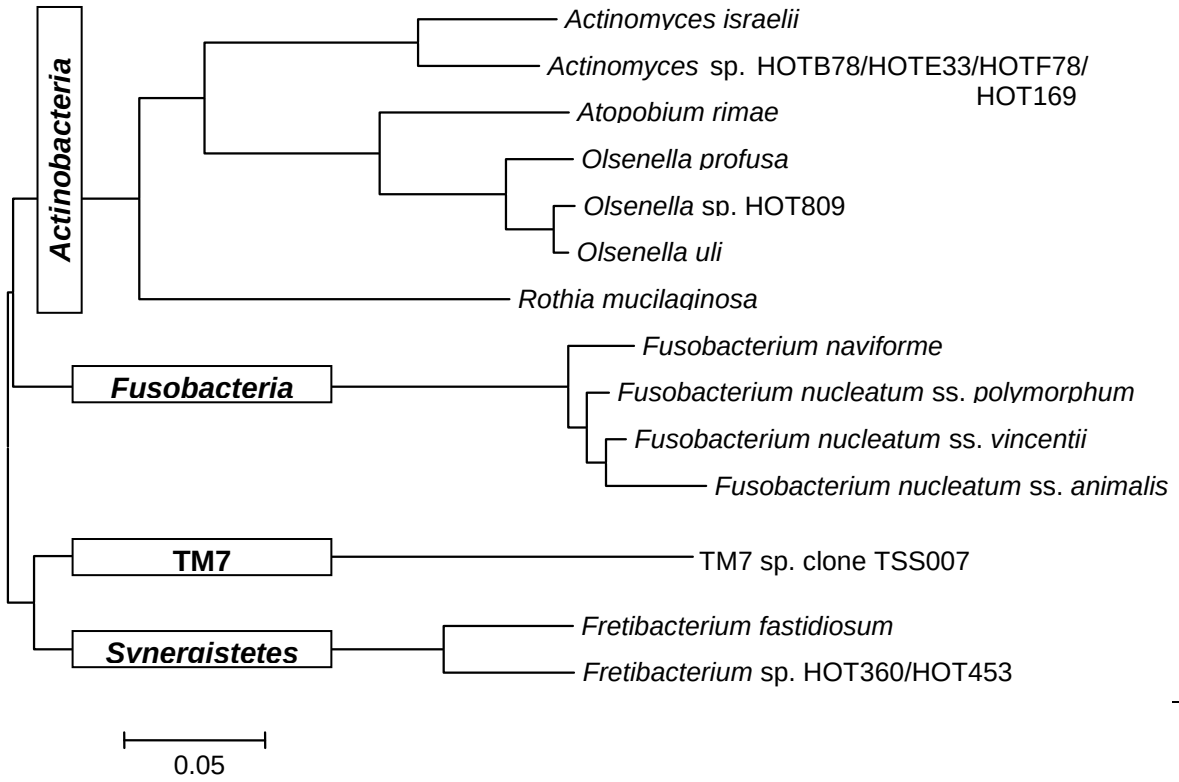
A1 py	A1 Sanger	A1 cult	A2 py	A2 Sanger	C py	C Sanger
2.03	4.4	4.4	0.21			
0.25						
			0.21			
3.10	3.3	6.7	4.46	8.9	3.19	6.3
1.39	2.2	1.1	0.68			
6.90	15.6	6.7	7.81	10.0	56.25	35.4
					13.84	6.3
			10.12	12.2	0.09	
7.03	13.3	7.8	7.78	15.6		
			0.03			
7.09	5.6	22.2	3.39	2.2		1.0
0.06						
0.76		3.3	0.47	1.1		
					0.09	
0.13	1.1		0.03	1.1		

Bacteroidetes



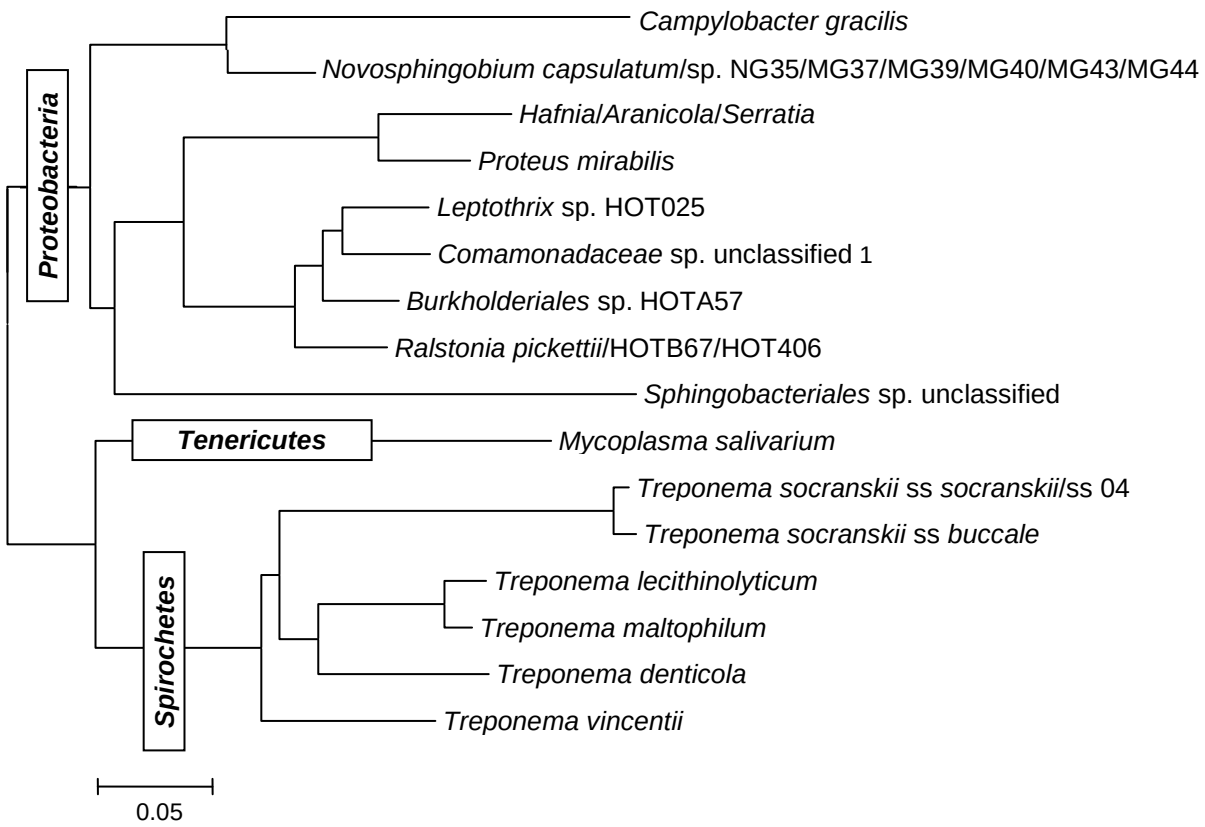
A1 py	A1 Sanger	A1 cult	A2 py	A2 Sanger	C py	C Sanger
0.06						
0.06						
0.13		1.1	0.47	1.1	0.09	
0.06						
9.50	5.6	7.8	19.12	21.1		
1.65		2.2	0.65		0.09	
0.06						
6.84	8.9		0.27		0.09	
0.06						
4.43	10.0		0.42	1.1		
0.25			0.27			
0.06						
1.90		2.2	0.06		0.09	
0.51			0.06			
3.80						
			0.50			
0.19			0.06			
4.11	2.2		2.85	2.2		
					0.09	14.6
					1.06	
0.38						
			8.85	12.2		

Actinobacteria – Fusobacteria – TM7 – Synergistetes



	A1 py	A1 Sanger	A1 cult	A2 py	A2 Sanger	C py	C Sanger
Actinomyces israelii						0.18	
Actinomyces sp. HOTB78/HOTE33/HOTF78/ HOT169						0.09	
Atopobium rimae	0.63		2.2	0.15			
Olsenella profusa				0.03			
Olsenella sp. HOT809	0.38			3.83	1.1		
Olsenella uli		2.2	20.0	0.03			
Rothia mucilaginosa	0.06						
Fusobacterium naviforme						0.71	
Fusobacterium nucleatum ss. *polymorphum*				0.03			
Fusobacterium nucleatum ss. *vincentii*				0.03		0.27	1.0
Fusobacterium nucleatum ss. *animalis*	2.59			5.05	1.1	13.49	25.0
TM7 sp. clone TSS007						0.09	
Fretibacterium fastidiosum	0.32	1.1		0.33		0.18	
Fretibacterium sp. HOT360/HOT453	0.13			1.90	2.2		

Proteobacteria – Spirochetes – Tenericutes



A1 py	A1 Sanger	A1 cult	A2 py	A2 Sanger	C py	C Sanger
0.38			1.01			
			0.03			
			0.03			
			0.03		0.18	
0.13			0.03			
0.06			0.06			
			0.06			
0.06			0.03			
0.19			0.18			
0.25		1.1				
0.13			0.21			
0.19						
			0.06			
			0.24		6.03	2.1
			0.06			
					3.19	8.3

The number of OTUs shared between samples, as analysed by mothur, is represented in Figure 12. The number of shared OTUs appears smaller than the number of shared taxa, as different samples might contain different variants, identified as separated OTUs by mothur.

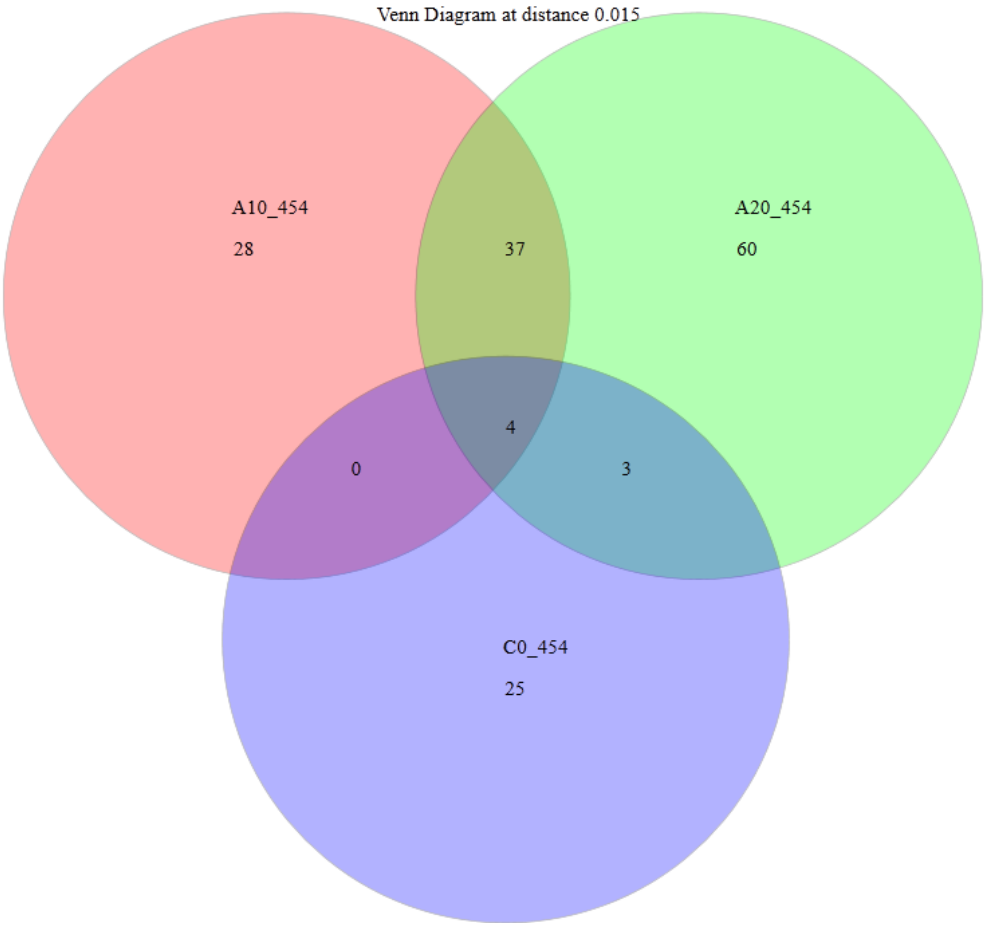


Figure 12. Venn diagram for Samples A1, A2 and C, analysed by 454 pyrosequencing. Numbers represent OTUs per sample and OTUs shared between samples.

The Jaccard index was calculated to compare the community membership for endodontic samples. The resulting distances between communities were visualised by hierarchical clustering in a dendrogram (Figure 13). For each sample, communities analysed by Sanger and pyrosequencing clustered together. Furthermore, Samples A1 and A2, obtained from the same patient, were more closely related between them than with the sample C. However, parsimony unweighted UniFrac analyses showed no significant differences between the samples or between patients (samples A1 and A2 versus sample C). The principal coordinate analysis (PCoA) of the Jaccard index distance matrix gave a similar clustering pattern (Figure 14). The genetic distance between the patients (A vs C) was found to be significant by Amova analysis ($p<0.001$).

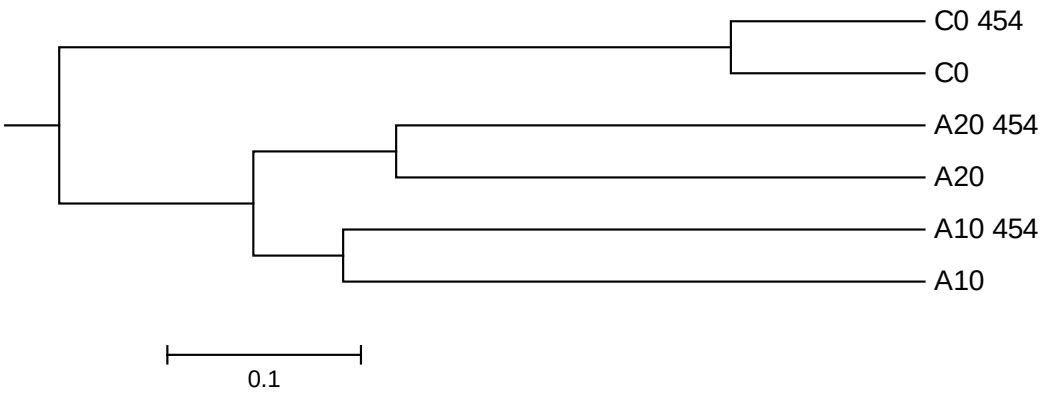


Figure 13. Dendrogram representing distances between endodontic samples communities calculated by the Jaccard index.

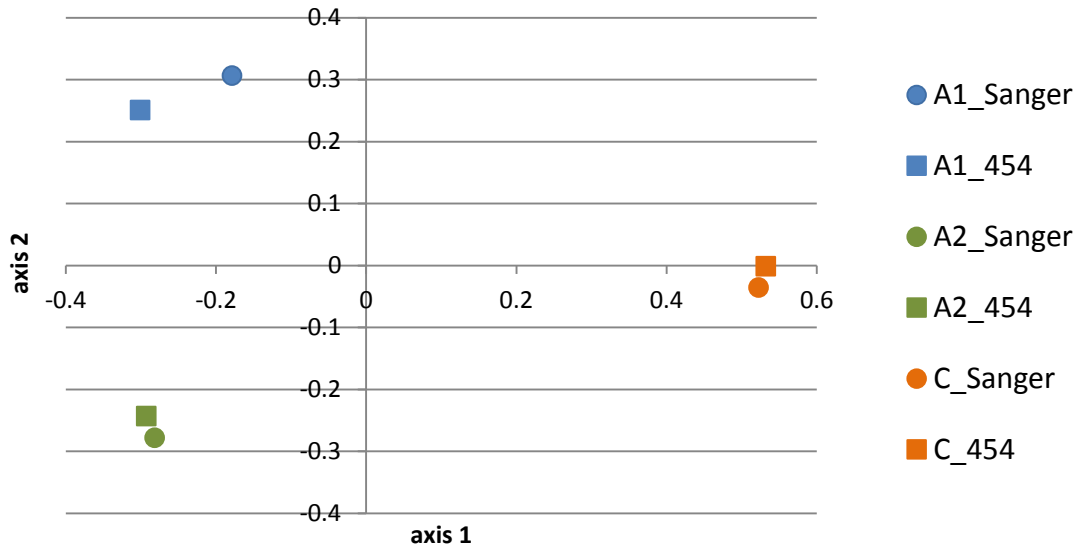


Figure 14. PCoA of the Jaccard index-calculated distances between the endodontic samples analysed by Sanger sequencing or 454 pyrosequencing.

The **corr.axes** command in mothur allows the calculation of the correlation coefficient between each OTU and the axes displayed in a PCoA file. It reveals the OTUs which drive the differences between certain samples. *Peptostreptococcus stomatis* and *Porphyromonas endodontalis* were the taxa responsible for driving the points coordinates in the positive and negative directions on the axis 1, respectively, with p values of 0 and 0.001. This would suggest that these OTUs are primarily responsible for the differences between patients A and C, with *P. stomatis* representative of the patient C and *P. endodontalis* of the patient A. Twenty one other OTUs, belonging to 5 phyla, however, had a p value less than 0.05 for axis 1. On axis 2 only *M. diversum* / *vescum* / *neglectum* had a p value of <0.03, driving the position in negative direction. Four other OTUs had a p value <0.05, all tending in the negative direction. Two were identified as *Filifactor alocis*, one as *Bacteroidetes* sp. HOT365 and one as unclassified *Flavobacteriales.* This would suggest that these

OTUs were associated with sample A2. The LEfSe analysis, however, found no OTUs significantly different between samples or patients.

The comparison of the community structure of samples was calculated with the theta YC metric. The dendrogram and PCoA obtained from this analysis were very similar to those obtained from the Jaccard index. The Amova analysis indicated significant difference not only between patients, but also between all three samples ($p<0.001$).

Samples were also compared based on their phylogenetic similarity by weighted and unweighted UniFrac analysis. The unweighted UniFrac analysis, comparing the community membership, showed a significant difference between patients (Amova $p<0.001$) but not between samples A1 and A2 ($p=0.344$). The weighted UniFrac analysis, comparing community structures by taking into account relative abundances of taxa, presented a significant difference between all patients and samples.

2.4.3 CMM culture of the endodontic samples

Samples A1 and A2 were cultured in CMM supplemented with serum, vitamin K and haemin, with passages every 10 d, up to 120 d of cumulative culture. At this point the interval between passages was increased to 30 d up to 210 d, after which it was reduced to 10 d, up to 480 d.

Sample C was cultured in CMM supplement with serum, vitamin K and haemin, with passages every 10 d up to 60 d, at which point two new vials were inoculated, one in the same conditions as before and one supplemented with

mucin. From this point onwards cultures of the sample C with and without mucin were analysed.

2.4.3.1 Statistical analysis for cloning / sequencing and 454 pyrosequencing

At 120 d, 1525 and 1094 sequences were obtained by pyrosequencing from samples A1 and A2, respectively. For sample C at 50 and 140 d, with and without mucin, 1369, 1678 and 840 sequences were analysed. The datasets were subsampled to 840 sequences for statistical analysis, but the phylogenetic analysis was performed on all sequences. Ninety sequences were obtained for each sample by the clone / Sanger analysis. Some time points from the CMM culture of the sample C had a lower number of sequences identified by Sanger sequencing and were subsampled to 46 sequences for statistical analysis.

The α-diversity analysis of datasets obtained by Sanger or pyrosequencing for endodontic samples and corresponding CMM cultures is presented in Table 5. For all samples the richness and the diversity of CMM cultures were lower than that of the endodontic samples from which they originated, with one exception discussed further. In all samples a further progressive loss of richness in culture was observed, with a reduction in the number of observed taxa, as well as a decrease of the CatchAll estimate, Chao1 and Inverse Simpson diversity index values. For samples A1 and A2 the largest reduction was observed between 120 and 150 d, corresponding to the increased incubation time. The subsequent reduction of the incubation time failed to re-establish the initial diversity. The inclusion of mucin in the sample C culture, however, increased the richness seen. At 110 and 140 d of culture with mucin the number of observed taxa (13 and 16), the diversity

estimated by CatchAll (15.3 and 19.5) and the Inverse Simpson diversity index (6.31 and 8.73) were higher than in the endodontic sample C (9 taxa, 9 and 4.51, respectively). It was also higher when compared to the corresponding samples without mucin, as the number of observed taxa were 8 at 110 and 140 d in this case, the same as CatchAll estimates; the Inverse Simpson diversity index was 2.51 at 110 d and 1.92 at 140 d.

The pyrosequencing data set of the culture of the sample C presents a different picture, with higher diversity observed at 140 d of culture, both with and without the addition of mucin, than in the original endodontic sample.

The Wilcoxon signed-rank test showed that values for all α-diversity indexes were significantly different between A1 and C CMM cultures, but only when values for C culture with mucin were not taken into account. All other compared sets returned no significant p values.

The rarefaction curves for Sanger analysis of the CMM cultures are represented in . Some of the curves, referring to cultures derived from samples A1 and A2, at 480 d and culture C at 30, 50 and 70 d, leveled out completely, suggesting that all the diversity was sampled. The rarefaction curves for the pyrosequencing analysed data, subsampled at 840 sequences, are represented in Figure 18. The curve for sample C CMM 50 d is the only curve to level out.

Table 5. α-diversity and richness of the endodontic infections A1, A2 and C and corresponding CMM cultures.

Samples and time points (d)		N seqs	S_{obs}	Good's coverage	CatchAll estimate	CatchAll cov. %	Chao1	invsimpson
Pyrosequencing data								
A1	0	840	54	0.98	114.3	52.49	75.86	16.69
	120	840	26	0.99	36.2	71.82	38	7.55
A2	0	840	57	0.97	109.9	56.41	120.25	14.05
	120	840	39	0.98	86.9	44.88	115.5	5.44
C	0	840	29	0.98	33	100	124	3.06
	50	840	17	0.99	22.8	74.56	22	4.37
	140	840	35	0.98	92.6	37.80	92	6.40
	140, mucin	840	33	0.98	77.1	42.80	52.5	10.22
Sanger data								
A1	0	90	16	0.98	19.5	82.05	16.2	9.89
	10	90	14	0.96	18.6	75.27	16	6.29
	20	90	14	0.94	17.3	80.92	16.5	6.62
	120	90	13	0.98	16.1	80.75	13.3	5.92
	150	90	9	0.96	9	100	12	3.35
	310	90	11	0.97	12.5	88.00	12.5	3.84
	480	90	5	1.00	ND	ND	5	1.55
A2	0	90	19	0.91	24.1	78.84	26	9.96
	10	90	18	0.91	24.6	73.17	32	6.68
	120	90	16	0.96	21.6	74.07	17.2	7.60
	150	90	13	0.94	15.6	83.33	23	6.42
	310	90	7	0.98	7	100	8	2.93
	480	90	5	0.99	5	100	5	1.50
C	0	90	9	0.98	9	100	9.5	4.51
	10	46	7	0.96	7	100	8	4.11
	20	46	7	1.00	7	100	7	4.28
	30	90	5	0.98	5	100	6	2.07
	50	46	5	0.98	5	100	5	2.39
	70	46	6	0.98	6	100	6	3.47
	70, mucin	46	5	1.00	5	100	5	4.04
	110	90	8	0.98	8	100	8.3	2.51
	110, mucin	90	13	0.95	15.3	84.97	19	6.31
	140	90	8	0.98	8	100	8.3	1.92
	140, mucin	90	16	0.93	19.5	82.05	21	8.73

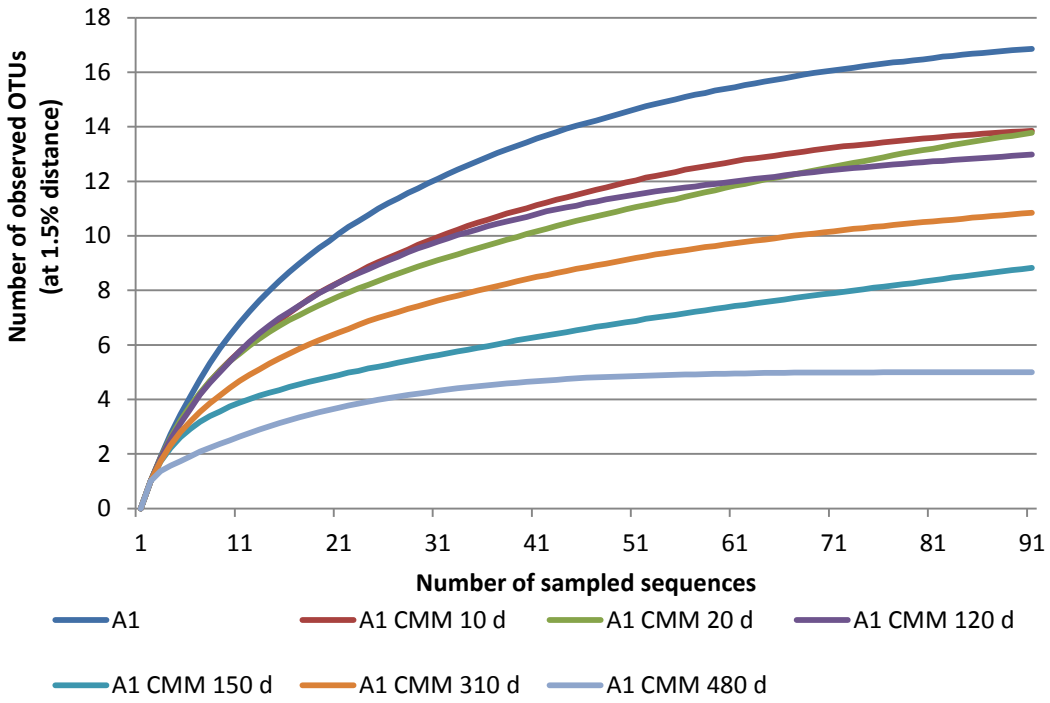


Figure 15. Rarefaction curves for the endodontic sample A1 and derived CMM cultures analysed by cloning / sequencing.

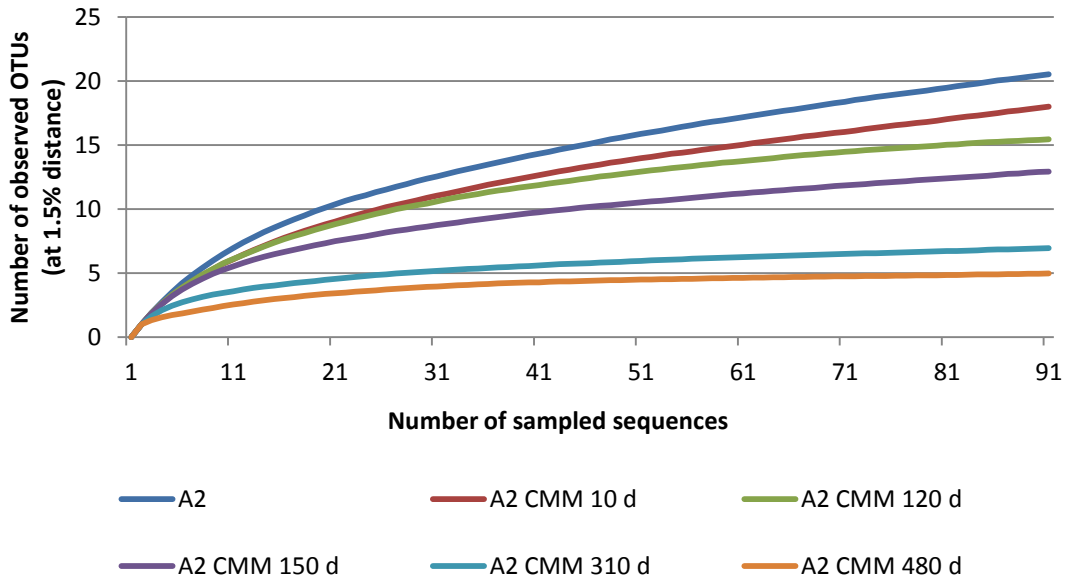


Figure 16. Rarefaction curves for the endodontic sample A2 and derived CMM cultures analysed by cloning / sequencing.

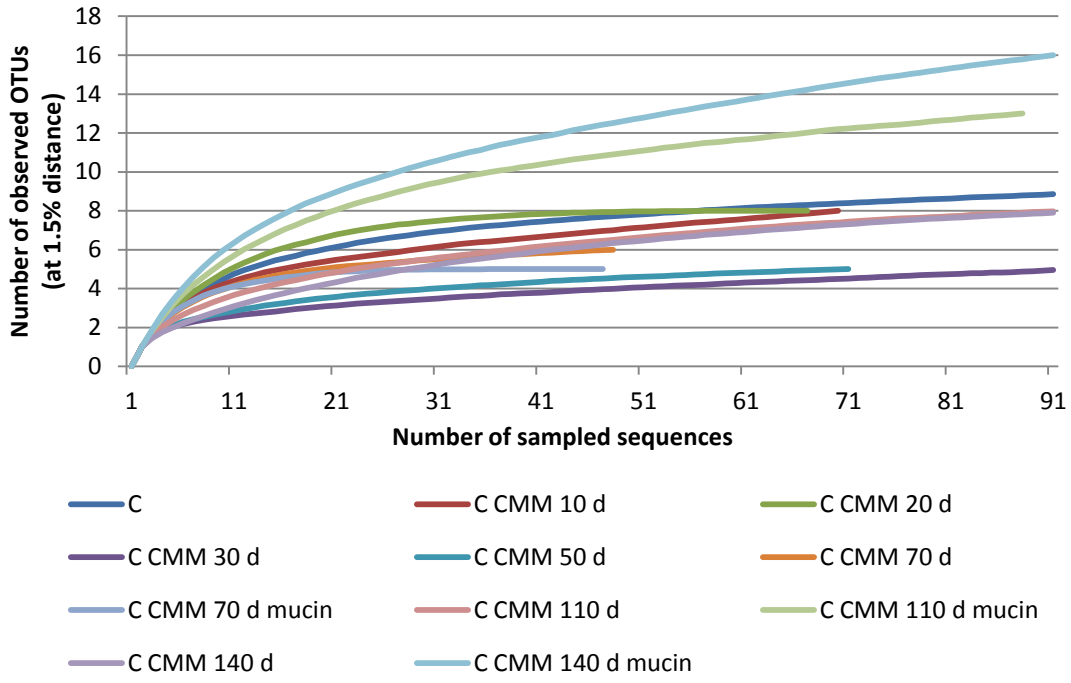


Figure 17. Rarefaction curves for the endodontic sample C and derived CMM cultures analysed by cloning / sequencing.

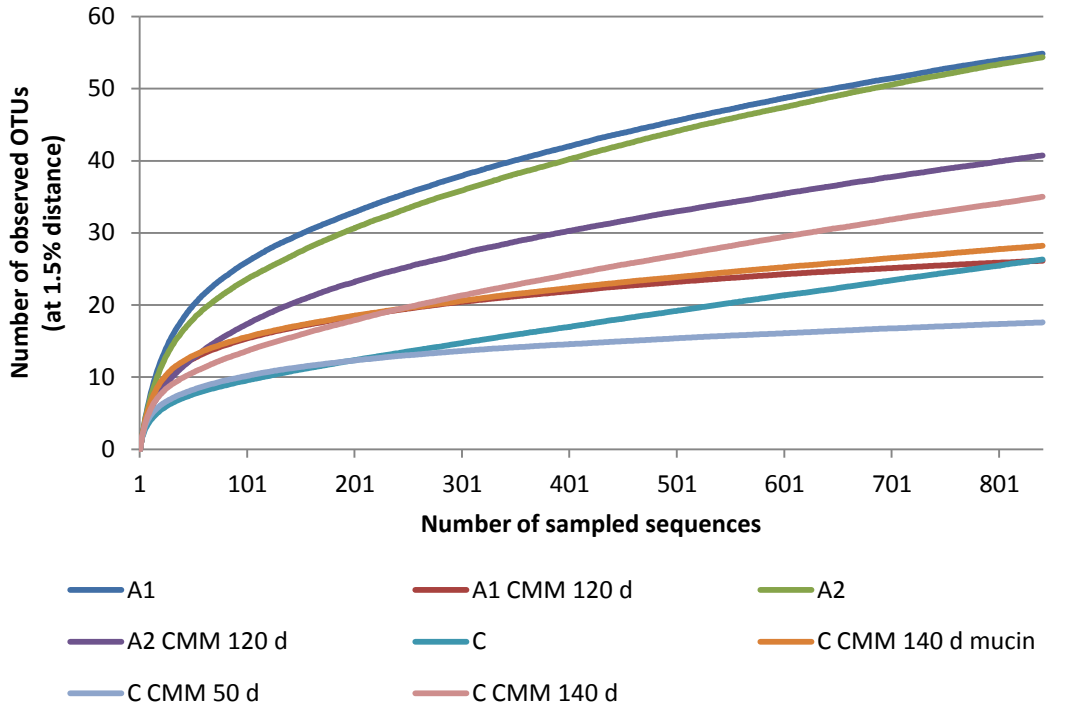


Figure 18. Rarefaction curves for the endodontic samples A1, A2 and C and derived CMM cultures analysed by pyrosequencing.

2.4.3.2 Phylogenetic analysis

The composition of the CMM culture samples over time is presented in Appendix 1.

2.4.3.2.1 CMM cultures of the samples A1 and A2

At 10 d culture A1 was analysed by Sanger analysis only. It was dominated by *Eubacterium nodatum*, with 30.0 % of sequences, followed by *Parvimonas micra* / HOT393 (22.2 %), *Pseudoramibacter alactolyticus* (11.1 %), *Mogibacterium* species (not distinguishable by 16S rRNA gene sequencing, 7.8 %), and *Peptostreptococcus stomatis* (5.6 %).

At 20 d, the numbers of *P. micra* remained high at 25.6 %, but those of *E. nodatum* were reduced to 12.2 %. Two other taxa increased in numbers at this time point, *Anaeroglobus geminatus*, at 18.9 %, and *Prevotella oralis* (17.8 %).

At 120 d, culture A1 was still dominated by *A. geminatus* and *P. oralis*, analysed by both Sanger and pyrosequencing. Other taxa found in significant numbers were *Dialister invisus* / HOTA97 (13.0 and 10 %), *Campylobacter gracilis* (9.0 and 2.2 %) and *Eubacterium infirmum* (8.0 and 10.0 %).

None of the taxa mentioned above were detected when the incubation time was increased to 30 d, at the 150 d time point, apart from *E. infirmum*, at 1.1 %. The predominant species were similar to those seen at 10 d of culture: *Mogibacterium* species (43.3 %) followed by *P. micra* (24.4 %) and *E. nodatum* (20.0 %). A similar distribution was also seen at 310 d, although the incubation time was reduced to 10 d. At 480 d *P. micra* made up 82.2 % of the total, with only 4 other species present:

Pseudoramibacter alactolyticus (6.7 %), *Eubacterium infirmum* (5.6 %), *Olsenella* sp. HOT809 (3.3 %) and *Mogibacterium* sp. (2.2 %).

The CMM culture derived from sample A2, at 10 d, was predominantly composed of *Mogibacterium* species (31.1 %), *Streptococcus constellatus* / *intermedius* / HOTE12 (15.6 %), *P. micra* (12.2 %), *Filifactor alocis* (12.2 %), *Pseudoramibacter alactolyticus* (6.7 %) and *Olsenella* HOT809 (6.7 %). After 120 d, the most frequently detected species were *Fusobacterium nucleatum* ss. *animalis* (33.4 % by pyrosequencing, 15.6 % by Sanger), *Anaeroglobus geminatus* (17.7 and 21.1 %, respectively), *S. constellatus* (9.9 and 22.2 %) and *P. micra* (6.21 and 12.2 %). The numbers of *Mogibacterium* sp. were reduced to 1 % of sequences by both methods at 120 d, but the proportions increased after the change to 30 d between passages, with 25.6 % at 150 d. Other species which also increased in numbers were *F. alocis* (23.3 %), *P. micra* (20.0 %) and *E. nodatum* (15.6 %). Reverting to 10 d incubation did not restore the bacterial composition to the equivalent of earlier stages. The diversity was further reduced to 5 species and dominated by S. constellatus (52.2 %) and *P. micra* (26.7 %). As with the A1 sample, at 480 d *P. micra* dominated, making up 80 % of sequences.

2.4.3.2.2 CMM cultures of the sample C

The culture derived from sample C was dominated by *Veillonella dispar* / *parvula* / HOTG30 species. Detected at less than 1 % in the original sample, it represented between 43.1 and 92.4 % of all sequences up to 110 d. At 50 d it made up 90.0 % of Sanger-analysed clones, but only 43.1 % of pyrosequencing sequences. Other species constituting a significant proportion of the culture were *Eubacterium*

yurii / HOT106 (23.2 and 16.9 %) and *F. nucleatum* ss. *animalis* (20.3 and 9.2 %) at 10 d and 20 d, respectively and *Streptococcus* sp. HOT058 (5.4 %) at 30 d. At 50 d, the composition was analysed both by pyrosequencing and Sanger sequencing. Only 3 other species were detected by Sanger sequencing apart from *V. dispar*: *Streptococcus* sp. HOT058 (5.7 %), *F. nucleatum* ss. *vincentii* (2.9 %) and *Peptostreptococcus stomatis* (1.4 %). *V. dispar* made up 43.1 % of pyrosequencing-analysed sequences, followed by *F. nucleatum* ss. *animalis* (22.3 %), *Streptococcus* sp. HOT058 (13.7 %), *E. yurii* (8.4 %) and *P. stomatis* (7.6 %). In CMM culture without the addition of mucin *V. dispar* dominated the culture up to 110 d, with *Streptococcus* sp. HOT058, *E. yurii* and *F. nucleatum* ss. *animalis* also present. At 140 d, however, the dominant species was *P. micra* (40.4 % of pyrosequencing sequences and 72.6 % of Sanger sequences). Other species detected by pyrosequencing mainly included *E. yurii* (19.3 %), *P. alactolyticus* (16.7 %) and *Clostridiales* sp. HOTC47 (13.1 %), while in the Sanger analysis the only other species above 10 % was *Peptoniphilus indolicus* (14.7 %).

After 60 d of culture a subsample was inoculated in CMM supplemented additionally with mucin. At 70 d only 3 taxa were identified in the mucin-supplemented culture: *V. dispar* (47.7 %), *E. yurii* (38.6 %) and *Streptococcus* sp. HOT058 (13.6 %). At 110 d, 11 species were identified, including *Peptostreptococcus stomatis* and *Streptococcus gordonii*. At 140 d the microbiome was dominated by *Clostridiales* sp. HOTC47 (25.5 % by pyro- and 23.3 % by Sanger sequencing) and *V. dispar* (22.3 and 24.4 %), with 11 other species detected. Compared to non-mucin CMM culture, at 140 d mucin-supplemented CMM presented no *P. micra* and *P.*

alactolyticus species but large numbers of *V. dispar* and *F. nucleatum* ss. *animalis*, absent in the former.

The Jaccard index analysis visualised by PCoA shows that samples A1 and A2 and cultures derived from them are indistinguishable, but well separated from the sample C (Figure 19). The addition of mucin to the CMM culture of sample C had only a minor effect on the composition of the community. The parsimony analysis and unweighted UniFrac confirmed the significance separation by patient (both with $p=0.001$) and the absence of significant separation between sets A1 and A2 when samples and CMM culture data were combined or between endodontic samples and corresponding CMM culture. Finally, the Amova test showed that endodontic samples were not significantly different from the corresponding CMM cultures (A1 $p=0.055$, A2 $p=0.209$, C $p=0.438$), but were significantly different from any other CMM culture ($p<0.001$). When the same time points were considered for CMM culture of sample C with and without the addition of mucin, the difference was not statistically significant ($p= 0.453$).

OTUs responsible for the increased values on the axis 1 ($p<0.001$) were *Parvimonas micra*, *Pseudoramibacter alactolyticus*, *Mogibacterium* sp., *Eubacterium infirmum*, *Olsenella* sp. HOT809 and *Granulicatella adiacens*. Those responsible for a negative x value were *V. dispar* / *parvula* and *Streptococcus* sp. HOT058. This separation corresponds to the species representative of samples A1 and A2 and corresponding CMM cultures, for the first group, and for the sample C and the corresponding CMM culture for the second group. However no OTUs were found to differ significantly between samples by LEfSe analysis.

The analysis of the structure of the communities by theta YC showed a significant difference between patients (parsimony p=0.027, unweighted UniFrac p=0.034), but not between sets A1 and A2 when samples and CMM culture data were combined or between endodontic samples and their corresponding CMM culture.

When samples were compared based on their phylogenetic similarity by weighted and unweighted UniFrac the significance of separation between samples was confirmed (p<0.001), but not for any other compared sets.

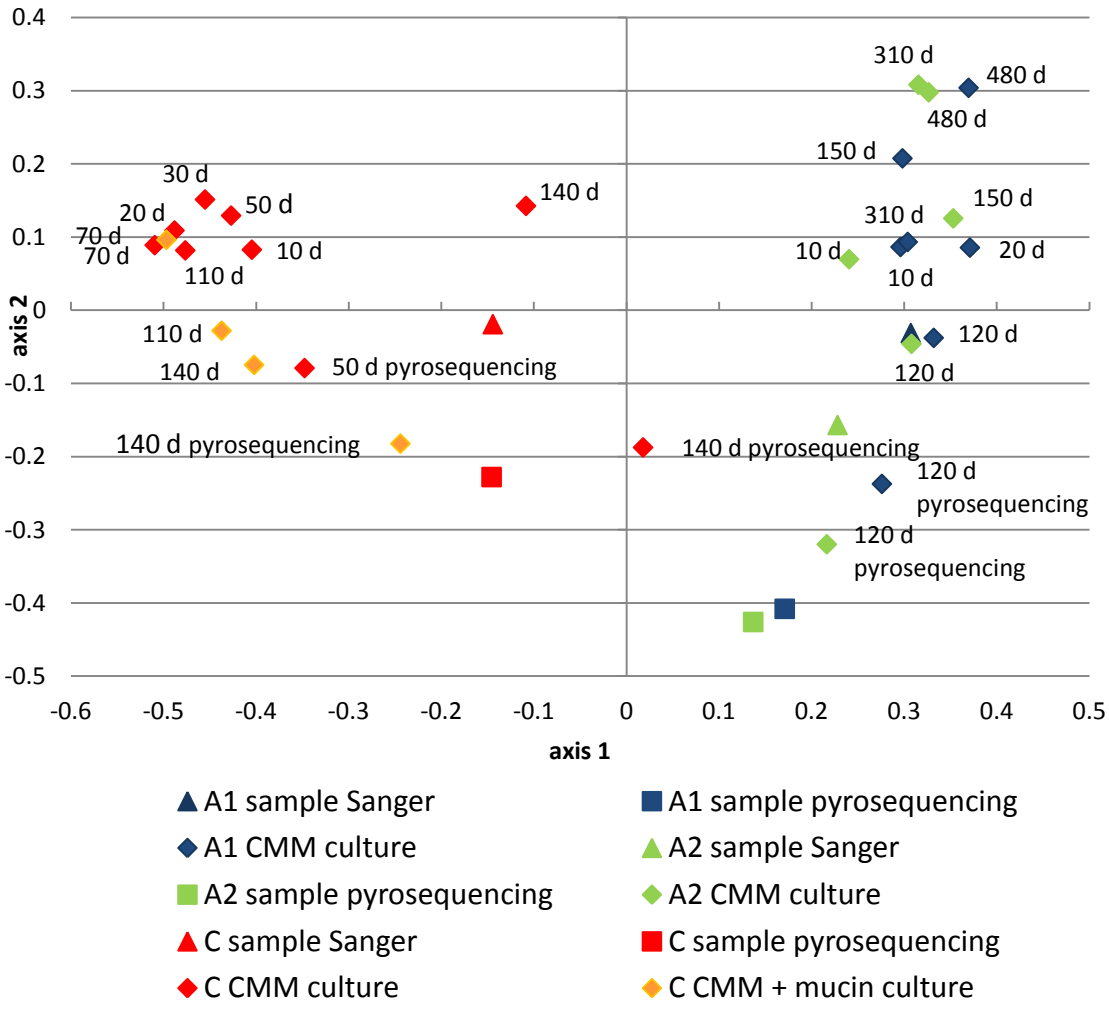


Figure 19. PCoA of the Jaccard index for endodontic samples and derived CMM cultures.

2.4.4 Uncultured bacteria in CMM culture

Several taxa of not-yet-cultured bacteria detected in the endodontic samples analysed in this study are described in Table 6. The table is separated in two parts, with the first including validated HOTs, well represented in molecular analyses but not cultivated. They are part of the main database of HOMD. The second part contains taxa that were detected in oral samples by other studies and are candidate HOTs, members of the extended set on HOMD. This section also includes taxa with only low similarity to any sequences in the database.

Some of these taxa persisted in the CMM culture, such as *Bacteroidetes* sp. HOT365 / HOTG44 / HOT281, detected at 120 d of CMM culture of sample A2 by pyrosequencing, at 0.20 %. *Peptostreptococcaceae* sp. HOT369 / HOT103 taxon was detected at 10 d of culture of sample A1 (2.2 %, Sanger) and at 120 d of culture of A2 (0.1 %, pyrosequencing) and *Actinomyces* sp. HOTB78 / HOTE33 / HOTF78 / HOT169 was detected at 120 d of A2 (0.1 %, pyrosequencing). *Prevotella* sp. HOT300 / HOT292 was also detected in the cultures of both A1 and A2, but it is not a genuine "uncultured" species, as an isolate identified as *Prevotella* sp. HOT300 by full-length 16S rRNA sequencing was isolated from the A1 endodontic sample in this study.

Some "uncultured" taxa were detected in CMM culture but not in the corresponding endodontic samples, such as *Actinomyces* sp. HOT172 and *Peptococcus* sp. HOTD92, both detected exclusively at 120 d of culture of the A2 sample (0.1 % of pyrosequencing sequences, both). In culture derived from sample C, some uncultured species made up a significant part of the microbiota. For

instance, in the pyrosequencing analysis, *Clostridiales* sp. HOTC47, *Finegoldia* sp. HOTD24 and *Treponema* sp. HOT258 were 25.46 %, 5.97 % and 4.63 % of the total, respectively.

Table 6. Uncultured species in the endodontic samples and their proportion of the total number of bacteria (%). *An isolate was identified during culture analysis of the A1 endodontic sample.

	A1		A2		C
Uncultured phylotypes	py	Sanger	py	Sanger	py
Bacteroidetes sp. HOT365 / HOTG44 / HOT281			8.85	12.2	
Clostridiales sp. HOT093			0.03		
Fretibacterium sp. HOT360 / HOT453	0.13		1.90	2.2	
Leptothrix sp. HOT025	0.13		0.03		
Peptostreptococcaceae sp. HOT369 / HOT103	0.25				
Prevotella sp. HOT300 / HOT292*	1.65		0.65		0.09
Prevotella sp. HOT315	0.25		0.27		
Prevotella sp. HOT526	4.43	10.0	0.42	1.1	
TM7 sp. clone TSS007					0.09
Actinomyces sp. HOTB78 / HOTE33 / HOTF78 / HOT169					0.09
Burkholderiales sp. HOTA57			0.06		
Capnocytophaga sp. HOT380 / HOTE54					1.06
Mucilaginibacter sp.			0.03		
Peptostreptococcaceae sp. HOTB61			0.21		
Ralstonia sp. HOTB67	0.06		0.03		
Selenomonas sp. HOTF21					0.09

2.5 Discussion

Culture-independent molecular methods offer a reliable way to detect the majority of bacteria present in different environments. But the potential biases associated with these methods cannot be disregarded. These can be related to DNA extraction, PCR amplification and sequencing itself. For DNA extraction, for example, the amount of DNA obtained from Gram-positive and Gram-negative strains could differ depending on the method used. Inadequate cell lysis could cause reduced representation of Gram-positive bacteria, while over rigorous disruption may lead to highly fragmented nucleic acids from Gram-negative bacteria, which in turn could be a source of artifacts during PCR amplification, such as chimera formation (Paabo, Irwin et al. 1990). The proportion of chimeric sequences was as high as 8.6 % during investigation of a subgingival plaque sample (Choi, Paster et al. 1994). Chimeric sequences can be eliminated before analysis using programs such as Chimera_Check on RDPII (Maidak, Cole et al. 2001), Mallard (Ashelford, Chuzhanova et al. 2006) or Uchime (Edgar, Haas et al. 2011). In this study a total of 45 sequences were found to be chimerae in the pyrosequencing derived datasets, of the total 12654 sequences, or 0.36 %. The frequency of such recombinants seems to be directly related to the number of PCR cycles, the length of extension period and the sequence similarity of mixed templates (Wang and Wang 1996). It could be partially explained by low number of cycles (25) and long extension step (90 s) during the PCR reaction, as well as quality of template DNA. It could also be related to lack of detection of such chimeric sequences, but the method used, uchime, is well documented and has been extensively used in studies analysing bacterial populations. For instance, a comparison of different chimerae

detection tools, found that for sequences between 100 and 600 nucleotides of length, uchime and Decipher performed the best (Wright, Yilmaz et al. 2012).

Furthermore, when studying a complex microbiota, the profile obtained will reflect the real relative abundance of species only if the amplification efficiency is the same for all sequences, which requires some assumptions. Firstly, all molecules should be equally accessible to primer hybridization. But DNA molecules with high G+C content are more difficult to denature during the PCR and could be underrepresented in the final amplicon mixture (Suzuki and Giovannoni 1996). In the present study, the phylum *Actinobacteria* was detected at a higher rate by culture than by the molecular methods, confirming earlier findings with oral samples (Munson, Banerjee et al. 2004, de Lillo, Ashley et al. 2006). Most members of this phylum have DNA of high G+C content, such as *Slackia exigua,* with 60 % G+C, and *Olsenella uli*, with 64.7 %.

Secondly, the PCR primers should bind to all target sequences with the same efficiency; the use of degenerate “universal” primers should enable this (Frank, Reich et al. 2008).

Moreover, the number of rRNA operons varies from one bacterium to another, affecting the proportions of amplicons obtained from components of a mixture (Farrelly, Rainey et al. 1995). The number of rRNA operons present in different species is not known even for all cultivated species, and is obviously unknown for not-yet-cultivated bacteria. This bias is therefore difficult to address. Nevertheless, it has been suggested that slow-growing bacteria have fewer copies than more rapidly growing organisms, although whether this is universally true has

yet to be confirmed (Krawiec and Riley 1990). This would have implications for this study, since the targets are predominantly slow-growing not-yet-cultivated bacteria. Furthermore, a single bacterial cell can harbour several copies of the genome. The case of *Epulopiscium* sp., with tens of thousands of copies of genome (Mendell, Clements et al. 2008), is extreme, but other species have been shown to contain more than one copy of their genome even when not undergoing cell division and DNA replication. For instance, *Deinococcus radiodurans* has been shown to possess four complete chromosomes during exponential growth and up to 16 genomes within the stationary phase (Levin-Zaidman, Englander et al. 2003). The pathogenic *Neisseria* species *N. gonorrhoeae* and *N. meningitidis* have also been shown to be polyploid, although, interestingly, the commensal *N. lactamica* was not (Tobiason and Seifert 2010).

Finally, Suzuki and Giovannoni (1996) have shown that the final concentration of amplicons in a mixed template PCR tended toward an equimolar ratio, independently of original concentration of gene sequences. In contrast to the rRNA operon number bias, this phenomenon would favour the detection of low represented taxa.

All these biases make it difficult to evaluate how close to reality is the predicted community structure. However, as all biases act equally on different datasets, the comparison of populations should be possible. Furthermore, for not-yet-cultivated bacteria, the detection of their 16S rRNA sequence should reflect the presence of the taxon in the community. The exception would be the persistence of extracellular DNA from dead cells, but in a batch culture model it should be quickly

diluted below detection levels. It could however be maintained if it was bound to other cells in the biofilm (Brundin, Figdor et al. 2010).

Cooked Meat Medium is a well established medium for the cultivation of anaerobic and aerobic organisms (Robertson 1916). Forgan-Smith *et al*. (1974) described some limitations of cooked meat medium for the recovery of anaerobic bacteria from small blood inocula, compared to some other commonly used media, as no bacteria were recovered by subculture after 1 d of CMM culture after inoculation with blood at 10^{-6} dilution, as opposed to 7 taxa recovered from USP thioglycollate medium. However, for longer incubation periods (7 d), CMM was more competitive and was surpassed only by fresh, home-made cooked meat medium; 9 taxa were recovered in CMM, 12 in the home-made medium and only 5 in USP thioglycollate medium. CMM seemed thus well suited to the objective of this study, namely, the establishment of a mixed batch culture with extended incubation times.

Haemin and vitamin K have been shown to enhance the growth of some anaerobic bacteria, including the black pigmented species of the genera *Porphyromonas* and *Prevotella*, previously classified as subspecies of *Bacteroides melaninogenicus* (Gibbons and Macdonald 1960), and including *P. endodontalis* (van Winkelhoff, van Steenbergen et al. 1985, Zerr, Drake et al. 2001). Despite the inclusion of these supplements, *P. endodontalis* was not detected in the mixed culture in CMM. *P. endodontalis* seems to be difficult to grow in mixed culture and its growth was shown to be inhibited by the presence of *P. micra* and *F. nucleatum* (Zerr, Cox et al. 1998). In a medium not supplemented with blood, the level of *P.*

endodontalis was reduced after 24 hours of culture and was not detected after 96 hours. In this study, *P. endodontalis* was detected by molecular methods in endodontic samples A1 and A2, but not in the corresponding CMM cultures, confirming the findings of the pilot study. The inclusion of these supplements in CMM seemed to increase the recovery rate for all bacterial species in the pilot study, but this difference was not statistically significant.

The aim of this experiment was firstly to establish an in-vitro culture from endodontic infection. There was no significant difference between the composition of the CMM cultures and the endodontic samples from which they were derived, confirming the usefulness and relevance of this culture medium.

Two of the studied endodontic infection samples originated from the same patient. These samples, A1 and A2, were more closely related than either were with sample C. This confirms recent data from the Human Microbiome Project showing that the individual host is the primary influence on microbiome composition (Segata et al. 2012). Nevertheless, when community structure was considered, taking into account relative abundance of taxa, statistically significant differences were observed between samples A1 and A2, presumably reflecting the different microhabitats present in the different teeth.

The A1 and A2 CMM cultures at 480 d were relatively simple as only 5 OTUs were observed for both samples and the community was well sampled. For the culture of sample C, middle-term points (up to 70 d) appeared similarly simple, but later points developed a richer community, even more so in the presence of mucin. The effect of mucin supplementation was not statistically significant in this study,

but too few samples were analysed and a better experimental design is needed to test the hypothesis that the inclusion of mucin would lead to an increase in species richness.

Several taxa of not-yet-cultivated bacteria were detected in endodontic samples, and some of them persisted in corresponding CMM cultures. For instance, *Bacteroidetes* sp. HOT365 / HOT281 / HOTG44, belonging to a deep branch with no cultivated representatives, was detected in CMM culture A2 up to 120 d. However, most of the uncultured taxa detected in endodontic samples, such as representatives of the Candidate Division TM7, were not detected in CMM cultures.

One taxon considered as uncultured in the HOMD database, *Prevotella* sp. HOT300, was isolated during culture analysis of the sample A1. Its growth was dependent on the presence of the "helper" strain, *P. acnes*, and would have been overlooked by culture studies not using helper strains.

The in-vitro communities established in CMM were maintained over an extensive period of time (up to 480 d) and were closely related to the corresponding endodontic infections. Several not-yet-cultured taxa were detected in CMM cultures even after long incubation. CMM seems to represent a suitable model for long-term in-vitro culture of oral bacterial communities.

Main findings:

1. Bacteria from endodontic infections were successfully established in long-term culture in CMM;
2. Bacterial compositions of endodontic samples from the same individual and derived CMM cultures presented a closer resemblance when compared to the unrelated sample;
3. CMM cultures were closely related to the endodontic samples from which they originated;
4. CMM cultures included several not-yet-cultured bacteria, such as *Bacteroidetes* sp. HOT365 / HOT281 / HOTG44, *Peptostreptococcaceae* sp. HOT369 / HOT103 and *Actinomyces* sp. HOTB78 / HOTE33 / HOTF78 / HOT169, up to 120 d;
5. During culture analysis, a previously uncultured bacterium *Prevotella* sp. HOT300 / HOT292 was isolated.

Chapter 3: Calgary Biofilm Device as a model for oral biofilms

Chapter 3. Calgary Biofilm Device as a model for oral biofilms

3.1 Introduction

Although, as discussed in the previous chapter, Cooked Meat medium is undoubtedly a useful medium for the culture of mixed communities that include anaerobic bacteria, it also suffers from some disadvantages. When used in a serial batch system, only a portion of the microbiota is transmitted to the new batch, which disrupts the stoichiometric dynamic, as in all batch system cultures. Thus it is likely that some of the slowest growing bacteria, often of interest in studies targeting not-yet-cultured taxa, will be lost. A possible way to overcome this problem is to use a continuous culture biofilm model. One of the simplest of these models consist of biofilms growing on a solid substrate, that are replenished with fresh growth media at intervals.

The Calgary biofilm device (CBD) was originally described by Ceri *et al.* (1999). It consists of a lid with 96 pegs that fits over a standard 96-well microtitre plate. It was primarily designed for antibiotic testing, which gave the device its alternative name, MBEC, for minimal biofilm eradication concentration. The CBD has been used to grow single species bacterial (Harrison, Ceri et al. 2005, Tomlin, Malott et al. 2005, Arias-Moliz, Ferrer-Luque et al. 2009) and yeast biofilms (Harrison, Rabiei et al. 2006), and mixed bacterial biofilms (Olson, Ceri et al. 2002).

The initial attachment of bacteria to the substrate is a key stage in biofilm formation. Hydroxyapatite (HA) has been extensively used as a material to

reproduce biofilm formation on enamel, as it is the principal inorganic component. It has been used in the form of discs for multispecies biofilm growth (Alvarez, Gonzalez et al. 2013) and for establishing a mixed biofilm from plaque (Shen, Qian et al. 2009). CBD pegs used in this study were coated with HA.

An important issue in the analysis of long term biofilm growth is the persistence of extracellular DNA from dead cells. This limitation can be addressed by the use of propidium monoazide (PMA)(Nocker, Sossa-Fernandez et al. 2007). This DNA intercalating agent, similar to ethidium bromide, cannot cross intact cellular membranes. It thus will only interact with DNA in the biofilm matrix or within damaged (and dead) cells. The application of a strong light source renders this interaction irreversible and the DNA crosslinked by PMA is rendered unreactive, and so will not be amplified by PCR.

Finally, one of the aims of this work was to assess the effect of presence of glucose in the medium on the oral biofilm formation. The presence of glucose might be predicted to favour the growth of fast-growing saccharolytic bacteria, to the possible detriment of slow-growing not-yet-cultivated bacteria. Some oral environments, such as the periodontal pocket and infected root canals, have low levels of carbohydrates, with proteins forming the main source of nutrients for bacteria.

3.2 Aim of the experiment

The aim of the experiments described in this chapter was to assess the suitability of HA coated pegs for the growth of a long term mixed oral biofilm and to evaluate different growth media for their capacity to sustain a mixed community with previously uncultured members.

The first experiment consisted of a longitudinal analysis of the biofilm composition every 8 days up to 88 days. The effect of mucin addition to the growth medium was also investigated.

In the second part, different growth media were compared after 24 days of growth, by 454 pyrosequencing.

3.3 Methods

3.3.1 Calgary biofilm device or MBEC

The HA coated MBEC (for "minimal biofilm eradication concentration") High-Throughput culture system (Innovotech Inc, Edmonton, Alberta, Canada) was used (Figure 20).

Figure 20. Calgary Biofilm Device with developing biofilm.

3.3.2 Longitudinal analysis of the biofilm composition on pegs

Unstimulated saliva was obtained from a periodontally healthy 27-year old female volunteer at least 2 hours after the last meal. Pegs were incubated in 200 µL of saliva overnight at 37 °C, anaerobically. The lid was then transferred to a new base plate containing 200 µL of growth medium per well. The medium was Brain Heart Infusion broth (BHI, Fluka), supplemented with vitamin K (0.5 µg/ml) and haemin (5 µg/ml), with or without pig gastric mucin (2.5 %, Type III, Sigma). In addition, a medium described for salivary bacteria recovery containing sheep blood,

SHI, was also used (Tian, He et al. 2010). The distribution of media on the plate is shown in Figure 21.

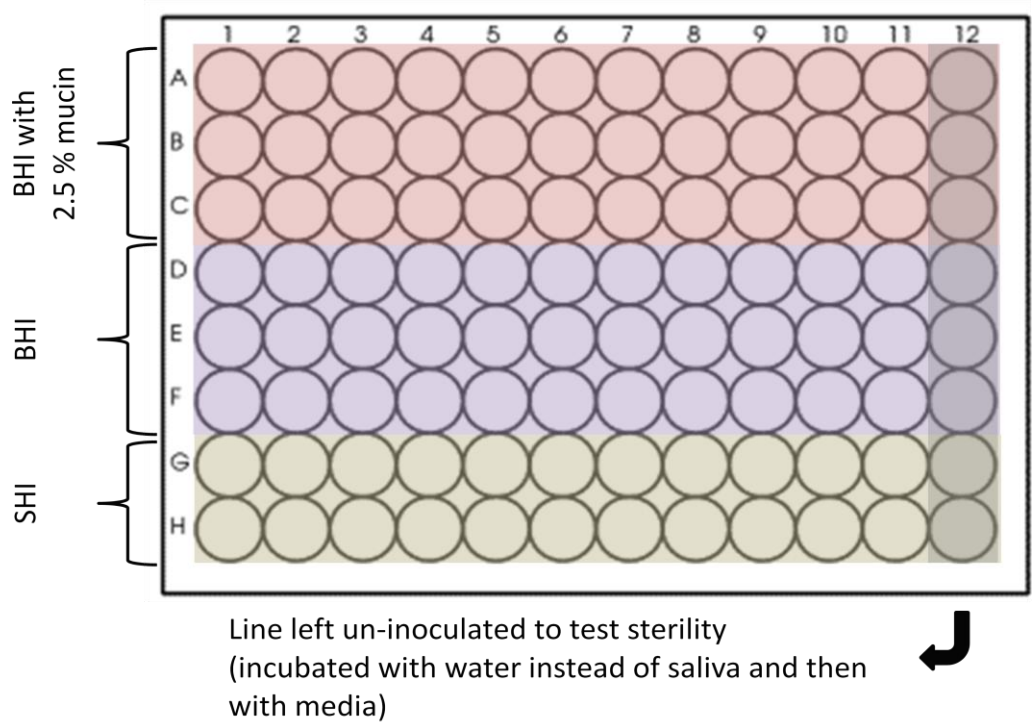


Figure 21. Schematic representation of the CBD anaerobic experiment.

The plate was incubated anaerobically at 37 °C and the media changed every 48 hours. The composition of the biofilm was analysed every 8 days by cloning and Sanger sequencing. Pegs were snapped off the plate with forceps and washed three times in PBS by dipping. Biofilms from 3 pegs were then carefully removed by scraping and placed in 500 µL of PBS in a 1.5 mL tube. DNA was extracted and 16S rRNA genes amplified, cloned and sequenced as described in section 2.3.2.5. At 24 days, the composition of the biofilms was also analysed by 454 pyrosequencing (section 2.3.2.5.5). In addition, a cultural analysis was performed at 48 days, with 39 isolates identified for BHI without mucin and 50 for BHI with mucin, on FAAB.

3.3.3 Validation of peg biofilm culture as a model for oral microbiota

The experiment was carried out in triplicate. Peg cultures were set up from a saliva inoculum as described in 3.3.2. Three different media were used: BHI with 2 % glucose, BHI without glucose and BMM (media composition described in page 135). The distribution of pegs to media is shown in Figure 22.

The plate was incubated in air + 5 % CO_2 at 37 °C and the medium changed every 48 hours. After 24 days of culture, biofilms from 6 pegs per medium were used for DNA extraction. Half of each sample was treated with PMA (3.3.5). Total DNA was extracted with the Genelute Bacterial Genomic DNA kit (Sigma), using the protocol for Gram positive bacteria, from PMA-treated and untreated cells, as described in section 2.3.2.5. The bacterial composition of the samples was determined by 454 pyrosequencing as described in section 2.3.2.5.5 and analysed as described in section 2.3.2.5.6.

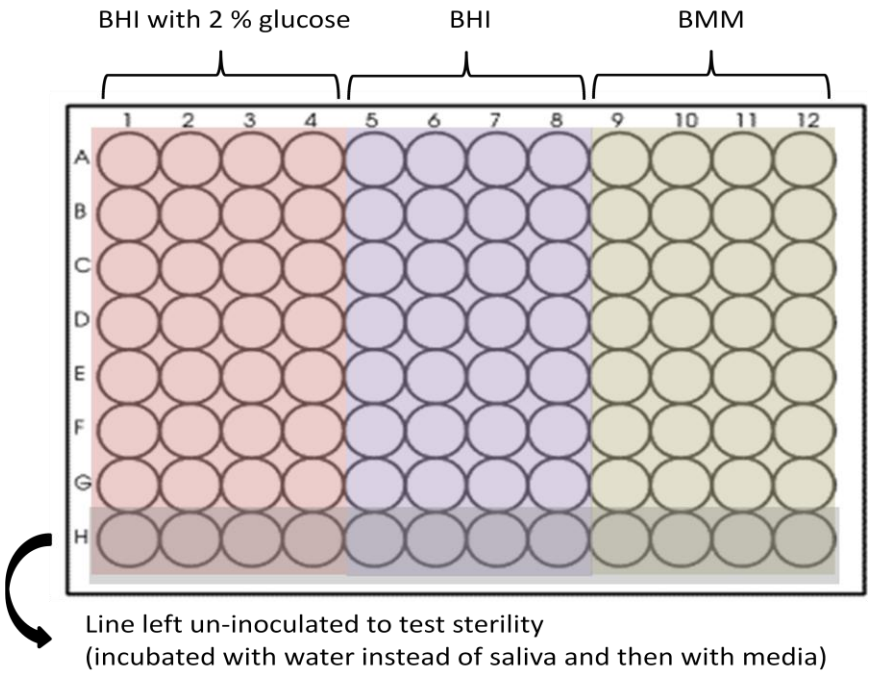


Figure 22. Schematic representation of the CBD aerobic experiment.

3.3.4 Media used in peg biofilm culture.

In the first experiment, pegs were incubated with commercially available BHI broth (Fluka), supplemented with vitamin K (0.5 µg/ml), haemin (5 µg/ml) and, for half of the pegs, with porcine stomach mucin (2.5 %, Type III, Sigma). The SHI medium composition for 1 L was as follows (Tian, He et al. 2010):

Proteose peptone	10 g
Trypticase peptone	5.0 g
Yeast extract	5.0 g
KCl	2.5 g
Sucrose	5 g
Haemin	5 mg
Vitamin K	1 mg
Urea	0.06 g
Arginine	0.174 g
Pig gastric mucin	2.5 g
Sheep blood	5 %
N-acetylmuramic acid	10 mg

The commercial BHI broth used in the first experiment contained 2 % glucose. For the second experiment, in order to assess the effect of glucose on biofilm composition, homemade BHI broth with and without glucose was prepared as follows:

BHI powder	17.5 g
Proteose peptone	10 g
NaCl	5 g
Na_2HPO_4	2.5 g
Vitamin K	0.5 mg
Haemin	5 mg
Pig gastric mucin	2.5 g

For the medium with glucose, 20 g of glucose (Merck) per L were added (2 %).

The BMM medium was prepared as described by Glenister *et al*. (1988):

Proteose peptone	10.0 g
Trypticase peptone	5.0 g
Yeast extract	5.0 g
KCl	2.5 g
Haemin	5 mg
Arginine	1 mmol
Vitamin K	1 mg
Urea	1 mmol
Pig gastric mucin	2.5 g

3.3.5 PMA treatment

Biofilm removed from pegs was resuspended in 500 µL PBS. Half (250 µL) was transferred to a new eppendorf and diluted to 500 µL. PMA, resuspended in 20 % dimethyl sulfoxide at 20 mM, was added to the cell suspension (1.25 µL, final concentration 50 µM). The tube was incubated in the dark with occasional shaking for 5 min and then exposed to the light source (500 W halogen lamp, 20 cm from the tube) for 5 min. During this time the tube was placed on ice to avoid excessive heating and shaken occasionally. The cell suspension was subsequently used for DNA extraction.

3.4 Results

3.4.1 Longitudinal analysis of the peg biofilm composition

The aims of this experiment were to assess the ability of the CBD system to support the growth of a mixed bacterial biofilm, and to monitor the changes in biofilm composition over time. The biofilm grown on pegs with BHI, BHI with mucin (BHIm) and SHI media, as described above, was visible to the naked eye as early as 2 d after inoculation. The SHI medium revealed itself impractical to use with this model because of its high viscosity, and the biofilm grown in this medium was found to be less taxon-rich than biofilms grown with BHI and BHIm. The analysis of the SHI biofilm was therefore not continued after 8 d.

3.4.1.1 Analysis of the α-diversity of the CBD anaerobic biofilms

Table 7 presents different measures of α-diversity and richness coverage for biofilms incubated with BHI, BHIm, and SHI, at different time points. The number of sequences identified by cloning and sequencing for each time point was randomly subsampled to 80 sequences for both statistical and phylogenetic analysis. Two samples, at 24 d of incubation, were analysed by pyrosequencing, producing 1465 sequences for BHI and 1211 for BHIm. The pyrosequencing data were randomly subsampled to 1211 sequences for each of the samples for statistical analysis, but the phylogenetic analysis was performed on the complete dataset. No chimeric sequences were identified by uchime executable for any dataset in this experiment.

Table 7. α-diversity of the anaerobic CBD biofilms incubated with BHI, BHIm and SHI, analysed at different time-points by Sanger or pyrosequencing (py).

Medium	Time (d)	N seqs	S_{obs}	Good's coverage	CatchAll estimate	CatchAll cov. %	Chao1	invsimpson
BHI	8	80	23	0.86	36.8	62.50	36.8	11.92
	16	80	14	0.94	23.3	60.09	16.0	5.56
	24	80	19	0.91	29.7	63.97	24.3	10.26
	32	80	17	0.93	20	85.00	20.0	3.09
	40	80	13	0.94	17.5	74.29	16.3	4.12
	48	80	20	0.89	26.7	74.91	27.2	9.84
BHIm	8	80	16	0.96	20	80.00	16.4	9.69
	16	80	17	0.95	23.1	73.59	18.5	8.19
	24	80	20	0.88	38.3	52.22	31.3	9.66
	32	80	20	0.90	29.5	67.80	29.3	11.49
	40	80	26	0.80	57.1	45.53	66.0	9.81
	48	80	24	0.81	57.3	41.88	59.0	10.36
	88	80	14	0.91	23	60.87	24.5	4.52
SHI	8	80	12	0.98	14.1	85.11	12.3	4.76
BHI py	24	1211	86	0.97	178.8	48.10	203.1	13.12
BHIm py	24	1211	95	0.96	287.2	33.08	189.0	12.62

The number of observed OTUs for Sanger analysed datasets ranged from 12 for the 8-d biofilm incubated with SHI to 26 for the 40-d biofilm with BHIm. There were no significant differences between the biofilms incubated with BHI and BHIm and between early (8-24 d) and late (32-48 d) stages of the biofilm incubation, as observed by Wilcoxon signed rank test using a cutoff of $p<0.05$. The sample incubated with SHI medium exhibited the lowest number of observed OTUs, and CatchAll and Chao1 estimates, but as only one time-point was analysed, no statistical test could be performed.

The Good's coverage values for all media and all time points range between 0.98 and 0.80, with an average of 0.91. These values were lower than those observed for the CMM culture (0.97), while the number of observed OTUs was

higher (27.3 vs 13.7), which indicates a higher α-diversity, confirmed by higher values for all other indicators. Student's *t*-test confirmed that the differences observed with CMM cultures were significant for the number of observed OTUs, Good's coverage and CatchAll, but not significant for Chao1 and the Inverse Simpson index (at the cut off of p=0.05).

Biofilms grown in BHI with and without mucin and sampled at 24 d were analysed by both Sanger and pyrosequencing. The number of OTUs observed by pyrosequencing was more than 4.5 times higher than for Sanger, which was reflected by a higher coverage value, increasing from 91 % to 97 % for BHI and from 88 % to 96 % for BHIm. Even larger numbers were observed for pyrosequencing CatchAll estimate values, 6 and 7.5 times larger than for Sanger sequencing.

The rarefaction curves for Sanger sequencing-generated datasets are presented in Figure 23.

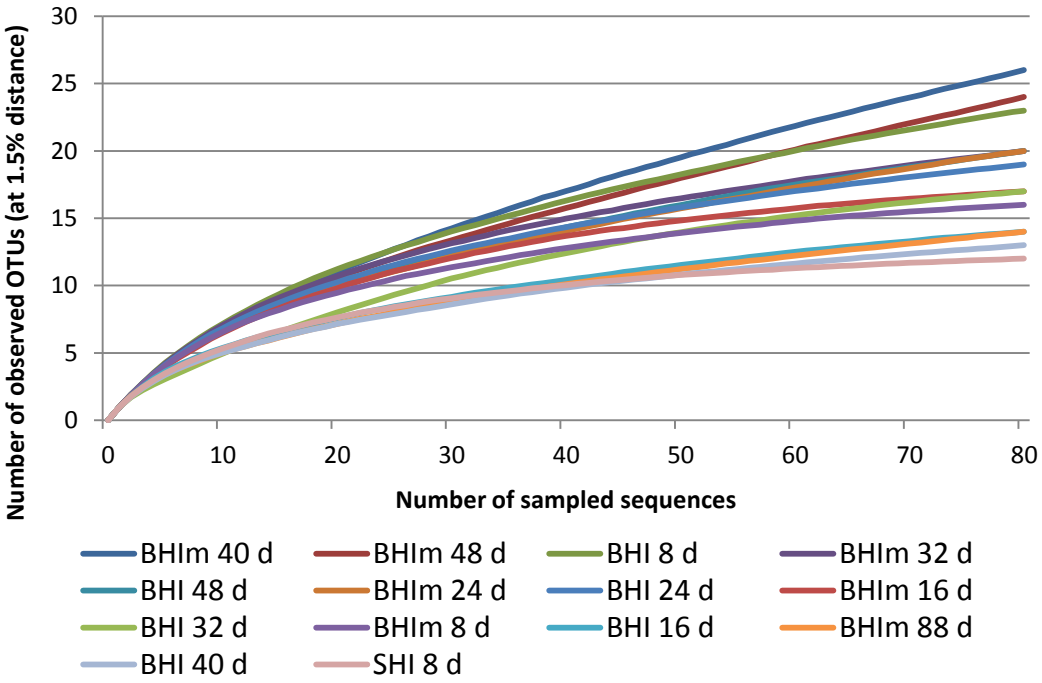


Figure 23. Rarefaction curves for anaerobic CBD biofilms.

3.4.1.2 Analysis of the β-diversity of the CBD anaerobic biofilms

3.4.1.2.1 OTU-based approach

The Jaccard index, which allows the comparison of community membership was calculated for the Sanger sequencing-obtained datasets. The distances obtained were plotted using PCoA (Figure 24). No visual difference could be seen between biofilms incubated with or without mucin. The only sample visually separated from others corresponded to the biofilm incubated with SHI. The parsimony and unweighted UniFrac values were not significant when biofilms were compared by culture medium. The visual evaluation was confirmed by the Amova which showed no significant difference between the BHI-based media, with all time points aggregated, but showed that the BHI media were significantly different to

the SHI-grown biofilm at the 8 d time-point (Table 8). The theta YC metric did not show any significant differences. Finally, the pyrosequencing analysis of the BHI and BHIm biofilms were found to be significantly different at 24 d (Amova, p <0.001).

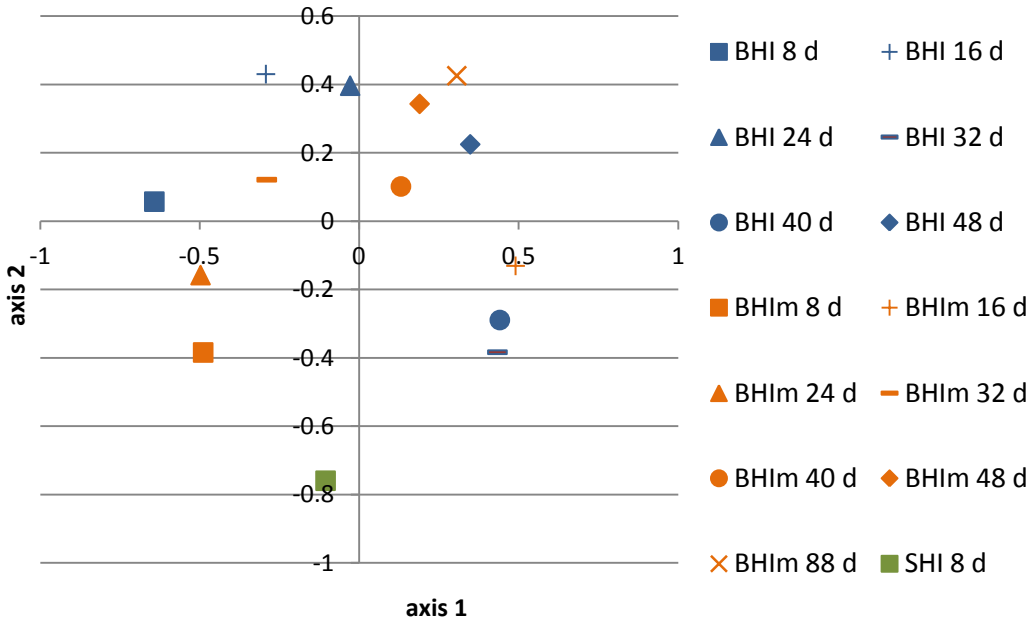


Figure 24. PCoA plot of Jaccard index distances for Sager analysis of anaerobic CBD biofilm samples.

Table 8. Amova analysis between media sets for anaerobic CBD experiment

Set of media compared	p value
BHI-BHIm	0.563
BHI-SHI	0.002
BHIm-SHI	<0.001

3.4.1.2.2 Phylogeny-based analysis

The UniFrac analysis was used to compare communities by their phylogenetic similarities. The comparison of the unweighted UniFrac distances for biofilms grown with BHI and BHIm gave statistically significant values by parsimony (p=0.013), unweighted UniFrac (p=0.018) and Amova (p=0.025). The weighted UniFrac test showed no significant differences.

3.4.1.3 Culture analysis of the bacterial composition of the biofilms incubated with BHI and BHIm

The bacterial composition of the biofilms incubated with BHI and BHIm was analysed by anaerobic culture after 48 d of growth. In total, 39 isolates were identified for the biofilm grown with BHI and 50 for that with BHIm. Isolates belonging to 5 phyla were identified : *Actinobacteria*, identified only from the BHIm biofilm, *Bacteroidetes*, *Firmicutes*, *Fusobacteria* and *Proteobacteria*. The communities isolated from both media were dominated by members of the *Firmicutes*, with 79.0 % for BHI and 72.9 % for BHIm. The most frequently detected species were *Streptococcus constellatus* / *intermedius*, *Anaeroglobus geminatus* and *Veillonella dispar* / *parvula* / HOTG30.

3.4.1.4 Taxonomic assignment of Sanger sequencing and pyrosequencing sequences

The evolution of the composition of the biofilms at phylum level is represented in Figure 25 and Table 9. In Figure 25 each circle corresponds to a time point, from the inner circle at 8 d of culture to the external one at 48 d. The final time point of 88 d for BHIm is not shown to keep the figures comparable. While

both cultures were dominated by the *Firmicutes* phylum, other phyla represented a higher proportion in the biofilm grown in presence of mucin than in BHI without mucin, totaling 53.8 % at 48 d. The most remarkable difference is probably the proportion of the sequences belonging to the *Synergistetes* phylum, making up 16.3 % of the total number of sequences in the BHIm biofilm at 48 d and totally absent from the BHI biofilm after 24 d.

A table representing all taxa identified, with corresponding proportions, can be found in the Appendix 2. The SHI-grown biofilm was the least taxon-rich, with only 8 taxa belonging to two phyla identified at 8 d. *Firmicutes* (66.3 %) were primarily represented by *Streptococcus* sp. HOTC56, with 41.3 % of the total number of sequences. The second phylum, *Spirochetes* (33.8 %), was dominated by *Treponema maltophilum*, with 32.5 % of all sequences.

In contrast, 20 taxa were identified in the BHI-grown biofilm and 10 for BHIm-grown biofilm at the same time point (8 d). At 48 d, 18 and 21 taxa were detected, respectively, while the pyrosequencing analysis at 24 d detected 50 and 59 taxa.

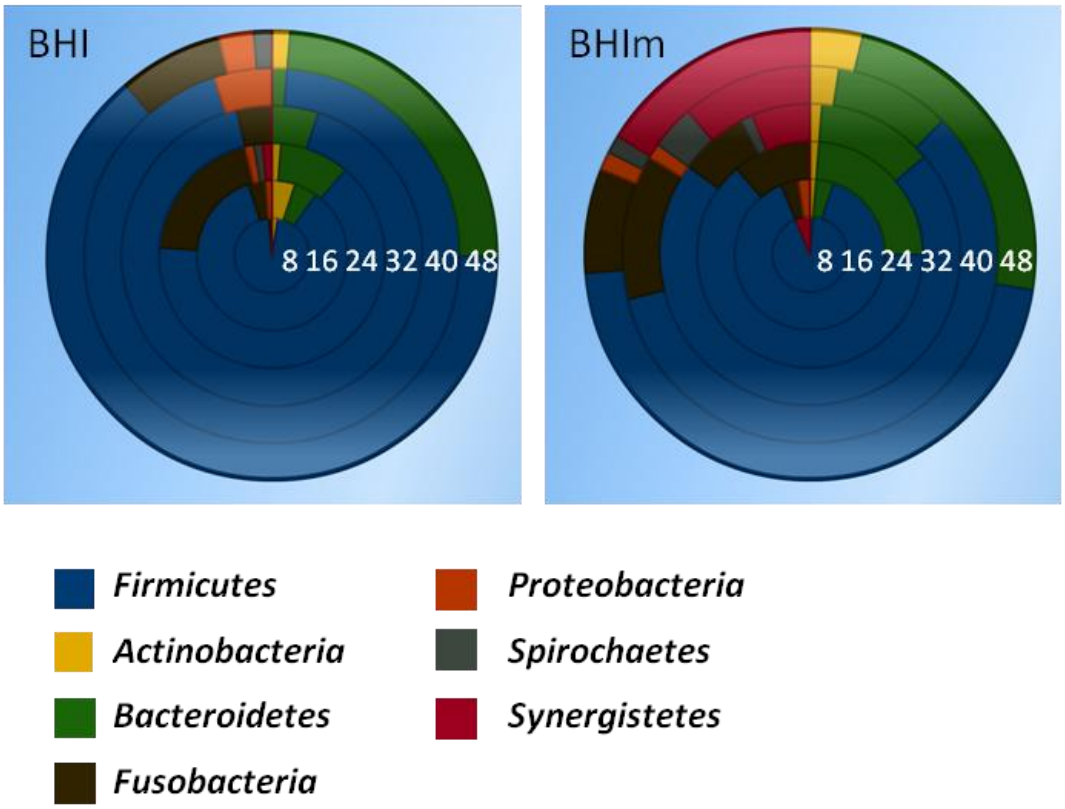


Figure 25. Evolution of biofilm composition for BHI without (left) and with (right) mucin, at phylum level. Each circle corresponds to a different time point of sampling, from 8 to 48 d.

After the first week of incubation, the biofilm in BHI was dominated by the *Veillonella dispar* / *parvula* / HOTG30 taxon, at 26.3 %, and *Streptococcus* sp. HOT058, at 17.5 %. *Streptococcus* sp. HOT058 was not detected at any later sampling point. *V. dispar* / *parvula*, with addition of *Anaeroglobus geminatus*, *F. nucleatum* ss. *animalis* and *Streptococcus constellatus* / *intermedius*, dominated the mixed population up to 48 d. Interestingly, *F. nucleatum* ss. *animalis* made up the highest proportion of the community at 24 d (20.0 % by Sanger and 17.5 % by pyrosequencing), when *S. constellatus* was at its lowest (7.5 and 6.7 %, respectively).

Table 9. Number of taxa detected and the percentage of the sequences for each phylum in CBD anaerobic biofilms at different time points.

Samples / Phyla	BHI 8 d	BHI 16 d	BHI 24 d	BHI 24 d py	BHI 32 d	BHI 40 d	BHI 48 d	BHI mucin 8 d	BHI mucin 16 d	BHI mucin 24 d	BHI mucin 24 d py	BHI mucin 32 d	BHI mucin 40 d	BHI mucin 48 d	BHI mucin 88 d	SHI 8 d	Total/ mean
Actinobacteria	1 2.5	1 5	1 1.3	2 0.2			1 1.3		1 1.3	1 1.3	2 0.2	1 1.3	2 2.6	2 3.8			6 1.30
Bacteroidetes		2 5	2 10	7 11.6	1 5	1 1.3	3 23.8	1 1.3	2 3.8	5 23.8	14 23	4 12.5	4 10	5 23.8	1 1.3		17 9.76
Firmicutes	18 96.3	11 85	11 65	30 62.5	12 91.3	10 93.8	11 63.8	8 92.5	10 88.8	16 63.8	32 61.9	13 71.3	10 58.8	10 46.3	7 45	6 66.3	49 72.03
Fusobacteria	1 1.3	1 3.8	1 20	4 19.1	1 3.8		1 7.5		2 3.8	1 11.3	3 12.2	1 7.5	1 12.5	1 7.5	1 2.5		5 7.05
Proteobacteria		1 1.3	1 1.3	4 5.6		1 5	1 2.5		1 2.5		3 0.5	0 0	1 1.3	1 1.3	2 18.8		5 2.51
Spirochaetes			1 1.3	1 0.4			1 1.3				2 0.4	1 1.3	2 3.8	1 1.3	1 32.5	2 33.8	3 4.76
SR1											1 0.1						1 0.01
Synergistetes			1 1.3	1 0.7				1 6.3			1 1.6	1 6.3	1 11.3	1 16.3			1 2.74
TM7				1 0.3							1 0.2						1 0.03
Total	20	16	18	50	14	12	18	10	16	23	59	21	21	21	12	8	

The bacterial composition of the BHIm biofilm after 8 d of incubation was dominated by *S. constellatus* / *intermedius*, at 41.3 %, and *A. geminatus*, at 30 %. These species remained among the principal members of the community up to 48 d, with *Veillonella dispar* / *parvula* / HOTG30, *Prevotella oralis, Streptococcus* sp. HOT058, *Fusobacterium nucleatum* ss. *animalis*, and *Fretibacterium fastidiosum.*

The BHIm culture was not sampled between 48 and 88 days of incubation and its composition changed dramatically during this time. At 88 d It was dominated by four species: *Treponema lecithinolyticum* (32.5 %), *Peptostreptococcaceae* sp. HOTE46 (21.3 %), *Campylobacter rectus* / HOTG43 (16.3 %) and *Streptococcus salivarius* (11.3 %). The three former taxa were detected only in reduced numbers, under 3 %, in earlier stages of the biofilm, while the latter was not detected at all before this time point.

3.4.1.5 Linear discriminant analysis (LDA) with LEfSe

The LEfSe analysis identified 6 taxa differentially abundant between the BHI and BHIm biofilms (Figure 26). The OTUs most prevalent in BHI biofilms were *Campylobacter showae* (OTU037) and *Parvimonas* sp. HOTC63 (OTU010), while those most abundant in BHIm were *Peptostreptococcus stomatis* (OTU041), *Prevotella baroniae* (OTU043), *Peptostreptococcaceae* sp. HOT369 / HOT103 (OTU040) and *Prevotella* sp. HOTB62 (OTU052) (in decreasing LDA score).

When all three media were compared, four OTUs were found to be statistically more abundant in the SHI biofilms: *Streptococcus* sp. HOTC65, *Lactobacillus oris*, *Streptococcus salivarius* and *Lactobacillus* sp. HOT461.

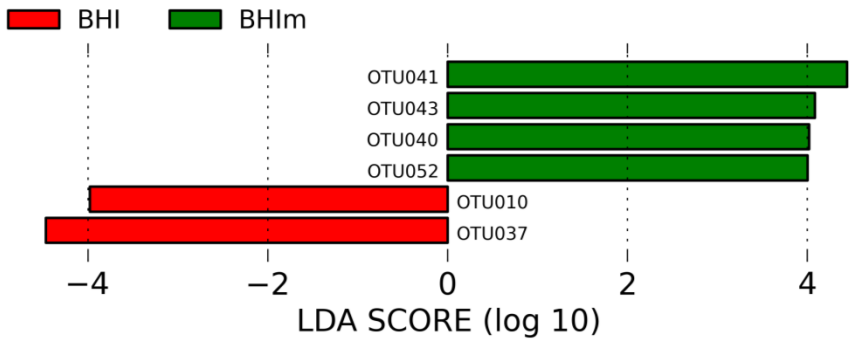


Figure 26. Linear Discriminant Analysis of anaerobic CBD biofilm communities grown with BHI and BHIm media.

3.4.2 Evaluation of the ability of CBD system to support the development of mixed oral biofilm and the role of glucose

To further assess the ability of the CBD system to support the growth of a complex natural biofilm, the second part of the experiment used pegs inoculated with the saliva of a healthy volunteer and incubated aerobically with 5 % CO_2, in order to replicate natural oral conditions. Three media were tested: BHI (without glucose), BHI_G (with glucose) and BMM. For each sample, half of the sample was treated with PMA before DNA extraction (labelled as -P, e.g., BHI-P for sample treated with PMA, and BHI for non-treated).

3.4.2.1 Analysis of the α-diversity of the aerobic CBD biofilms

A total of 63698 sequences were obtained from the pyrosequencing analysis after de-noising and trimming. The uchime program detected 683 chimeric sequences (1.07 %), which were removed. The pyrosequence data were normalised by random sub-sampling to 9166 sequences per sample, although phylogenetic analysis was carried out on the complete dataset.

A summary of the α-diversity of the biofilm samples analysed by pyrosequencing is shown in Table 10. The number of observed OTUs ranged from 145, for the biofilm incubated with BHI without glucose and not treated with PMA, to 421, for the biofilm incubated with BMM and treated with PMA. The Good's coverage values were all above 98 %, suggesting that the community was well sampled in each case. Nevertheless, the CatchAll estimate suggests a much higher richness than the number of species observed (Wilcoxon signed-rank test $p<0.001$) or those predicted with the Chao1 estimator ($p<0.001$). When samples were compared regarding PMA treatment, the only statistical difference observed was for the Inverse Simpson index (Student's *t*-test p=0.02).

Table 10. α-diversity of the aerobic CBD biofilms. Biofilm samples are named by incubation medium (BHI, BHI_G and BMM) and the possible PMA treatment is indicated by -P.

Sample	N seqs	S_{obs}	Good's coverage	CatchAll estimate	CatchAll cov. %	Chao1	invsimpson
BHI	9166	145	0.99	332.5	43.61	240.4	8.95
BHI-P	9166	219	0.99	577.9	37.90	364.3	8.11
BHI_G	9166	208	0.99	457.4	45.47	322.2	9.69
BHI_G-P	9166	170	0.99	375.0	45.33	267.7	6.75
BMM	9166	212	0.99	469.3	45.17	350.1	10.50
BMM-P	9166	421	0.98	899.6	46.80	744.9	6.23

The rarefaction curves for aerobic CBD biofilms are presented in Figure 27. All curves, with the exception of the one corresponding to the BMM-P sample, seem to follow a similar pattern.

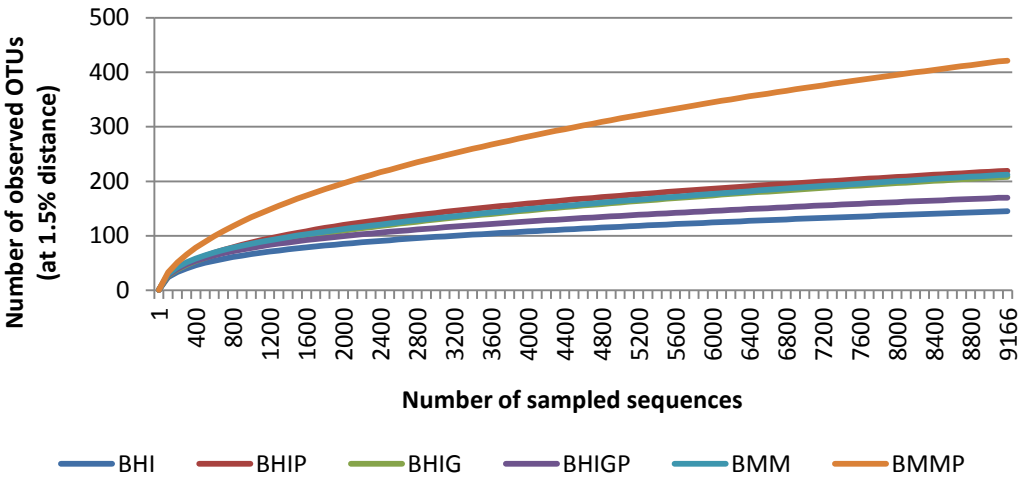


Figure 27. Rarefaction curves for aerobic CBD biofilms analysed by pyrosequencing.

3.4.2.1.1 OTU-based approach

No significant differences were seen in the composition of the biofilm samples when compared using parsimony and unweighted UniFrac analyses of a dendrogram constructed using the Jaccard index (data not shown). The community structures were then compared with the theta YC metric, which takes into account the relative abundances of OTUs. The dendrogram obtained is shown in Figure 28. The clustering pattern observed between PMA treated and untreated samples was found to be significant by Amova ($p<0.001$) but not by parsimony ($p=0.069$) or unweighted UniFrac ($p=0.102$).

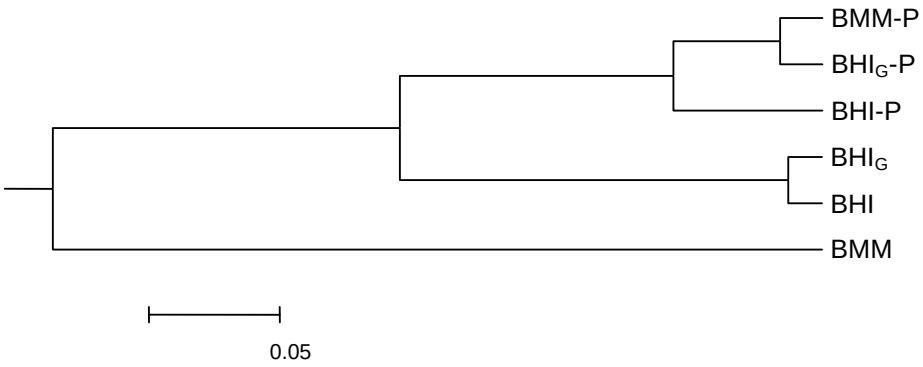


Figure 28. Dendrogram representing theta YC distances for aerobic CBD biofilms.

3.4.2.1.1 Phylogeny-based analysis

Biofilm communities were also compared based on their phylogenetic similarities by weighted and unweighted UniFrac analysis. No significant differences were found by the unweighted UniFrac test, but the weighted counterpart presented a statistically supported clustering of PMA treated samples (Figure 29), resembling the clustering obtained in Figure 28 (Amova $p<0.001$).

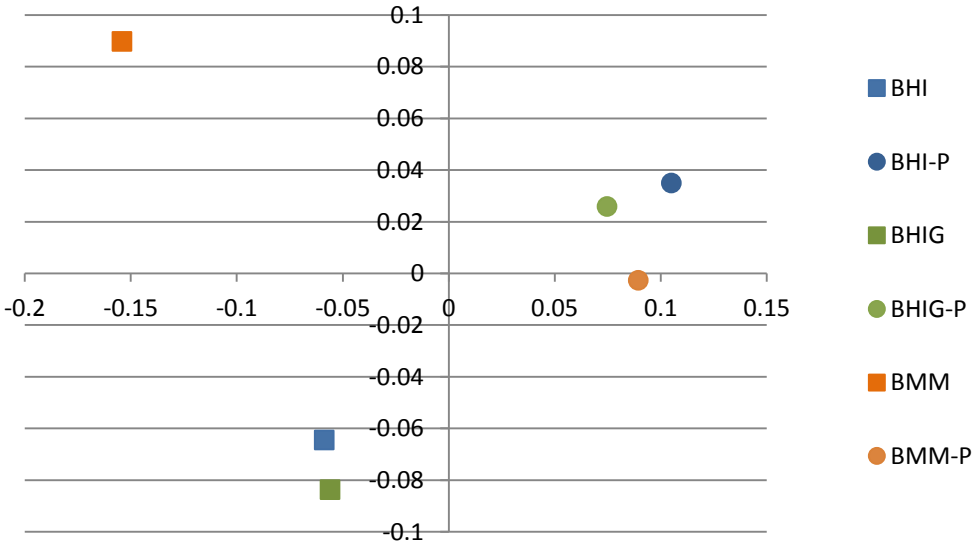


Figure 29. PCoA plot of weighted UniFrac distance for aerobic CBD biofilms.

3.4.2.2 Linear discriminant analysis with LEfSe

No significant difference in OTU abundance was found between biofilms incubated with BHI with or without glucose. Two OTUs were found to be enriched in BMM medium: *Streptococcus* sp. HOT071 and *Clostridiales* sp. HOT085, with LDA scores between 2.5 and 3.

When PMA treated and untreated samples were compared, 25 OTUs showed a differential abundance (Figure 30). Nine were more abundant in untreated biofilms while 16 were higher in PMA treated samples (Table 11). The *Flavobacteriales* sp. found to be the most correlated with PMA treatment seem to represent a new taxon. The closest match on the HOMD was for *Flavobacteriales* sp. HOT321, with 86.2 % over 450 bp. No hit presenting over 98 % identity was found by NCBI BLASTN. This taxon was found in biofilms grown with all media and in all independent replicates.

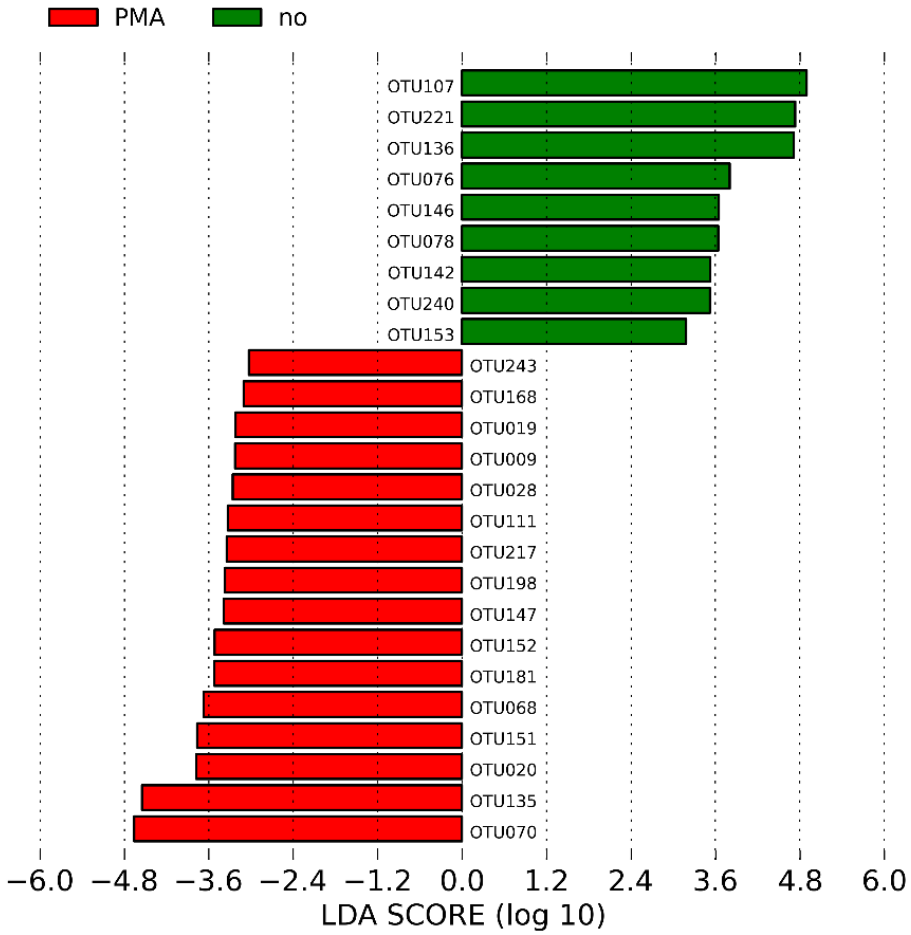


Figure 30. Linear Discriminant Analysis of aerobic CBD biofilm communities treated or not with PMA.

Table 11. OTUs differentially abundant in PMA treated and untreated aerobic CBD biofilms, by decreasing absolute LDA values.

PMA treated samples	Untreated samples
Flavobacteriales sp. uncultured	*Lactobacillus oris*
Neisseria elongata	*Staphylococcus* sp. NBRC 13889
Aggregatibacter paraphrophilus / *aphrophilus*	*Neisseria flava* / *mucosa* / *pharyngis* / *sicca*
Peptostreptococcus stomatis / HOTE46	*Fusobacterium nucleatum* ss. *polymorphum*
Eubacterium yurii	*Parvimonas* sp. HOT110
Prevotella sp. HOT781	*Fusobacterium periodonticum*
Porphyromonas catoniae / HOT279	*Oribacterium sinus*
Parvimonas sp. HOTC63	*Streptococcus* sp. HOTC04
Selenomonas noxia / HOTG67	*Porphyromonas endodontalis* / HOT395 / HOTG50
Solobacterium moorei	
Lautropia mirabilis	
Bacteroidetes sp. HOT274	
Actinomyces odontolyticus / *meyeri*	
Actinomyces sp. HOTB78 / HOTE33 / HOTF78 / HOT169	
Prevotella oris	
Tannerella forsythia	

3.4.2.3 Phylogenetic analysis of aerobic CBD biofilms

All biofilm communities analysed in this experiment were dominated by the *Firmicutes* phylum (mean proportion 61.3 %), followed by *Proteobacteria* (25.3 %), *Bacteroidetes* (8.2 %) and *Fusobacteria* (4.18 %). Other phyla detected were *Actinobacteria* (0.6 %), TM7 (0.15 %), *Spirochetes* (0.05 %) and SR1 (0.01 %).

A total of 267 taxa were identified in all aerobic CBD biofilms (Appendix 2), but only 11 represented over 5 % in any of the samples (Table 12).Two *Lactobacillus* species were the most numerous: *Lactobacillus paracasei* and *Lactobacillus oris*,

making up a mean of 13.6 and 9.8 % of all sequences for all biofilms, respectively. *Campylobacter showae* / HOTE65 taxon represented a mean of 7.5 % through all samples.

The biofilms grown in BHI without glucose were dominated by *Neisseria flava* / *mucosa* / *pharyngis* / *sicca* (18.4 %) and *Lactobacillus oris* (15.3 %) for PMA untreated samples and by *Lactobacillus paracasei* (18.8 %), *Flavobacteriales* sp. uncultured (15.6 %) and *Neisseria elongata* (10.5 %) for PMA-treated samples. For the biofilms incubated in presence of glucose, *Staphylococcus* sp. NBRC 13889 (19.4 %), *Lactobacillus paracasei* (17.0 %) and *Lactobacillus oris* (13.5 %) were the most represented species in untreated samples and *Lactobacillus paracasei* (26.5 %) was the only species making over 10 % in PMA treated samples. Finally, the BMM biofilms were dominated by *Lactobacillus oris* (25.9 %) in untreated biofilms, but this taxon made up only 0.2 % of treated biofilms. There was no taxon with relative abundance over 10 % in the PMA-treated BMM biofilms. The most numerous taxa were *Campylobacter showae* / E65 (9.7 %), *Streptococcus constellatus* / *intermedius* / HOTE12 (8.4 %), *Lactobacillus paracasei* (8.2 %) and *Neisseria elongata* (8.0 %).

Table 12. The most abundant taxa identified in aerobic CBD biofilms, treated with PMA or untreated (NT), with corresponding relative abundance (%).

Samples	BHI_G		BHI		BMM	
Taxa	NT	PMA	NT	PMA	NT	PMA
Lactobacillus paracasei	16.98	26.53	9.63	18.84	1.18	8.17
Lactobacillus oris	13.51	3.45	15.33	0.10	25.88	0.24
Campylobacter showae / HOTE65	4.72	4.86	8.41	9.98	7.55	9.71
Neisseria flava / *mucosa* / *pharyngis* / *sicca*	9.60	1.43	18.37	2.11	9.71	2.10
Neisseria elongata	1.60	9.86	3.17	10.45	4.47	8.04
Flavobacteriales sp. uncultured	0.50	6.78	1.16	15.61	2.95	6.37
Parvimonas micra	2.80	5.75	6.20	4.51	8.35	3.44
Gemella morbillorum / *haemolysans*	2.24	1.93	3.87	1.77	1.57	5.78
Staphylococcus sp. NBRC 13889	19.37	0.11	8.07	0.10	0.82	0.07
Streptococcus constellatus / *intermedius* / HOTE12	2.64	5.53	3.25	3.58	4.35	8.39
Veillonella atypica / *dispar* / *parvula* / HOTE53 / HOTG30	3.23	6.06	3.29	2.50	4.70	5.53

3.4.2.4 Uncultured bacteria in CBD anaerobic and aerobic biofilms.

Several uncultured phylotypes, as defined in the HOMD database, were detected in both aerobic and anaerobic CBD experiments (Table 13). In the anaerobic CBD biofilm 16 not-yet-cultivated taxa were detected, belonging to the *Firmicutes* (10 taxa), *Bacteroidetes* (3), *Proteobacteria*, SR1 and TM7 (1 for each) phyla. The aerobically incubated CBD biofilms included at least 94 not-yet-cultivated taxa between them. The distribution between phyla was as follows : *Firmicutes* 42, *Bacteroidetes* 14, TM7 12, *Fusobacteria* 9, *Proteobacteria* 8, *Actinobacteria* 6, SR1 2 and *Spirochetes* 1.

For biofilms incubated in BHI with and without glucose addition, more uncultured taxa were detected in the absence of glucose, 38 against 26. In BMM 82 uncultured taxa were detected.

Table 13. Uncultured taxa detected in CBD biofilms.

CBD conditions	Anaerobic			Aerobic			
Uncultured taxa	BHI	BHIm	BHI_G	BHI	BMM	+PMA	-PMA
Actinobacteria							
Actinomyces sp. HOTC25				x	x	x	
Actinomyces sp. HOTD50					x	x	
Actinomyces sp. HOTE63					x	x	x
Actinomyces sp. HOTE91			x		x	x	x
Atopobium sp. HOT416				x	x	x	
Propionibacterium sp. HOT194					x	x	
Bacteroidetes							
Bacteroidetes sp. HOT365 / HOT281 / HOTG44		x		x	x	x	x
Bacteroidetes sp. HOT511				x	x	x	
Bergeyella sp. HOT322				x	x	x	
Capnocytophaga sp. HOTB79					x	x	
Capnocytophaga sp. HOTH18					x	x	
Flavobacteriales sp. uncultured			x	x	x	x	x
Porphyromonas sp. HOT275 / HOT278 / HOTB43			x	x	x	x	x
Prevotella sp. clone BL216					x		x
Prevotella sp. HOT300/HOT292	x	x			x	x	
Prevotella sp. HOT526		x			x	x	
Prevotella sp. uncultured 1					x	x	
Prevotella sp. uncultured 2				x	x	x	
Tannerella sp. HOT286				x	x	x	x
Tannerella sp. uncultured					x	x	
Firmicutes							
Burkholderiales sp. HOTA57	x	x					
Catonella sp. HOT451					x	x	
Centipeda sp. HOTB01				x			x
Clostridiales sp. HOT075					x	x	
Clostridiales sp. HOT085					x	x	x
Clostridiales sp. HOT093		x			x	x	
Corynebacterium sp. HOTA16			x				x
Erysipelothrichaceae sp. HOTA18		x					
Erysipelotrichales sp. HOTC62		x					
Eubacterium sp. uncultured 1			x	x	x	x	x
Eubacterium sp. uncultured 2			x		x	x	x
Lachnospiraceae sp. HOT083				x		x	
Lachnospiraceae sp. HOT100			x		x	x	
Lachnospiraceae sp. HOTA61 / HOTE59	x		x	x	x	x	x
Lachnospiraceae sp. HOTB32					x	x	
Lachnospiraceae sp. uncultured 1			x	x	x	x	x
Mitsuokella sp. HOT521					x	x	

CBD conditions	Anaerobic		Aerobic				
Uncultured taxa	BHI	BHIm	BHI_G	BHI	BMM	+PMA	-PMA
Mobiluncus sp. uncultured			x	x	x	x	x
Moraxella sp. HOTB07					x	x	
Parvimonas sp. HOTC63			x	x	x	x	x
Peptostreptococcaceae sp. HOT081	x	x	x	x	x	x	x
Peptostreptococcaceae sp. HOT091	x	x					
Peptostreptococcaceae sp. HOT369 / HOT103	x	x	x		x	x	x
Peptostreptococcaceae sp. HOTE46		x					
Selenomonas sp. HOT126				x	x		x
Selenomonas sp. HOTE20					x	x	
Selenomonas sp. HOTE39				x	x	x	
Selenomonas sp. HOTF30			x	x	x	x	x
Selenomonas sp. HOTF82			x		x	x	x
Selenomonas sp. HOTF83/HOTH63				x	x	x	x
Selenomonas sp. HOTF85					x	x	
Selenomonas sp. HOTF87			x	x	x	x	x
Selenomonas sp. HOTF96			x		x	x	x
Selenomonas sp. HOTG51					x	x	
Selenomonas sp. HOTG55				x	x	x	x
Selenomonas sp. HOTH23				x	x	x	x
Selenomonas sp. HOTH30					x	x	x
Selenomonas sp. HOTH32			x		x	x	x
Selenomonas sp. HOTH66					x	x	
Solobacterium sp. HOTA05			x	x	x	x	x
Streptococcus sp. HOT064/HOTE41			x	x	x	x	x
Streptococcus sp. HOTB66					x		x
Streptococcus sp. HOTC04			x	x	x	x	x
Streptococcus sp. HOTC14					x	x	
Streptococcus sp. HOTC56	x	x					
Streptococcus sp. uncultured			x				x
Veillonellaceae sp. HOT150			x		x	x	
Xanthomonadaceae sp. uncultured				x			x
Fusobacteria							
Fusobacteria sp. HOTA71					x	x	
Fusobacterium sp. HOTH27					x	x	
Leptotrichia sp. HOT212			x		x	x	x
Leptotrichia sp. HOT215					x	x	
Leptotrichia sp. HOT392/HOT217				x	x	x	
Leptotrichia sp. HOT417					x	x	
Leptotrichia sp. HOTA45					x	x	
Leptotrichia sp. oral clone 19-33					x	x	

CBD conditions	Anaerobic			Aerobic			
Uncultured taxa	BHI	BHIm	BHI_G	BHI	BMM	+PMA	-PMA
Leptotrichia sp. uncultured				x		x	
Proteobacteria							
Aggregatibacter sp. HOTG01					x	x	
Haemophilus sp. HOTD10 / HOT035					x	x	
Johnsonella sp. HOT166					x	x	
Kingella sp. HOT012					x	x	
Kingella sp. HOTD49					x	x	
Lautropia sp. HOTA94				x	x	x	x
Leptothrix sp. HOT025	x	x					
Neisseria sp. HOT018					x	x	
Neisseria sp. HOTD61				x		x	
Spirochetes							
Treponema sp. HOT237					x	x	
SR1							
SR1 sp. HOT345		x		x		x	
SR1 sp. uncultured 1				x		x	
TM7							
TM7 phylum sp. oral clone 13-10					x	x	
TM7 sp. HOT346					x	x	
TM7 sp. HOT348					x	x	
TM7 sp. HOT349					x	x	
TM7 sp. HOT350				x		x	
TM7 sp. HOT351					x	x	
TM7 sp. HOT352 / HOT353			x				x
TM7 sp. HOT355	x	x	x	x	x	x	x
TM7 sp. HOT437 / HOT356				x	x	x	x
TM7 sp. uncultured 1				x		x	
TM7 sp. uncultured 2					x	x	
TM7 sp. uncultured 3					x	x	
Total	9	15	26	38	82	86	40

3.5 Discussion

In this chapter, mixed bacterial communities were successfully established on HA-coated pegs of the Calgary Biofilm Device from an inoculum of human saliva. A diverse community of oral bacteria was maintained in the model biofilms up to 88 days. Although there were trends apparent in the change of the composition of the biofilms over time, these were not statistically significant. This work has shown that this model is a convenient way of generating large numbers of oral biofilms of a composition of in-vivo relevance. The use of 454 pyrosequencing resulted in significantly higher estimates of species richness than the conventional cloning and Sanger sequencing analysis, showing the value of next generation sequencing for deep coverage analysis of complex bacterial communities.

The CBD biofilms from saliva were dominated by *Lactobacillus* and *Veillonella* species, genera associated with dental caries (Loesche and Syed, 1973). Lactobacilli, in particular, are typically found in late-stage caries, due to their preference for a low pH habitat (Gross et al. 2010). Bacteria interacting in a complex environment form sometimes mutually profitable relationships. One such cooperation was described between *Streptococcus* and *Veillonella* species (Mikx and Van der Hoeven 1975). Streptococci, early colonizers of tooth surface, produce lactic acid, which can be used by *Veillonella* species, who therefore might help to maintain a neutral pH, and be beneficial to oral health (Kumar, Griffen et al. 2005). Lactobacilli are also lactate producers, and it is possible that a similar interaction was in play in the CBD biofilms (Hojo, Nagaoka et al. 2009). Whether *Veillonella* does play a protective role in caries is questionable, however; *Veillonella* numbers have been shown to be

correlated with caries activity and their presence may indicated active lactic acid production but they may not be able to effecting a clinically-relevant environmental raising of pH (Gross, Beall et al. 2012).

Although the biofilm cultivated on the CBD was in some ways typical of a caries-associated plaque, many of the components have previously been shown to be health-associated. Lactobacilli themselves have been shown to be antagonistic to *S. mutans* and Gram-negative species associated with periodontal disease and to have anti-inflammatory properties, and have thus been proposed for use as oral probiotics (Staab, Eick et al. 2009, Soderling, Marttinen et al. 2011, Teanpaisan, Piwat et al. 2011). Other bacteria associated with health are members of the *Proteobacteria* phylum, such as *Neisseria flavescens* (Crielaard, Zaura et al. 2011). *Proteobacteria* made up a relatively high proportion of CBD biofilms, with 25.3 % of all sequences. The CBD biofilms also included many of the putative periodontal pathogens, but in low proportions; they were typically detected at less than 1 % of the total number of sequences. The important oral genus *Fusobacterium* represented only 4 % of the biofilm population, but were quite diverse, with 23 taxa detected. *Fusobacterium* species coaggregate with a wide range of other species enabling them to play an important bridging role in oral biofilm development (Bolstad, Jensen et al. 1996). In addition, they contribute to the establishment of anaerobic conditions in the biofilm, relevant to those experiments here which were performed aerobically (Bradshaw, Marsh et al. 1998).

One of the aims of these experiments was to evaluate the importance of mucin for the establishment of an oral biofilm, its richness, and the presence of

uncultured bacteria. No statistical difference was observed regarding α-diversity of biofilms established in BHI with or without mucin. The compositions of communities analysed by phylogeny taking into account only membership, however, presented a statistically significant difference. The taxa differentially present in mucin supplemented biofilms were *Peptostreptococcus stomatis*, *Prevotella baroniae*, *Peptostreptococcaceae* sp. HOT369 / HOT103 and *Prevotella* sp. HOTB62. Furthermore, *Fretibacterium fastidiosum*, the only representative of the *Synergistetes* phylum, was not detected after 24 days in BHI biofilms, while it was still largely present in BHIm biofilms up to 48 d. All of these species are obligate anaerobes, which are typically nutritionally demanding, confirming previous findings that mucin contributes to the formation of a multi-species biofilm which is conducive to the growth of fastidious anaerobes (Bradshaw, Homer et al. 1994).

The SHI medium was developed to support a mixed saliva-derived bacterial community *in vitro* (Tian, He et al. 2010). It was reported that SHI was superior to commonly used media, including BHI, BMM and chopped meat medium. The culture was analysed only 24 h after inoculation, however, which appears insufficient to allow the establishment of a fully developed community. The authors also suggested that SHI could be used to study previously uncultured species, such as TM7. In practice, however, TM7 made up 6.48 % of the salivary microbiota but represented only 0.03 % in SHI culture.

In the present study, biofilms grown in SHI were statistically different from those established in BHI-based media, with a simpler composition and higher proportions of *Streptococcus*, *Treponema* and *Lactobacillus* species identified at 8 d

of culture. The use of this medium was however discontinued because the high viscosity of the medium, probably due to the inclusion of blood, made the handling and the removal of biofilms difficult. While several agar media include blood as a nutrient supplement, no commonly used broth media contain blood. This could be related to the difficulty of visualising and quantifying bacterial growth in the resulting opaque medium, but could also be related to viscosity-associated handling problems.

Glucose is included as a major energy and carbon source in most currently used bacterial culture media. However, the availability of glucose in many oral habitats, such as periodontal pockets and endodontic infections, is low. The inclusion of glucose in growth media trying to replicate these conditions is therefore questionable. Indeed, glucose has been shown to inhibit some metabolic pathways, the phenomenon known as catabolic repression (Gorke and Stulke 2008). It has been shown to inhibit some inducible enzymes in *E. coli* (Jirešová, Janeček et al. 1981) and bioluminescence in marine luminous bacterium *Photobacterium fischeri* (Nealson, Eberhard et al. 1972), for instance. Bacteria that are able to use both glucose and an alternative carbon source will typically favour glucose utilisation. In this case, the metabolites associated with the use of the alternative source are lost to the community, while some other species may rely on them in the natural environment. As mentioned above, the mixed community successfully grown using the CBD was dominated by members of the genera *Lactobacillus* and *Veillonella*. Both of these genera are associated with sugar metabolism, with *Lactobacillus* forming lactic acid from sugars and *Veillonella* then

using lactate as an energy source. It would appear then, the biofilm created was typical of a sugar-rich environment, and while this might be relevant to caries-associated biofilms, it would be of less relevance to the periodontal pocket or endodontic infections. For this reason, a version of BHI was formulated with reduced glucose to see if this would influence the composition of the community derived from saliva.

No difference in α-diversity was observed in biofilms grown in the presence or absence of glucose in this study, and no OTUs were found to statistically differ between BHI with and without glucose. However, representatives of the SR1 phylum were found only in BHI without glucose, as well as representatives of the *Bacteroidetes* HOT365 / HOT281 / HOTG44 and HOT511, uncultured *Prevotella* and *Tannerella* species and *Clostridiales* sp. HOT075, HOT085 and HOT093. Generally, only 26 taxa of uncultured bacteria were detected in BHI with glucose, versus 38 for BHI without glucose. So while reducing glucose did not appear to have a significant influence on the overall composition of the community, the growth of a number of anaerobic species was enhanced. Although glucose itself was specifically excluded from the medium, there were likely to have been significant residual sugar in the medium. Complex bacterial growth media are crude extracts of animal or plant tissues, and, in this case, BHI will have contained free glucose and glycogen from the constituent brains and hearts. The fermentable carbohydrate content in growth media for the modeling of the growth of oral bacterial communities is an important consideration.

The composition and structure of biofilms incubated with BMM medium were not significantly different from those incubated with the BHI-based media, although two OTUs were found to be enriched in BMM medium: *Streptococcus* sp. HOT071 and *Clostridiales* sp. HOT085. The significance of this finding is unclear.

PMA treatment was the factor which most influenced the perceived biofilm composition in this study, with 25 OTUs presenting a differential abundance. The species whose detection was lower in PMA treated biofilms included *Lactobacillus oris*, *Staphylococcus* sp. NBRC 13889 and *Neisseria* sp. This difference could be explained by the rapid growth of these taxa in the early stages of biofilm development, with a subsequent reduction in proportions. DNA from dead cells may not have been degraded but become integrated as a constituent of the biofilm. Another possible origin of extracellular DNA may be its active production by some species, which has been shown to play a role in the adhesion of bacteria to surfaces (Das, Sharma et al. 2010), micro-colony formation (Dominiak, Nielsen et al. 2011) and the spatial organisation of the biofilm (Bockelmann, Janke et al. 2006).

Main findings:

1. Bacteria from saliva samples were successfully established in long-term culture on HA-coated CBD pegs;
2. No statistical difference was observed regarding α-diversity of biofilms established in BHI with or without mucin and glucose;
3. PMA treatment, allowing to remove extracellular DNA, was the most influential factor regarding the perceived composition of the CBD biofilms;
4. Several uncultured taxa were detected in CBD biofilms, 16 taxa for anaerobically and 94 taxa for aerobically incubated biofilms;
5. Among uncultured bacteria were detected 2 taxa of SR1 and 12 taxa of TM7 Candidate Divisions.

Chapter 4: In-vitro culture of the TM7 Candidate Division

Chapter 4: In-vitro culture of the TM7 Candidate Division

4.1 Introduction

Among oral bacteria at least 34 % of taxa are as yet uncultured (Dewhirst, Chen et al. 2010). Despite the advances of metagenomic and metatranscriptomic analyses, which are contributing to a new understanding of the functional potential of microbiomes, in-vitro culture remains essential for the assessment of the function of individual species, and their role in human health and disease. Oral uncultivable bacteria include both relatives of readily cultivable species and complete branches of the phylogenetic tree including the phyla TM7, SR1 and GN02.

As has been discussed in Chapter 1, there are numerous reasons as to why individual species cannot be cultured in the laboratory, but a common theme for species which naturally form part of a multi-species community appears to be dependence on interactions with other community members. Vartoukian *et al.* (2010) were able to culture a previously uncultivated member of the phylum *Synergistetes* by hybridisation-directed enrichment in co-culture. The strain represents a species, now named *Fretibacterium fastidiosum* (Vartoukian, Downes et al. 2012), the members of which are unable to grow independently, but can grow in co-culture with a range of other oral bacterial species including *Fusobacterium nucleatum* and *Parvimonas micra*.

Attempts have been made to culture a representative of the phylum TM7 which is widely distributed in the environment and forms part of mammalian

microbiomes. Only limited success has been achieved with cultures being short-lived or viable only as micro-colonies (Hugenholtz 2002, Ferrari, Binnerup et al. 2005, Abrams, Barton et al. 2012). No TM7 cultures have been deposited with the culture collections, but the partial success previously reported suggests that the cultivation of a representative of this group should be achievable. Possible culture techniques could include the use of antibiotics as selective agents, as it has been predicted that TM7 bacteria are resistant to streptomycin due to an uracil residue in position 911 of their 16S rRNA, highly uncommon in *Bacteria* but responsible for streptomycin resistance in *Archaea* (Hugenholtz, Tyson et al. 2001). Furthermore, although the most frequently described TM7 morphotype in wastewater were rod-shaped cells forming long chains, with cell diameter and length ranging from 0.3 to 0.5 and 0.6 to 1.7 µm, respectively (Hugenholtz, Tyson et al. 2001), an analysis of rhizosphere microbiota passed through 0.45 µm filter was found to be enriched in TM7 cells, suggesting that the dominant morphotype in this environment consisted of cells smaller than 0.45 µm (Tabei and Ueno 2010). Both strategies, streptomycin inclusion and filtration of the substract, were tested in this study.

4.2 Aims

1. To design and validate specific oligonucleotide probes and primers for Division TM7.

2. To use specific primers designed for the Division TM7 to detect these organisms in samples from endodontic infections and saliva, and Cooked Meat Medium, Calgary Biofilm Device and Fastidious Anaerobe Agar cultures.

3. To use colony hybridisation enrichment to attempt to obtain pure cultures of representatives of the TM7 phylum.

4.3 Methods

4.3.1 Detection of TM7 bacteria by PCR

4.3.1.1 Design of TM7-specific primers

All sequences identified as belonging to the TM7 phylum by the Ribosomal Database project - II (rdp, (Maidak, Cole et al. 2001)) were used to obtain an alignment with representatives of oral genera for primers design. Sequences were filtered for length (minimum 500 bp) and aligned using BioEdit software (Hall 1999). A phylogenetic tree was constructed with the MEGA4 program (Tamura, Dudley et al. 2007). The tree was used to further reduce the number of sequences, by keeping one representative of each major cluster and keeping all unique sequences detected in human-related samples, where this information was available. For each cluster, the longest sequence with the fewest ambiguities was chosen. The edited alignment included 280 sequences (Appendix 3). The alignment was then augmented with representatives of all of the genera listed in HOMD (62 sequences) and realigned. The combined alignment was manually inspected for TM7-specific regions. Seven potential primers were selected on the basis of having one or less mismatch to the TM7 phylum members and at least 3 mismatches to the representatives of other oral genera (Table 16). Due to the extreme diversity of the TM7 16S rRNA genes, 3 of the primers had to include one degenerated nucleotide. For the same reason, it was difficult to apply strict G + C content criteria and the G + C content of the primers ranged from 45 to 67 %. The primers were checked for self-complementarity using Oligo Calc (Kibbe 2007). The primers were synthesised by Eurofins MWG Operon (ecom.mwgdna.com).

4.3.1.2 Validation of designed primers

The specificity of selected primers was confirmed *in silico* by submitting primers sequences to the rdp probe match program with 0 mismatches allowed.

As there are no cultivable relatives for the TM7 phylum, a broad-spectrum validation panel was used, which included bacteria from the five predominant phyla of the oral microbiota (*Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Fusobacteria* and *Proteobacteria* (Paster, Olsen et al. 2006)) and *Synergistetes*. The strains were cultured as described in 2.3.1.1. DNA was extracted using Genelute bacterial genomic DNA extraction kit (Sigma Aldrich) as described in 2.3.2.5.1.

Plasmid DNA from a cloned 16S rRNA gene from TM7 HOT352 was used as a positive control and DNA from a dental caries lesion, previously shown to contain TM7 oral taxa, was used to test the sensitivity of the primers. Both the plasmid DNA and caries lesion DNA were graciously donated Kathrin Schulze-Schweifing.

The DNA extracted from reference strains, positive plasmid DNA and mixed DNA from carious lesion were amplified using TM7-specific primers in conjunction with appropriate (forward or reverse) universal primers (27F-YM and 1492R, Table 2).

The PCR reaction consisted of 23 µl of Thermoprime *Taq* polymerase master mix (Abgene), 1 µl of template, and 0.5 µl (0.2 µM final concentration) of each primer. Initial denaturation, 95 °C for 5 min, was followed by 35 cycles of denaturation at 95 °C for 1 min, annealing for 1 min at annealing temperature (see below) and extension at 72 °C for 2 min, with a final period of extension at 72 °C for

5 min. Annealing temperatures from 55 to 62 °C were tested (see result section 4.4.1.1).

Reference strains used for testing specificity of the TM7 primers:

Actinobacteria

Actinomyces naeslundii (NCTC 10301)

Atopobium rimae (ATCC 49626)

Slackia exigua (ATCC 700122)

Bacteroidetes

Tannerella forsythia (FDC 338)

Porphyromonas gingivalis (ATCC 33277)

Prevotella denticola (ATCC 35308)

Prevotella oris (ATCC 33573)

Firmicutes

Parvimonas micra (ATCC33270)

Streptococcus mutans (NCTC10449)

Lactobacillus casei (ATCC393)

Eubacterium minutum (ATCC700079)

Shuttleworthia satelles (DSM 14600)

Bulleidia extructa (DSM 13220)

Proteobacteria

Aggregatibacter actinomycetemcomitans (ATCC 33384)

Campylobacter rectus (ATCC 33238)

Neisseria mucosa (NCTC 10777)

Fusobacteria

Fusobacterium nucleatum ss *nucleatum* (ATCC 25586)

Synergistetes

Pyramidobacter piscolens (DSM 21147)

4.3.1.3 Validation of previously described TM7 primers

In addition to primers designed in this study, two previously described TM7-specific primers were tested: TM7-580F (Hugenholtz, Tyson et al. 2001) and TM7-1177R (Brinig, Lepp et al. 2003) (Table 19).

TM7-580F primer, in conjunction with universal primer 1492R, was tested in two sets of conditions, those (a) described by Hugenholtz *et al*. (2001) and those (b) adapted by Kuehbacher *et al*. (2008). PCR conditions (a) consisted of a denaturation step at 96 °C for 10 min, followed by 30 cycles of 1 min denaturation at 94 °C, 1 min annealing step at 60 °C and 2 min elongation at 72 °C. A final elongation step at 72 °C for 5 min was performed. In PCR conditions (b) the initial denaturation step of 5 min at 95 °C was followed by 35 cycles of 1 min denaturation at 95 °C, 1 min annealing at 50 °C and 2 min extension at 72 °C, before the final elongation step at 72 °C for 5 min.

Primer TM7-1177R was tested using the conditions described by Brinig *et al*. (2003), consisting of an initial denaturation at 96 °C for 5 min, 35 cycles of 1 min denaturation at 94 °C, 1 min annealing at 64 °C and 2 min elongation at 72 °C, finishing with a final elongation step of 3 min at 72 °C.

Primer sensitivity was tested with TM7 16S rDNA carried by a plasmid and DNA from a carious lesion which contained TM7 representatives (4.4.1.2). The specificity was tested against the bacterial panel previously described (4.3.1.2) and confirmed by the sequencing of the amplified product, as previously described (2.3.2.5.4).

4.3.1.4 Detection of uncultured bacteria with specific PCR

The presence of targeted not-yet-cultured species was assessed by PCR amplification with TM7-specific primer set TM7-580F/1492R and PCR conditions (b). Template DNAs tested were: a saliva sample from a healthy volunteer (sample S-TM7-1), endodontic samples A1, A2 and C and CMM cultures derived from these samples at all time points, aerobic CBD biofilms at all time points and anaerobic CBD biofilms (24 d).

Products generated by PCR were cloned and sequenced (as described in sections 2.3.2.5.3 and 2.3.2.5.4) for saliva and endodontic samples, CMM cultures A1 and A2 at 120 d and C at 50 d, for aerobic and anaerobic CBD biofilms at 24 d.

4.3.2 FISH

4.3.2.1 Design of TM7-specific probes

Oligonucleotide probes targeting oral TM7 were designed by visual inspection of the alignment described in section 4.3.1.1. The criteria for probe development included an exact match to HOT352 / HOT353, at least 2 mismatches to all non-TM7 groups in the alignment and a brightness class I to III as described by Fuchs *et al.* (1998). The brightness class was evaluated by comparing the position of potential probes on the TM7 16S rRNA putative secondary structure (Podar, Abulencia et al. 2007) to that of *E. coli*. Two potential probes and their exact specificity to oral TM7 taxa are described in Table 22. For HOT346 and HOT350, however, the reference sequences were too short to determine the specificity of the TM7-1431 probe.

4.3.2.2 Validation of newly and previously designed probes

TM7 targeting probes described in Table 22 were evaluated against a panel of oral bacteria (see result section 4.4.2.1):

Actinobacteria

Actinomyces naeslundii (NCTC 10301)

Slackia exigua (ATCC 700122)

Bacteroidetes

Tannerella forsythia (FDC 338)

Prevotella oris (ATCC 33573)

Firmicutes

Parvimonas micra (ATCC33270)

Streptococcus mutans (NCTC10449)

Lactobacillus casei (ATCC393)

Bulleidia extructa (DSM 13220)

Proteobacteria

Campylobacter rectus (ATCC 33238)

Neisseria mucosa (NCTC 10777)

Fusobacteria

Fusobacterium nucleatum ss *nucleatum* (ATCC 25586)

4.3.2.3 FISH microscopy

For FISH detection, 10 µL of bacterial suspension in PBS was applied to each 6 mm-diameter well of a 0.075 % gelatine-coated Shandon Multi-Spot microscope slide (Thermo Electron Corporation). Cells were allowed to dry and were fixed by incubation with 30 µL of 1:1 PBS / 100 % ethanol for 2 h at 4 °C in a humid chamber. Slides were washed with PBS and dehydrated by successive immersions in 50 %, 80 % and 96 % ethanol for 3 min each. The hybridisation was carried out at 50 °C for 2

h in the dark in a humid chamber. The hybridisation buffer included 18 % v/v 5M NaCl, 2 % v/v 1M Tris-HCl (pH=8.0), 0.1 % v/v of 10 % SDS and formamide (0 to 40 %). The hybridisation mix included 8 µL of pre-heated hybridisation buffer and 0.5 µL of each probe (16 µM) per well. The slide was then washed twice in post-hybridisation buffer, composed of 4.2 % v/v 5M NaCl, 2 % v/v 1M Tris-HCl, 1 % v/v 0.5M EDTA and 0.1 % v/v of 10 % SDS, for 15 min at 52 °C. Slides were rinsed in PBS and allowed to dry, before being covered with Vectashield mounting medium (Vector Laboratories Inc.) and sealed with a coverslip and varnish.

The detection of fluorescently-labelled cells was undertaken using a Leica SP2 confocal laser scanning system (Leica microsystems) fitted with argon/argon-krypton laser (operating at 488 nm), krypton laser (568 nm) and helio-neon laser (633 nm), with a Leica DMIRE2 inverted microscope (x100 objective).

4.3.3 Colony hybridisation

Colony-hybridisation was performed using digoxigenin (DIG) labelled oligonucleotide probes targeting 16S rRNA from bacterial cells transferred to nylon membrane (Table 14). The detection of the hybridised probe was undertaken by an anti-DIG antibody conjugated to alkaline phosphatase and subsequent chromogenic reaction (Table 15).

Table 14. Colony Hybridisation protocol, part I - colony lift, rRNA fixation and hybridisation of the DIG-labelled probes

Stage	Conditions	Time
Preparation of colony lift		
1. Positively-charged nylon membrane disc placed carefully on agar plate culture avoiding air bubbles[1]	In anaerobic cabinet	1 min
2. 3 orientating pin-holes made through membrane on plate prior to removal of membrane		
3. 1 µl of each positive control applied to membrane (DIG-labelled DNA (DIG Nucleic acid detection kit, 100x) and 16S rRNA PCR product of the target, denatured)		
4. Membrane baked for cell lysis and RNA fixation (Braun-Howland, Vescio et al. 1993)	80 °C	40 min
Membrane pre-hybridisation		
5. Membrane moistened in 2x saline sodium citrate (SSC) solution and placed in 125 ml roller bottle[2]		
6. Pre-hybridised with 10 ml pre-heated DIG Easy Hyb hybridisation buffer[3]	In hybridisation oven at hyb. T	1 h
Membrane hybridisation		
7. Hybridised with 10 ml pre-heated DIG Easy Hyb hybridisation buffer with 20 nM of probe	As for pre-hybridisation	2 h
Post-hybridisation		
8. Washed twice with 25 ml Low Stringency Wash Buffer: 2x SSC + 0.1% SDS	At RT, with agitation	2x 5 min
9. Washed twice with 25 ml pre-warmed High Stringency Wash Buffer: 0.1x SSC + 0.1% SDS	At post-hyb. T, with agitation	2x 15 min

[1] 80 mm diameter, 0.45 µm pore size (Amersham Hybond, GE healthcare Ltd), presterilised by autoclaving between filter paper sheets

[2] Thermo Hybaid

[3] Roche Diagnostics

Table 15. Colony Hybridisation protocol, part II - detection of hybridised probe by chromogenic reaction.

Stage	Conditions	Time
Immunological reaction between DIG and anti-DIG		
10. Washed with 15 ml Washing Buffer	At RT, with agitation	5 min
11. Blocked with 30 ml Blocking Solution	At RT, with agitation	30 min
12. Antibody solution (3 µl Anti-DIG-Alkaline Phosphatase conjugate in 15 ml Blocking Solution) applied to membrane	At RT, with agitation	30 min
13. Washed twice with 30 ml Washing Buffer	At RT, with agitation	2x 15 min
14. Equilibrated with 15 ml Detection Buffer	At RT, with agitation	5 min
Detection of DIG/ anti-DIG conjugates		
15. Membrane covered with NBT/BCIP solution: 80 µl NBT/BCIP stock solution in 4 ml Detection Buffer	RT, in the dark, no agitation	16 h
16. Reaction stopped with 10 ml 1x TE buffer		NA

The criteria for the selection of hybridisation conditions were: hybridisation of 1431-DIG probe with positive controls and lack of hybridisation between the non-sense NON338-DIG probe and any templates, and the 1431-DIG probe and the strains in the validation panel (4.3.2.2). The positive controls were (i) DIG-labelled control DNA, pBR328, 1 µL (DIG Nucleic Acid Detection kit, Roche Diagnostics) at 50 pg/ µL and (ii) heat-denatured (5 min, 95 °C) amplicon of the full length 16S rDNA of TM7 HOT352 / HOT353. The optimum conditions were a temperature of 33 °C for hybridisation and 38 °C for post-hybridisation steps.

4.3.4 Targeted enrichment of TM7

4.3.4.1 Growth conditions

To assess optimal growth conditions plates were inoculated with saliva from a healthy volunteer (samples S-TM7-3).

The experimental conditions were:

(i) FAAB plates inoculated with 100 µL of whole saliva, anaerobic incubation;

(ii) BA plates inoculated with 100 µL of whole saliva, aerobic incubation with 5 % CO_2;

(iii) FAAB plates with streptomycin (10 µg/mL) inoculated with 100 µL of whole saliva, anaerobic incubation;

(iv) FAAB plates inoculated with 100 µL of saliva passed through 0.45 µm filter, anaerobic incubation.

The presence of TM7 was detected by PCR with TM7-580F/1492R primers (section 4.3.1.3) using DNA extracted from a 1/4 of the culture plate after 10 d incubation.

4.3.4.2 TM7 culture

Five series of TM7-targeted culture were performed. 100 µL of saliva samples obtained from the same volunteer on different occasions (S-TM7-4 to S-TM7-8) were inoculated on FAAB plates and incubated anaerobically for 12 d, after which colony hybridisation was performed as described in section 4.3.3. The following day the region(s) corresponding to positive detection of TM7 were harvested and resuspended in 250 µL of pre-reduced RTM (2.3.2.1). 100 µL of the suspension was used for DNA extraction and assessment of the presence of TM7 by PCR with primers TM7-580F/1492R; 50 µL was fixed on slides for FISH detection of TM7 and 100 µL was used to inoculate fresh culture plates. After passage 4 new plates received a streak of helper strains *Propionibacterium acnes* and *Staphylococcus aureus*.

4.4 Results

4.4.1 PCR-detection of TM7 bacteria

4.4.1.1 Design and evaluation of TM7-specific oligonucleotides

A total of 1435 16S rRNA gene sequences, designated as belonging to the Division TM7 in the Ribosomal Database Project database were aligned and used to construct a phylogenetic tree. Using the tree as a guide, the number of sequences in the alignment was reduced to 281, to include representatives of all major clusters seen in the larger tree (Appendix 3). The phylogenetic tree showed two main subdivisions, and most of human and animal related sequences fall into subdivision 2. Two sequences make a figure of exception, GQ263527 and GQ097299. Both were detected in human skin samples, in the same study (Grice, Kong et al. 2009).

The alignment was then augmented with representatives of all of the genera listed in HOMD and realigned. The combined alignment was manually inspected for TM7-specific regions using the primer selection criteria listed in Section 4.3.1.1. Three forward and four reverse primers were selected in this way and are shown in Table 16.

Table 16. Potential TM7-specific primers, numbered according to *the Escherichia coli* 16S rRNA (Brosius, Palmer et al. 1978) (IUPAC notation of degenerate bases: M = A or C; Y = C or T; R = A or G; W = A or T).

TM7 primers	Sequence (5'-3')	Length	(G + C) %	Tm (°C)	Ref.
TM7-207F	TGAGGRATAACTGCCCGAAAGGG	23	52 to 57	60	This work
TM7-233F	TAATRCCGCATATGGTCTTCGG	22	45 to 50	55.8	This work
TM7-560F	CCGGAGTGACTGGGCGTAAAGA	22	59	60.9	This work
TM7-1158R	CCTTCCTCNCCGTTACCG	18	61 to 67	56.7	This work
TM7-1174R	ATACTGACCTGACATCATCCCCTCC	25	52	59.6	This work
TM7-1405R	CTTCGGGTGTTGGTCACTTTCATGG	25	52	60.3	This work
TM7-1417R	ACGAATCGGACTTCGGGTGTTGG	23	57	61.3	This work
TM7-580F	AYTGGGCGTAAAGAGTTGC	19	47 to 53	58.0	(Hugenholtz, Tyson et al. 2001)
TM7-1177R	GACCTGACATCATCCCCTCCTTCC	24	58	60.4	(Brinig, Lepp et al. 2003)

The primers were used in combination with the universal primers 27F-YM and 1492R and initially tested using plasmid DNA from a cloned 16S rRNA gene from TM7 HOT 352, and a sample from a dental caries lesion, previously shown to contain TM7 oral taxa. Using an annealing temperature of 58 °C, all primers produced a PCR product from the cloned DNA template (Table 17), and primers pairs TM7-560F / 1492R and 27F-YM / TM7-1405R gave product from the clinical sample. The primers negative at this annealing temperature were re-tested at 55 °C, but either a product of the incorrect size or multiple size products were seen.

Table 17. Sensitivity assessment of the TM7-specific primers.

Annealing T	Ta = 58 °C		Ta = 55 °C	
TM7 primers	TM7 DNA	mixed DNA	TM7 DNA	mixed DNA
TM7-207F	+		+	multiple bands
TM7-233F	+		+	multiple bands
TM7-560F	+	+	NA	NA
TM7-1158R	+	incorrect size	+	incorrect size
TM7-1174R	+	incorrect size	+	incorrect size
TM7-1405R	+	+	NA	NA
TM7-1417R	+		+	multiple bands

The primers were then tested in various combinations with template DNA obtained from a variety of oral bacteria (Table 18). All primer combinations gave product with one or more of the panel bacteria. At 58 °C, 27FYM / TM7-1405R was particularly cross-reactive, giving product with 15 of the 19 species tested. Raising

the annealing temperature to 60 °C and 62 °C did not completely inhibit the cross-reactivity and at 62 °C, the TM7-positive sample no longer gave a product. Primers TM7-560F / 1492R gave product with the *A. naeslundii* template only, so an alternative annealing protocol of 5 cycles at 65 °C, followed by 30 cycles at 60 °C was tested but a product was still obtained.

None of the designed primers was found to be specific and sensitive enough to specifically amplify TM7 16S rRNA sequences from a mixed template.

Table 18. Assessment of the specificity of the TM7-specific primers. Positive (+) and negative (-) reactions are reported.

PCR conditions	58 °C		60 °C		62 °C				
Template	TM7-560F /1492R	TM7-1405R /1492R	TM7-560F /1492R	27F-YM /TM7-1405R	TM7-560F /1492R	TM7-560F /TM7-1158R	TM7-560F /TM7-1174R	TM7-560F /TM7-1405R	TM7-207F /TM7-1405R
Parvimonas micra	-	+	-	-	-	-	-	-	-
Prevotella denticola	-	+	-	+	-	-	-	-	-
Streptococcus mutans	+	+	-	+	-	-	-	-	-
Prevotella oris	-	+	-	-	-	-	-	-	+
Lactobacillus casei	-	+	-	-	-	-	-	-	+
Actinomyces naeslundii	+	-	+	-	+	-	+	+	-
Eubacterium minutum	-	+	-	+	-	-	-	-	-
Atopobium rimae	+	-	-	-	-	-	-	-	-
Shuttleworthia satelles	-	+	-	-	-	-	-	-	-
Slackia exigua	+	-	-	-	-	+	+	-	-
Bulleidia extructa	+	+	-	+	-	+	+	+	-
Aggregatibacter	-	+	-	-	-	-	-	-	-
Tannerella forsythia	-	+	-	-	-	-	-	-	+
Campylobacter rectus	-	+	-	+	-	-	-	-	-
Porphyromonas gingivalis	-	+	-	+	-	-	-	-	-
Neisseria mucosa	-	+	-	+	-	-	-	-	-
Fusobacterium nucleatum	-	-	-	-	-	-	-	-	+
Pyramidobacter piscolens	-	+	-	-	-	-	-	-	-
16S rDNA	+	+	+	+	+	+	+	+	+
Carious lesion DNA	+	+	+	+	-	-	-	-	-

4.4.1.2 Evaluation of previously described TM7 primers

Two primers specific for the TM7 phylum previously described in literature were assessed (Table 19).

Table 19. Previously described TM7-specific primers.

Primers	Sequence (5'-3')	Tm (°C)	Reference
TM7-580F	AYTGGGCGTAAAGAGTTGC	58.0	(Hugenholtz, Tyson et al. 2001)
TM7-1177R	GACCTGACATCATCCCCTCCTTCC	60.4	(Brinig, Lepp et al. 2003)

The primer TM7-1177R was first tested using the previously described conditions with a 64 °C annealing temperature (Brinig, Lepp et al. 2003), but no product was obtained even from TM7 rDNA. A range of annealing temperatures (56 to 64 °C, 1 °C increments) was then tested with TM7 rDNA and TM7-positive mixed DNA. A product for mixed template DNA was observed at the maximal temperature of 62 °C. When these conditions were tested against the panel bacteria, a faint product was observed for *Eubacterium minutum*. When this primer set was used to amplify TM7 from endodontic samples A1 and A2, described in Chapter 2, a positive product was observed. It was cloned and sequenced, with 48 clones per sample. The sequencing revealed, however, that the product was mixed (Table 20) and TM7 sequences were obtained only for the A2 sample. TM7 taxa detected with this primer were TM7 sp. HOT349 and HOT437. This primer was not used further.

Table 20. Taxa detected in endodontic samples A1 and A2 with TM7-specific primer TM7-1177R (48 clones)

Taxa Samples	A1	A2
Eubacterium infirmum	3	4
Eubacterium nodatum	1	
Filifactor alocis		7
Mogibacterium diversum / vescum / neglectum	3	7
Mogibacterium pumilum	3	
Peptostreptococcaceae sp. HOTD17	6	2
Peptostreptococcus stomatis	6	
Solobacterium moorei	6	16
TM7 sp. HOT349		1
TM7 sp. HOT437		4
Treponema socranskii ss 04		4
Treponema socranskii ss *buccale*		3
Treponema sp. HOT268	1	

The TM7-580F primer, described by Hugenholtz *et al*. (2001), was tested using PCR conditions originally described and validated for TM7 detection in waste water, and in the conditions modified by Kuehbacher *et al*. (2008) for the detection of the division among the intestinal microbiota. At an annealing temperature of 60 °C, TM7-580F / 1492R gave a product with the plasmid TM7 DNA but not from the TM7-positive caries sample. Product was obtained from the templates, however, at an annealing temperature of 50 °C, although the caries sample gave rise to extra bands in addition to the one of the correct size. TM7-580F / 1492R was then tested with the panel of oral bacteria and the band of the correct size was not seen for any strain.

When tested with endodontic samples, exclusively TM7 sequences were detected (4.4.1.3). This primer was used to assess the presence of TM7 bacteria for the remainder of the study.

4.4.1.3 Detection of TM7 in saliva, endodontic, CMM and CBD samples

Primer set TM7-580F / 1492R was used to detect TM7 in endodontic samples A1, A2 and C and derived CMM cultures, as well as in saliva sample S-TM7-1 and CBD biofilms. The range of TM7 phylotypes detected in these samples and their proportions are shown in Table 21 and Figure 31.

Six TM7 taxa were detected in the saliva of a healthy volunteer: HOT346, HOT348, HOT349, HOT352 / HOT353, HOT356 / HOT437 and HOTA56.

Six TM7 taxa were detected in endodontic samples. Sample A1 was the most diverse, with all 6 taxa detected, while only 2 taxa were detected in sample C. The TM7 taxa detected in CMM cultures resembled those found in the endodontic infection samples with which the cultures were inoculated. The only taxon detected in CMM culture which was not seen in the endodontic samples was TM7 sp. HOT355. Conversely, TM7 sp. HOT488 was found in samples A1 and A2 but was not detected in CMM cultures. Samples A1 and A2, as well as corresponding CMM cultures, were dominated by the taxa HOT356 / HOT437 and HOT352 / HOT353. Sample C and derived CMM culture were dominated by taxa HOT352 / HOT353 and HOT348.

In the CBD biofilms, 12 TM7 taxa were detected, including 3 potentially novel taxa. The dominant phylotype was HOT355. The most diverse was the TM7 population in biofilms grown in BMM, with 8 taxa including 2 potential novel taxa. Only biofilms incubated with BHI containing glucose included HOT351 and HOT352 / HOT353 taxa. The CBD biofilm in BHI without mucin was dominated by HOT355,

but also presented a phylotype closely related (99 % identity) to the canine oral taxon 363 (COT363).

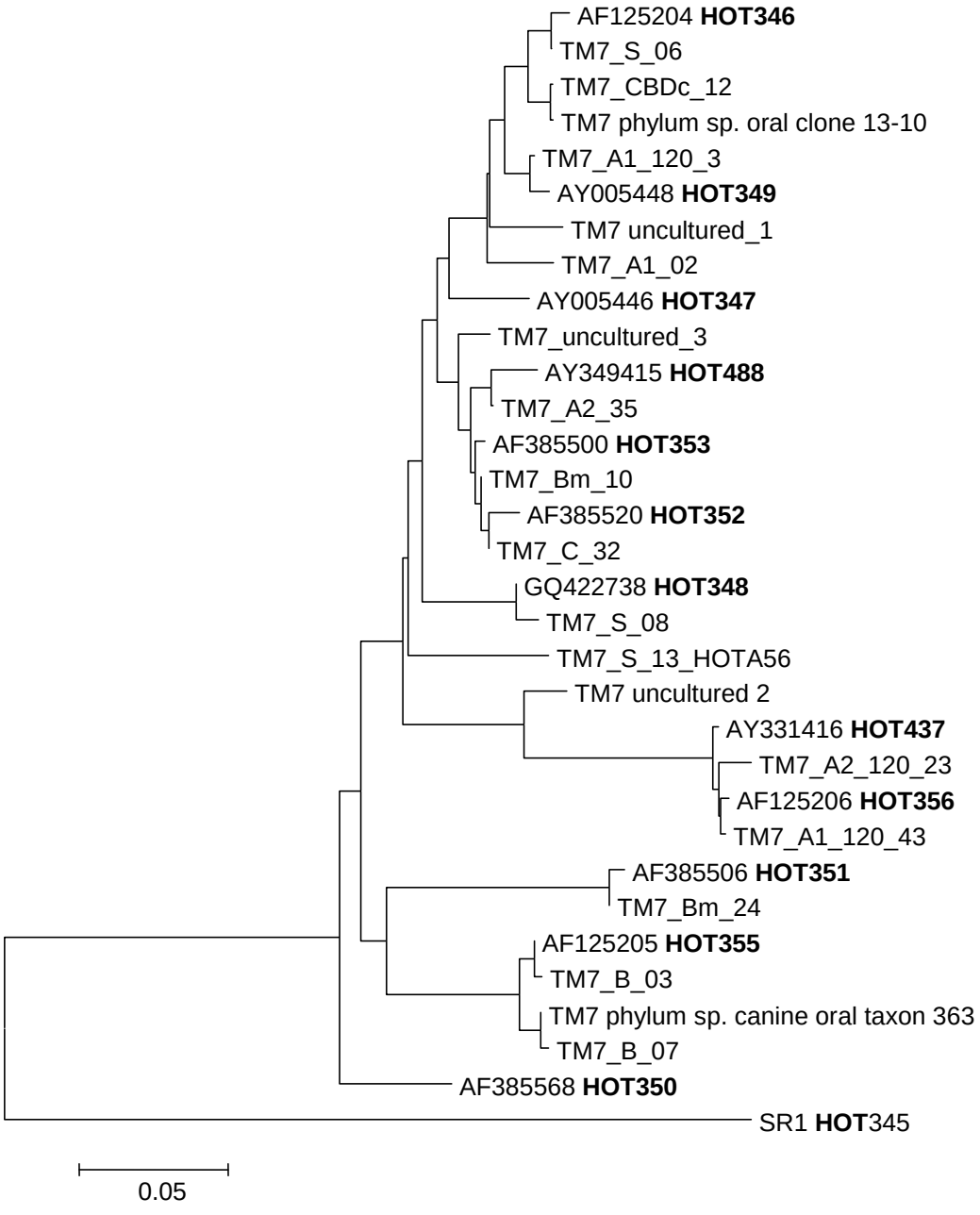


Figure 31. Phylogenetic tree of TM7 taxa detected in this study with reference sequences.

Table 21. TM7 taxa detected with TM7-580F primer, with number of clones (out of 48) identified.

Samples	Endodontic samples			CMM culture			Saliva	Anaerobic CBD biofilms		Aerobic CBD biofilms		
TM7 taxa	A1	A2	C	A1	A2	C		BHI	BHIm	BHIG	BHI	BMM
HOT346							10					4
HOT348	6		21			10	8					6
HOT349	5			12			5					13
HOT350											5	1
HOT351									5	9		2
HOT352 / HOT353	7	9	27	11	23	38	13		16	9		
HOT355				3				45	27	30	37	12
HOT356 / HOT437	23	33		21	25		7				6	
HOT488	3	6										
uncultured 1											5	
uncultured 2												6
uncultured 3												2
oral clone 13-10												2
HOTA56							5					
novel_A1_02	4			1								
COT363								3				

4.4.2 FISH detection of TM7 bacteria

4.4.2.1 Design and validation of TM7-specific probes.

Using the alignment described in 4.3.1.1, 2 new oligonucleotides were designed to be used as fluorescently labeled probes for FISH (Table 22), using the criteria described in 4.3.2.1. Furthermore, a previously described probe, TM7-905 (Hugenholtz, Tyson et al. 2001), was also tested.

The TM7-specific probes were then evaluated against a panel of oral bacteria (Section 4.3.2.2). First, the hybridisation was performed in the conditions described for TM7-905, i.e. hybridisation temperature of 46 °C and 20 % formamide. Non-specific hybridisation to several members of the panel were however observed in these conditions (Table 23). When the temperature was raised to 50 °C, TM7-1431 did not hybridise with any of the bacteria in the panel. TM7-905 and TM7-892, on the other hand, still exhibited non-specific binding. Increasing the formamide concentration to 40 % did not eliminate non-specific binding. However, the addition of a pre-hybridisation step, 15 min incubation with hybridisation buffer in hybridisation conditions but without the addition of the probes, abrogated non-specific binding for TM7-905 but not for TM7-892.

Table 22. TM7 FISH probes. (Hybridisation temperature 50 °C, * pre-hybridisation step required)

Probe	Sequences (5'-3')	5' fluorophore	Length	G+C %	Brightness class (Fuchs, Wallner et al. 1998)	Targeted taxa	Formamide %	Reference
TM7-892	AGCCTTGCGGCCGCACTCCACA	Cy5 and Cy3	22	68	III	TM7 HOT347, 348, 349, 353/353, 356/437, 488	NA	This study
TM7-1431	CCCACCTTAGGCCGACGAATCGG	Cy5 and Cy3	23	65	III	TM7 HOT347, 348, 349, 352/353, 355, 488,	20	This study
TM7-905	CCGTCAATTCCTTTATGTTTTA	Cy5 and Cy3	22	32	IV	TM7 universal	20*	(Hugenholtz, Tyson et al. 2001)
EUB338	GCTGCCTCCCGTAGGAGT	Cy5 and Cy3	18	67	III	universal	0 - 40	(Amann, Binder et al. 1990)
NON338	TGAGGATGCCCTCCGTCG	FITC	18	67	-	-	-	Reverse sequence of EUB338

Table 23. Summary of results of hybridisation conditions optimisation for TM7 probes. B.e. = *Bulleidia extructa*, A.n. = *Actinomyces naeslundii*, S.e. = *Slackia exigua*, F.n. = *Fusobacterium nucleatum* ss. *nucleatum*, P.m. = *Parvimonas micra*, L.c. = *Lactobacillus casei*, C.r. = *Campylobacter rectus*.

		Hybridisation conditions			
Probe	Sequence	46 °C, 20 % formamide	50 °C, 20 % formamide	50 °C, 40 % formamide	50 °C, 20 % formamide pre-hybridisation
TM7-905	5'-CCGTCAATTCCTTTATGTTTTA-3'	non specific B.e., A.n., S.e.	non specific B.e., A.n., S.e.	non specific B.e., A.n., S.e.	specific
TM7-1431	5'-CCCACCTTAGGCCGACGAATC GG-3'	non specific F.n., P.m.	specific	specific	specific
TM7-892	5'-AGCCTTGCGGCCGCACTCCACA-3'	non specific P.m., L.c., A.n., B.e., C.r.	non specific P.m., A.n., B.e.	non specific P.m., A.n., B.e.	non spec P.m., A.n., B.e.

Some artifacts were seen after FISH staining. In some preparations, even when the hybridisation between panel bacteria and TM7-specific probes was clearly negative, some structures, not related in shape to the bacteria tested, gave a positive signal. When the NON338 probe was added, it also hybridised to these structures (Figure 35). The structures, which were probably PTFE coating particles, were mostly coccoid in shape and could potentially have been mistaken for bacterial cells. For this reason, a NON338 control probe was included in every experimental run.

TM7 cells were detected in saliva of a healthy volunteer (sample S-TM7-1) with TM7-1431 and TM7-905 probes. All cells hybridising with TM7-1431 and most of those reacting with TM7-905 presented were coccal cells, ranging in diameter from 0.6 to 1.2 µm (Figure 32, panels A and B). However, some different shaped cells hybridised with TM7-905 probe, including short rods present as single cells or forming chains (Figure 32, panels C and D).

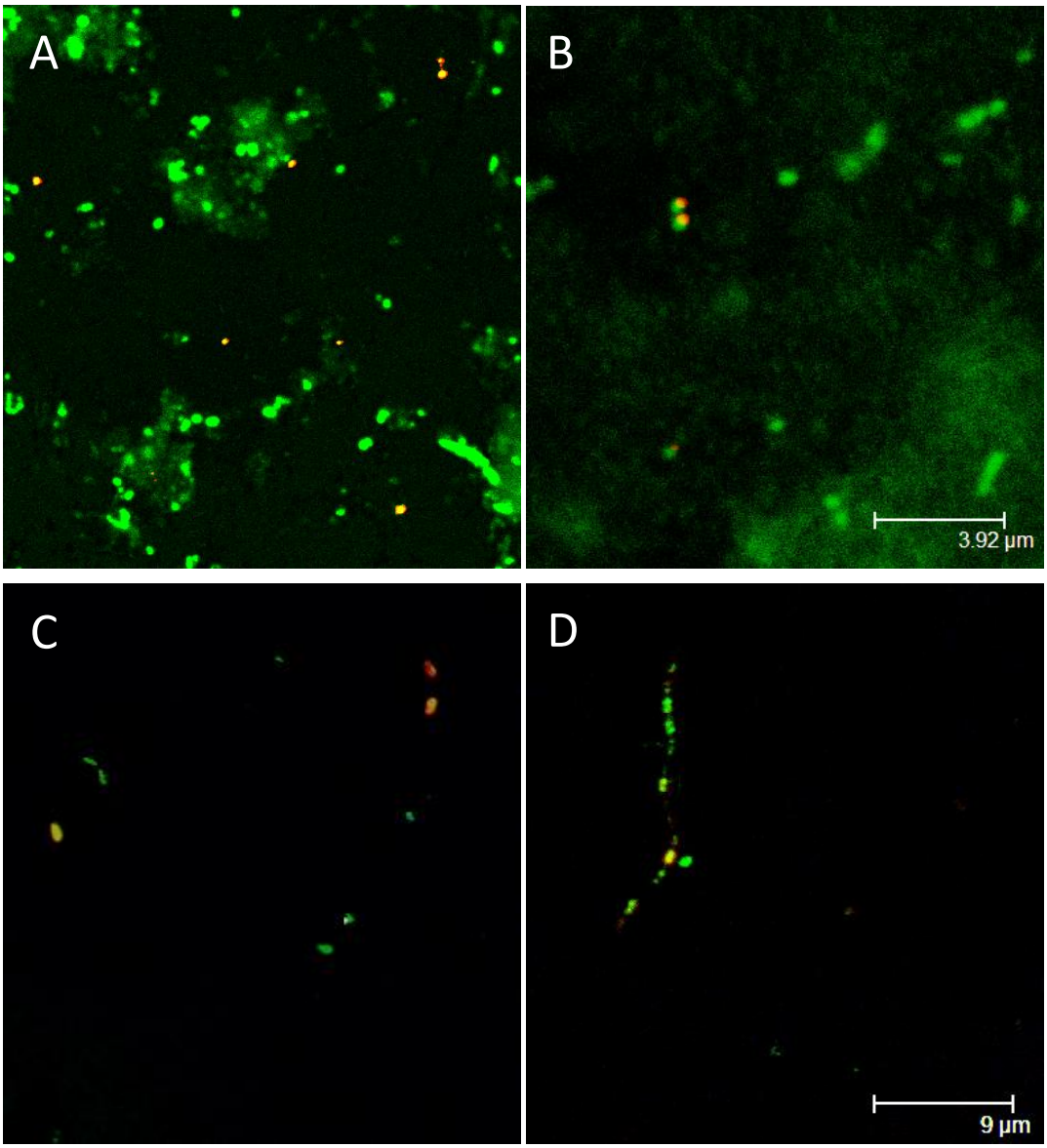


Figure 32. FISH micrograph of TM7 cells in saliva presenting an overlay of total bacteria in green (Cy5-EUB338 probe), non specific hybridisation in blue (FITC-NON338 probe) and TM7 cells in red (A, Cy3-TM7-1431 probe, B, C and D, Cy3-TM7-905 probe).

4.4.3 TM7 bacteria culture from saliva

4.4.3.1 Culture medium and conditions for TM7 culture

In this experiment, saliva was used to inoculate agar plates in an attempt to culture TM7 under the four conditions described in the methods section: untreated saliva on FAAB incubated anaerobically and FAAB supplemented with 10 µg/ml streptomycin, saliva passed through a 0.45 µm filter on FAAB and BA incubated in air + 5 % CO_2.

After incubation for 10 d, bacterial growth harvested from the plates was tested for the presence of TM7 by PCR with primers TM7-580F/1492R. A strong PCR product of the correct size was seen for the unfiltered saliva grown on FAAB and a weak band from the growth on FAAB with streptomycin. No products were seen from the filtered saliva plates or those incubated aerobically. The PCR product from the FAAB plate was cloned as described in section 2.3.2.5.3 and 24 clones partially sequenced and identified. All clones were identified as TM7 HOT352 / HOT353. Cloning was attempted for the weak product obtained from the streptomycin plate, but failed. Anaerobically incubated FAAB was therefore chosen as the method to attempt TM7 isolation.

4.4.3.2 Colony hybridisation enrichment for TM7

Saliva was cultured on FAAB anaerobically for 12 d, after which the plate was blotted and hybridised with the TM7-1431 probe. After blotting, one colony gave a positive hybridisation signal. The corresponding region on the original plate was harvested and used to inoculate an FAAB plate. After 10 days of growth, colony hybridisation was performed and two positive signals were seen.

This procedure was repeated 8 times. After each incubation, the growth was tested for the presence of TM7 by PCR and found to be positive up until the final passage when TM7 could not be detected.

After passage 4, the growth was examined for the presence of TM7 by FISH using probes TM7-905 and TM7-1431. Both probes hybridised to coccal cells (Figure 33), which made up less than 1 % of the total cells seen with the universal EUB-338 probe. A clone library was also prepared from the TM7-specific PCR product obtained from this growth. All clones sequenced were found to be TM7 HOT352 / HOT353.

From passage 4, the plates were streaked with *P. acnes* and *S. aureus*. Colony hybridisation showed TM7-positive signals in a region corresponding to the edge of the *S. aureus* streak (Figure 34).

Following passage 6, micro colonies were visible around the *S. aureus* streak and these were collected and used as the inoculum for passage 7. All of the resulting colonies had a similar morphology and gave a positive product in TM7-specific PCR. 48 clones were sequenced from a library prepared from this product and all were identified as TM7 HOT352 / HOT353. In addition a library was prepared with universal primers 27F-YM/1492R and all 48 clones sequenced from this library were identified as *Slackia exigua*. After passage 8, the TM7 colony hybridisation gave no positive signals.

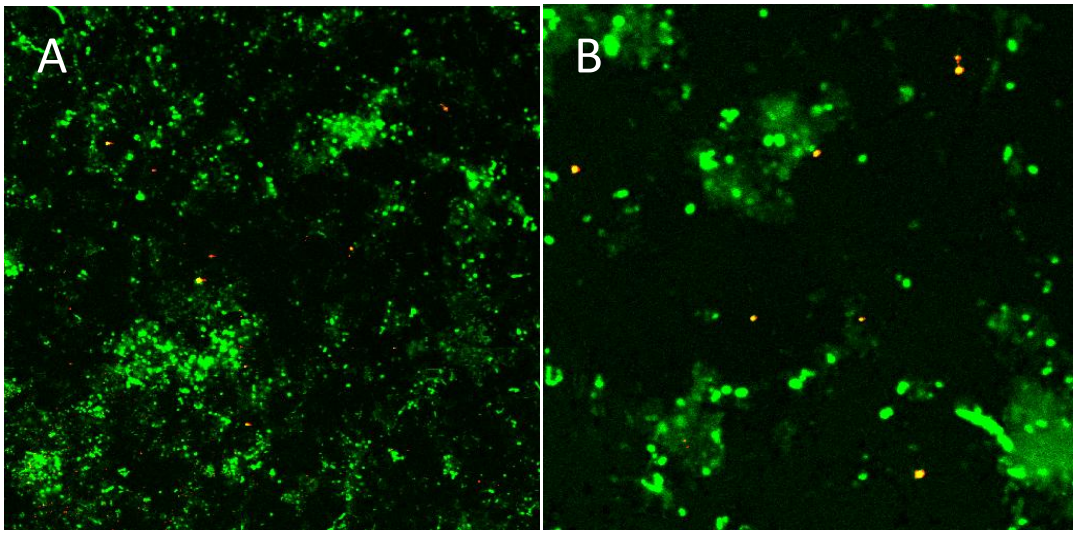


Figure 33. Confocal FISH micrographs of mixed population from FAAB plates containing TM7 bacteria. Overlay showing total bacteria in green (probe Cy5-EUB338) and TM7 cells in red (A probe Cy3-TM7-905, B probe Cy3-TM7-1431).

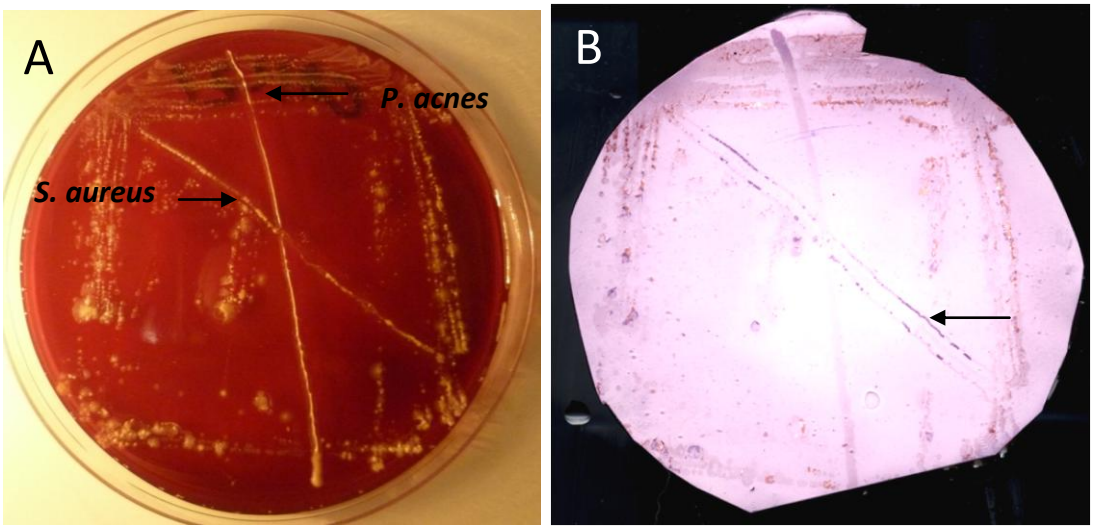


Figure 34. Photographs of A - FAAB plate at CH passage 5, and B - correspondent membrane hybridised with TM7-1431-DIG probe. The arrow indicates positive region around *S. aureus* streak.

The complete experiment was repeated on four further occasions. On three occasions TM7 was detected by colony hybridisation up to three, three and four passages respectively.

On the fourth occasion, the first blotting revealed two positive colonies and each was passaged to a new FAAB plate. One of the subsequent plates did not show a positive hybridisation signal after 12 d of incubation. On the second plate, a number of positive signals were seen and the most intense was selected for passaging. This procedure was repeated and after the third passage, a *S. aureus* streak was added to the newly inoculated plate. This time however no hybridisation at the edges of the streak was observed, but a number of signal-positive colonies were seen. A library was created with universal primers 27F-YM/1492R and all 72 clones were identified as *Atopobium parvulum*. A library created with TM7-specific primers was also created from the same material and all clones were identified as TM7 HOT352 / HOT353. FISH performed with TM7-905, EUB338 and NON338 probes revealed clusters of cells positive with TM7-905 probe (Figure 35). After the fifth passage, however, this culture was lost following a failure of the anaerobic workstation.

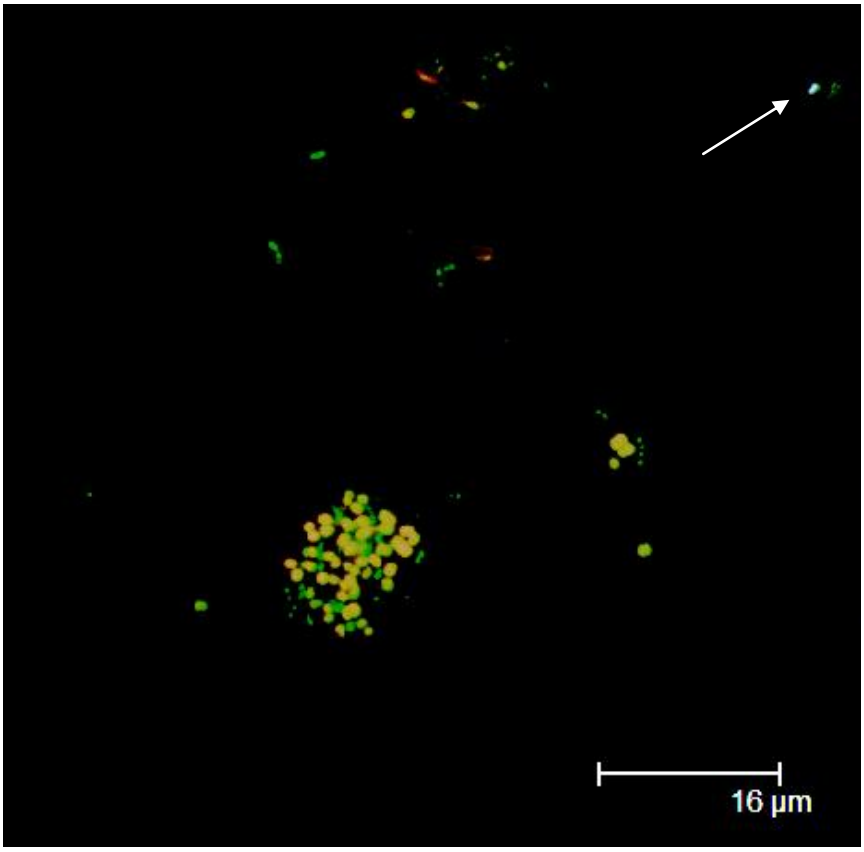


Figure 35. FISH micrograph of cell clusters observed after 4 passages. Overlay of total bacteria in green (Cy5-EUB338 probe), TM7 cells in red (Cy3-TM7-905 probe) and non specific hybridisation in blue (FITC-NON338 probe). The arrow shows a spherical shape hybridising with NON338 probe, probably coating particle.

4.5 Discussion

The development of specific oligonucleotides for TM7 was difficult, for a number of reasons. Firstly, the V1 hypervariable region of 16S rDNA of some phylotypes of TM7 included an insertion of variable length up to 190 bp, between *E. coli* positions 92 and 93, making automated alignments unreliable. Secondly, the high sequence variability among members of the division made it hard to find division-specific regions of homology, suitable for primer design. The intra-phylum sequence variability was evaluated at 17 % by Hugenholtz *et al*. (2001), which is an underestimate following the incorporation of new taxa within the division. Finally, the validation of primers and probes targeting uncultivated taxa is challenging. While some studies have used techniques such as catFISH to validate probes at a single nucleotide discrimination (Ouverney, Armitage et al. 2003), for some others validation was rather perfunctory (Brinig, Lepp et al. 2003). In this study, the validation of FISH probes was complicated by the non-specific reaction with the PTFE coating of the wells on the microscopy slides.

The phylogenetic tree of TM7 sequences presented in Appendix 3 shows two main subdivisions, as has been reported previously (Dinis, Barton et al. 2011). All human and animal related sequences were found to cluster together in the subdivision 2, while the subdivision 1 included exclusively sequences from environmental origin, with the exception of two sequences related to skin samples, originating from the same study. The subdivision 2 included also some sequences of environmental origin, and attempts were made to target an environmental TM7 bacterium which would be closely related to human-associated TM7 (Dinis, Barton

et al. 2011). One such bacterium, with 16S rRNA sequence 98.6 % similar to the human oral taxon 348, was found in high proportion in wastewater samples. However, the high 16S rRNA similarity does not guarantee metabolic similarity in bacteria, as sometimes even different strains of the same species, whilst undistinguishable by their 16S sequences, can present dissimilar behaviour. Finally, the separation of the all sequences in two subdivisions suggests that Candidate Division TM7 may represent more than one phylum.

A total of 16 TM7 phylotypes were identified in this study. The taxon HOT346, largely found in healthy subjects (Paster, Boches et al. 2001, Brinig, Lepp et al. 2003), was detected only in saliva and CBD biofilms grown with BMM. The taxon dominating endodontic infections A1 and A2 in this study was HOT356 / HOT437, and it was maintained in high proportions in derived CMM cultures. It was also detected in saliva, but not in the sample from endodontic infection C. This taxon was found to be associated with periodontitis (Paster, Boches et al. 2001), but was also found in health with 71 % prevalence (Kumar, Griffen et al. 2003). TM7 HOT352 / HOT353 was first identified as being associated with halitosis (Kazor, Mitchell et al. 2003). In this study, however, it was found in all endodontic samples, CMM cultures derived from them, saliva, and most CBD biofilms. It was however not found in CBD biofilms grown without mucin or without glucose, suggesting a requirement for these substrates. This taxon was also the only one identified from communities grown on agar plates. The CBD biofilms incubated with BHI-based media were dominated by TM7 HOT355 taxon. It comprised up to 93.8 % of the TM7 population in anaerobic CBD biofilm without mucin. Finally, the BMM CBD

biofilm included the most rich TM7 community, with 9 taxa, including 2 possible novel. No representatives of the human oral taxon 347 were detected in this study.

The predominant morphology of TM7 cells observed in this study differed from what has been previously described, as only a limited number of cells observed presented the morphotype often described as a "filament", but which in fact were chain-forming rods in a sheath. The most frequently detected morphotype was coccoid cells between 0.6 and 1.2 µm of diameter. This finding could be explained by the fact that TM7-1431 probe did not target the totality of the TM7 diversity, as cocci were the only morphotype detected with this probe. But even when the TM7-905 probe, targeting the whole TM7 division, was used, cocci were predominantly detected. It may be related to the fact that only one subject was used to provide saliva samples and the TM7 diversity in the studied subject was more rich in TM7 representatives of this morphotype. But it is also possible that since the "filament" morphotype is easier to identify, some previous studies may have disregarded the coccoid morphotypes. One HOT identified as presenting the "filament" morphotype, HOT356 (Ouverney, Armitage et al. 2003), was detected in the saliva sample representing 14.6 % of TM7 clones.

On two occasions, a simple community containing only one other species other than TM7, was established, but lost. The two simple cultures were obtained with *Slackia exigua* and *Atopobium parvulum*, both members of the *Actinobacteria* phylum and the *Coriobacteriaceae* family. A more detailed study of the genomes of these species, as well as a metabolic analysis of the mixed cultures, may reveal clues as to the exact mechanism of the interaction in place. Surprisingly,

Staphylococcus aureus also seemed able to support the growth of TM7 bacteria. In this case a comparative analysis of metabolic pathways of *S. exigua* and *A. parvulum* on the one hand and *S. aureus* on the other may be useful. But another possible explanation is that *S. aureus* was in fact supporting and stimulating *S. exigua* growth. Indeed, *S. exigua* is known to be a fastidious and slow growing organism, and a recent study reported that *S. exigua* found in wounds was never found as a sole present species (Kim, Rowlinson et al. 2010). If *S. aureus* was indeed stimulating *S. exigua*, and not TM7 cells directly, it could explain the fact that no similar interaction was observed when TM7 growth was supported by *A. parvulum*.

When aerobic culture was tested for growth of a mixed population supporting TM7 bacteria, no TM7 growth was detected. This could suggest a strict requirement for anaerobic conditions for oral TM7 bacteria, but not necessarily so. Indeed, if the TM7-supporting species are anaerobic and did not survive in the test conditions, TM7 cells, while able to survive the presence of oxygen, would not grow. *S. exigua* and *A. parvulum*, for instance, are both strict anaerobes. The predicted streptomycin resistance of TM7 did not provide a useful tool for community simplification for the oral microbiota samples tested. Indeed, streptomycin resistance appears to be relatively common among oral bacteria, and a single point mutation may be sufficient to confer resistance.

When the plate-grown community included only 2 species, only single colony types were seen, suggesting that TM7 cells were intimately mixed with the other species in the co-culture. The proportion of TM7 cells to the main cell type was low, under or at 1 %, and no TM7 sequences were detected when 27F-YM/1492

amplified 16S rRNA was sequenced. If a simple community could be obtained in future experiments, a deeper analysis of the diversity, by next generation sequencing techniques such as 454 pyrosequencing, for instance, may reveal the presence and the proportion of TM7 bacteria. This type of simple community may also be analysed by proteomic analysis (VerBerkmoes, Denef et al. 2009, Schneider and Riedel 2010, Cantarel, Erickson et al. 2011), or a whole transcriptome shotgun sequencing, for example using RNA-seq technology (Giannoukos, Ciulla et al. 2012), to reveal the molecular basis of the observed support of the TM7 growth.

Main findings:

1. Specific primers and probes targeting TM7 sp. HOT352 / HOT353 were desiged and validated;
2. TM7 sp. HOT352 / HOT353 was detected in all three models of in-vitro culture assessed (CMM, CBD biofilms and agar plates);
3. The colony hybridisation technique allowed the establishment of a simple community including TM7 bacteria with members of the *Coriobacteriaceae* family on two occasions.
4. A closer metabolic analysis of these simple co-cultures may yield clues to the mechanisms of this relationship and provide keys to the pure culture of TM7.

Chapter 5: General discussion

Chapter 5: General discussion

The overall aim of the work described in this thesis was to attempt to culture previously uncultivated oral bacterial taxa *in vitro*. Two laboratory models were used: Cooked Meat Medium and the Calgary Biofilm Device. The use of both models resulted in the successful establishment and maintenance of diverse oral bacterial microbial communities, whose composition was not significantly different from the original samples from which they were derived. The CBD biofilms resembled those formed using the hydroxyapatite (HA) disc model (Madhwani and McBain 2011). The latter model is commonly analysed after up to 10 days of growth as they are often used to study the early stages of dental plaque biofilms or can be sampled and used as a secondary inoculum (Shaddox, Alfant et al. 2010), which was not attempted in this study with the CBD biofilm. Rudney *et al*. (2012) used a biofilm reactor to establish biofilms on HA discs from saliva and plaque inocula. The design of their study had some similarities to the work performed in this study, although the biofilm was grown in BMM aerobically, without the addition of CO_2; and the biofilms were analysed after only 48 h of growth. Replicate inocula were obtained from the same subjects at different times, and it was reported that the bacterial composition of the inocula and the biofilms derived from them were stable, although the composition of the communities obtained from saliva and subgingival plaque samples were significantly different. In this study only saliva samples were used to inoculate the CBD biofilm model, and the use of supra- and sub-gingival samples may extend the clinical relevance of the model.

In addition, Rudney *et al*. (2012), analysed the composition of the biofilms by means of the Human Oral Microbial Identification Microarray (HOMIM) system (Colombo, Boches et al. 2009) and not by sequencing the 16S rRNA gene. The use of HOMIM was recently compared to the 454 pyrosequencing analysis of oral wash samples (Ahn, Yang et al. 2011). The results obtained by both methods highly correlated at phylum and genus levels, particularly for dominant members of the community. The correlation was weaker for rarer genera. Thus the use of HOMIM could significantly speed up the process of analysing the composition of inocula and in-vitro biofilms. It is however less suited for the detection of uncultured bacteria, which are frequently present in low numbers, and, if novel, will not have a detection probe on the array.

One limitation of the experimental design used in these studies was that complex media were used, which by their very nature are not chemically defined. The use of defined media would allow for a more precise analysis of the effect of different constituents, but few are available even for individual oral bacterial species, let alone mixtures, because of their nutritionally fastidious nature. Wong et al. (2001) have described a defined medium with mucin which they found to allow growth of plaque biofilms at a similar rate and composition to BMM. The use of this medium could possibly have allowed for a better control over carbohydrates level, as it was reported that sucrose pulses had more substantial effects in this medium than in BMM (Sissons, Anderson et al. 2007). An alternative is to attempt to mimic the natural habitat of the target organisms. One such attempt resulted in the creation of the SHI medium (Tian, He et al. 2010). When used with CBD biofilms in

this study, SHI however did not outperform other media, a finding confirmed by Rudney *et al*. (2012).

To improve media and culture conditions, an improved understanding of the natural environments is required. Proteomics could be used to analyse the proteins present in an environment and related bacterial activity at the same time. A recent study of the human salivary supernatant from six healthy subjects combined protein dynamic range compression, multidimensional peptide fractionation, and high-mass accuracy MS/MS with a novel two-step peptide identification method (Jagtap, McGowan et al. 2012). The study compared the results to a database of human proteins and those translated from bacterial genomes. Twenty pathways, as described in Kyoto Encyclopedia of Genes and Genomes, were identified, with carbohydrate metabolism, amino acid metabolism, energy metabolism, translation, membrane transport, and signal transduction predominant. A combination of metagenomics and metaproteomics can provide valuable insights into the community structure and physiology of different phylogenetic groups present in a specific environment. It has been applied to the gut environment (Verberkmoes, Russell et al. 2009), and allowed for identification of novel pathways for microbial metabolism and human immune response. Proteomic analyses have also been performed on oral habitat samples, such as the acquired pellicle (Siqueira, Custodio et al. 2012) and human oral epithelial cells (Ghosh, Yohannes et al. 2012). These studies may allow a better understanding of the natural environment of oral bacteria and help to recreate it in laboratory conditions. To better understand

which metabolic pathways are in action in a specific community would enable those nutrients implicated to be supplied in artificial culture media.

The growth rate of uncultured bacteria may be another crucial point in obtaining a community including uncultivated species. Some success in the culture of novel species has resulted from the use of extended incubation times. Thus a mesophilic representative of the *Thermotogales* order was isolated for the first time after a 2-month incubation (Ben Hania, Ghodbane et al. 2011). Unlike its use for single species growth (Holman 1919), CMM does not seem to be adapted to extremely long incubation times of mixed cultures. In this study, the standard incubation time between passages was of ten days, but it was speculated that if the target species did not attain a certain proportion within the 10 days of incubation, it would be lost to the subsequent batch. The incubation time was thus increased to 30 days, in an attempt to enrich for slower-growing organisms. Unfortunately, the composition of the community changed dramatically and previously persistent not-yet-cultivated species were lost. This may have been due to a lack of nutrients or the accumulation of toxic by-products produced by the community. In the CBD, on the other hand, there was no need to subsample the community at every change of media and the slowest growing representatives were not lost over time.

The depth of the biofilm was not controlled in the CBD model used in these studies, which can lead to heterogeneity between biofilms. Although in this study the pooling of biofilms from several pegs was used to alleviate the problem, another solution would be the use of the constant-depth film fermenter (CDFF) model (Hope and Wilson 2003). Recently, the CDFF was used to recreate in-vitro

dental plaque (Zaura, Buijs et al. 2011) and saliva (Hope, Bakht et al. 2012) microcosms. In the latter study the variability between independent experiments in the CDFF was evaluated, and it was concluded that experimental variation could be minimised by running experiments in parallel in two independent CDFFs. The advantage of the CBD device is that different conditions can be tested on the same plate, reducing inter-experiment variation.

The CBD device establishes a bacterial community as a biofilm, in which extracellular DNA has been reported to play an important structural role (Branda, Vik et al. 2005). The presence of DNA presents a problem for molecular analyses however, because it will be amplified by universal PCR-based assays and thus give a misleading impression of community structure. One way to overcome this is to use RNA as the starting point for the analysis in place of DNA, because of its substantially shorter half-life (Arraiano, Yancey et al. 1988, Keer and Birch 2003). The development and validation of protocols using PMA to degrade free DNA have greatly simplified molecular assays and allowed the discrimination of DNA from live cells and that in cells with compromised membranes or extracellular DNA (Nocker, Sossa-Fernandez et al. 2007). The protocol has also been validated for use with 454 pyrosequencing (Nocker, Richter-Heitmann et al. 2010) and multispecies biofilms (Alvarez, Gonzalez et al. 2013). It has also been adapted to be used on filter membranes and to use 460 nm LEDs rather than high-wattage halogen lamps, which would prevent damage to previously intact cells (Hellein, Kennedy et al. 2012). Ethidium monoazide can also be used in a similar manner (Nogva, Dromtorp et al. 2003), but was reported to be able to penetrate intact membranes of some

bacterial species (Nocker, Cheung et al. 2006). In this study PMA treatment had a statistically significant effect on community composition, and more uncultured species were revealed, probably by removing the DNA from dead cells of fast-growing species.

The inclusion of several taxa of uncultured bacteria in in-vitro cultures shows the success of mixed communities in supporting the growth of their uncultured members. Diverse mixed communities, however, are not easy to analyse and it can be difficult to determine the role of individual species in the mixed community. Several models have attempted to isolate bacteria in pure culture while at the same time keeping them in interaction with the source community using semi-permeable membranes (Zengler, Toledo et al. 2002, Ferrari, Binnerup et al. 2005). For example, a multi-chamber set-up yielded successful isolation of several previously uncultured marine organisms (Kaeberlein, Lewis et al. 2002). Microfluidic confinement was also shown to allow isolated cells to display normal quorum sensing behaviour (Boedicker, Vincent et al. 2009). Several new lineages remotely related to previously cultivated strains, with one lineage in particular presenting less than 84 % identity to any published sequences (Zengler, Toledo et al. 2002) were isolated using high-throughput cultivation of cells isolated in gel micro-droplets, with 10^7 droplets analysed, and sea-water and soil as inocula. This type of high-throughput culture study seems to be particularly adapted to poorly-sampled environments, such as sea and fresh water and some soil communities, but its utility in relatively well described habitats, such as the oral cavity, is less clear. A high-throughput study combining different techniques to grow previously uncultured oral bacteria

resulted in the successful cultivation of three new genera and several new species (Sizova, Hohmann et al. 2012). However, the new species, as described in the study, were often closely related to previously published or unpublished isolates, sometimes with 16S rRNA identity over 99 %. The new genera all fell into the *Firmicutes* phylum, the most represented oral phylum. While this work is valuable in isolating representatives of previously uncultured taxa within otherwise well cultured phyla, these methods may be unlikely to lead to the culture of representatives of deep uncultured branches or phyla.

It is for this reason that the last part of this work was focused on the targeted culture of representatives of the TM7 phylum. From five attempts, two simple co-cultures were obtained on agar plates. While these cultures were lost, these results were encouraging. If another simple co-culture can be obtained, several recently developed techniques could be applied to better understand the nature of interactions in place, such as metatranscriptomic analysis (Frias-Lopez and Duran-Pinedo 2012). Metagenomics, or whole metagenome shotgun analyses (Riesenfeld, Schloss et al. 2004, Chen and Pachter 2005), can now be performed to a depth sufficient to analyse such a community, even if the TM7 representative is present in small numbers compared to the helper strain, thanks to the development of next generation techniques, such as Illumina MiSeq paired end sequencing. This approach would allow the sequencing and assembly of the genome of a representative of the TM7 division. While several TM7 genome sequences are deposited in the databases (Marcy, Ouverney et al. 2007, Podar, Abulencia et al. 2007), the quality and coverage of these remain low.

The culture of previously uncultured bacteria remains a high priority for the understanding of the role of complex bacterial communities in health and disease. The further development and refinement of the approaches developed in the work described in this thesis, augmented by the technological advances of the “omics” era should enable the culture of a wide range of currently poorly characterised organisms.

Main findings:

1. CMM batch culture and CBD biofilm were successfully used to established a long-term mixed bacterial communities *in vitro*, but best results were obtained with CBD model;
2. Both models included not-yet-cultivated bacteria as part of the mixed communities;
3. PMA treatment represents an advantageous tool for discrimination between live and dead cells in molecular analyses;
4. The colony hybridisation technique allowed the establishment of a simple community including TM7 bacteria with members of the *Coriobacteriaceae* family on two occasions.

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Appendix 1: Composition of the endodontic samples and derived CMM culture.

Table 24. Composition of the endodontic sample A1 and derived CMM culture.

Samples	A1 sample			A1 CMM culture						
	py	Sanger	Isolates	10d	20d	120d-py	120d-Sanger	150d	310d	480d
Actinobacteria										
Actinomyces sp.HOTB78 / HOTE33 / HOTF78 / HOT169			2.2							
Atopobium rimae	0.63		2.2	3.3		0.16				
Bifidobacterium dentium					1.1	0.32		1.1		
Olsenella sp. HOT809	0.38			3.3	1.1	1.51	2.2	5.6		3.3
Olsenella uli		2.2	20.0					1.1		
Rothia mucilaginosa	0.06									
Bacteroidetes										
Alloprevotella tannerae	0.19									
Porphyromonas endodontalis	4.11	2.2								
Prevotella denticola /HOTG57 / HOTG70	0.13		1.1							
Prevotella enoeca	3.80									
Prevotella histicola	0.06									
Prevotella marshii	0.06									
Prevotella melaninogenica / HOT313 / HOTE10	0.06									
Prevotella nigrescens	9.50	5.6	7.8							
Prevotella oralis	1.90		2.2		17.8	16.02	13.3		4.4	
Prevotella oris	6.84	8.9			2.2	5.42	4.4		1.1	
Prevotella pallens	0.06									
Prevotella pleuritidis	0.51									

Samples	A1 sample			A1 CMM culture						
	py	Sanger	Isolates	10d	20d	120d-py	120d-Sanger	150d	310d	480d
Prevotella salivae / HOTE13	0.06									
Prevotella sp. HOT300 / HOT292	1.65		2.2			0.40	1.1			
Prevotella sp. HOT315	0.25									
Prevotella sp. HOT526	4.43	10.0								
Prevotella sp. unclassified	0.06									
Tannerella forsythia	0.38									
Firmicutes										
Anaeroglobus geminatus					18.9	23.51	36.7			
Catonella morbi	0.06					0.08				
Dialister invisus / HOTA97	19.30	21.1	4.4	3.3	2.2	12.99	10.0		1.1	
Dialister pneumosintes / HOT D97 / HOT 502	2.72	3.3	2.2			3.19	4.4			
Eubacterium infirmum	2.03	4.4	4.4	4.4	1.1	7.97	10.0	1.1	12.2	5.6
Eubacterium nodatum / HOTG32	1.39	2.2	1.1	30.0	12.2	0.56		20.0	1.1	
Lactobacillus catenaformis	3.80					0.40				
Mogibacterium diversum / *vescum* / *neglectum*	3.10	3.3	6.7	7.8	8.9	2.15	3.3	43.3	26.7	2.2
Oribacterium sp. HOT372 / HOT078 / HOTA41	0.13	1.1		3.3						
Parvimonas micra / HOT393	7.09	5.6	22.2	22.2	25.6	4.70	4.4	24.4	44.4	82.2
Peptostreptococcaceae sp. HOT369 / HOT103	0.25			2.2						
Peptostreptococcus stomatis	6.90	15.6	6.7	5.6	1.1	0.32		2.2	6.7	
Pseudoramibacter alactolyticus	7.03	13.3	7.8	11.1	6.7	3.67	6.7		2.2	6.7
Selenomonas sputigena / HOT134 / HOTC23	0.19									
Shuttleworthia satelles / HOTG69	0.76		3.3	2.2	1.1	0.72		1.1		
Solobacterium moorei	4.43		3.3			1.27				

Samples	A1 sample			A1 CMM culture						
	py	Sanger	Isolates	10d	20d	120d-py	120d-Sanger	150d	310d	480d
Streptococcus infantis / HOT065	0.06									
Streptococcus mitis bv 2 / HOTC56	0.06									
Veillonella rogosae	0.06									
Veillonellaceae sp. HOT155	0.76									
Veillonellaceae sp. HOT132 / HOT129 / HOTB19	0.32									
Fusobacteria										
Fusobacterium nucleatum ss. *animalis*	2.59			1.1		4.86	1.1			
Proteobacteria										
Campylobacter gracilis	0.38					9.00	2.2			
Comamonadaceae sp. unclassified	0.06									
Leptothrix sp. HOT025	0.13									
Ralstonia sp. HOTB67	0.06									
Sphingobacteriales sp. unclassified	0.19									
Spirochetes										
Treponema socranskii ss. *buccale*	0.19									
Treponema socranskii ss. *socranskii* / ss 04	0.13					0.48				
Synergistetes										
Fretibacterium sp. HOT360 / HOT453	0.13									
Fretibacterium fastidiosum	0.32	1.1				0.32				
Tenericutes										
Mycoplasma salivarium	0.25		1.1							

Table 25. Composition of the endodontic sample A2 and derived CMM culture.

	A2 sample		A2 CMM culture					
	py	Sanger	10d	120d- py	120d-Sanger	150d	310d	480d
Actinobacteria								
Actinomyces sp. HOT172				0.10				
Actinomyces sp. HOTB78 / HOTE33 / HOTF78 / HOT169				0.10				
Atopobium rimae	0.15							
Bifidobacterium dentium			1.1	0.70				
Olsenella profusa	0.03							
Olsenella sp. HOT809	3.83	1.1	6.7	0.50			1.1	2.2
Olsenella uli	0.03					2.2		
Bacteroidetes								
Alloprevotella rava	0.50							
Alloprevotella tannerae	0.06							
Bacteroidetes sp. HOT365 / HOTG44 / HOT281	8.85	12.2		0.20				
Porphyromonas endodontalis	2.85	2.2						
Prevotella denticola / HOTG57 / HOTG70	0.47	1.1						
Prevotella melaninogenica / HOT313 / HOTE10				0.10				
Prevotella nigrescens	19.12	21.1						
Prevotella oralis	0.06			6.11	3.3			
Prevotella oris	0.27			0.60	1.1			
Prevotella pleuritidis	0.06							
Prevotella sp. HOT300 /HOT292	0.65				2.2			
Prevotella sp. HOT315	0.27							

	A2 sample		A2 CMM culture					
	py	Sanger	10d	120d- py	120d-Sanger	150d	310d	480d
Prevotella sp. HOT526	0.42	1.1						
Firmicutes								
Anaeroglobus geminatus			2.2	17.74	21.1			
Clostridiales sp. HOT093	0.03							
Dialister invisus / HOTA97	8.52	5.6		6.01	4.4	1.1		
Dialister pneumosintes / HOT D97 / HOT 502	4.21	1.1		2.40				
Eubacterium infirmum	0.21		3.3	2.20	2.2			6.6
Eubacterium nodatum / HOTG32	0.68			0.30		15.6	3.3	
Filifactor alocis	10.12	12.2	12.2	2.30	3.3	23.3		
Granulicatella adiacens				0.10				
Lactobacillus catenaformis	0.92			1.00				
Mogibacterium diversum / *vescum* / *neglectum*	4.46	8.9	31.1	1.00	1.1	25.6	5.6	
Mucilaginibacter sp. unclassified	0.03							
Oribacterium sp. HOT372 / HOT078 / HOTA41	0.03	1.1	1.1	0.20		2.2		
Parvimonas micra / HOT393	3.39	2.2	12.2	6.21	12.2	20.0	26.7	80.0
Peptococcus sp. HOTD92				0.10				
Peptostreptococcaceae sp. HOT369 / HOT103				0.10				
Peptostreptococcaceae sp. HOTB61	0.21							
Peptostreptococcus stomatis	7.81	10.0	1.1	1.60	1.1	3.3	1.1	
Pseudoramibacter alactolyticus	7.78	15.6	6.7	2.71	6.7	4.4	10.0	2.2
Selenomonas sp. HOT136				0.10				
Selenomonas sputigena / HOT134 / HOTC23	0.36							
Shuttleworthia satelles / HOTG69	0.47	1.1	4.4	0.40		1.1		

	A2 sample		A2 CMM culture					
	py	Sanger	10d	120d- py	120d-Sanger	150d	310d	480d
Solobacterium moorei	3.68		1.1	0.30				
Streptococcus constellatus / *intermedius* / HOT E12	0.03		15.6	9.92	22.2	1.1	52.2	8.9
Streptococcus mitis bv 2 / HOTC56				0.10				
Streptococcus sp. HOT058	0.03							
Veillonella dispar / *parvula* / HOTG30			1.1					
Veillonellaceae sp. HOT132 / HOT129 / HOTB19	0.03							
Fusobacteria								
Fusobacterium nucleatum ss. *animalis*	5.05	1.1		33.37	15.6			
Fusobacterium nucleatum ss. *polymorphum*	0.03							
Fusobacterium nucleatum ss. *vincentii*	0.03							
Proteobacteria								
Burkholderiales sp. HOTA57	0.06							
Campylobacter gracilis	1.01			3.11	3.3			
Comamonadaceae sp. unclassified	0.06							
Hafnia / Aranicola / Serratia	0.03							
Leptothrix sp. HOT025	0.03							
Neisseria pharyngis				0.10				
Novosphingobium capsulatum / sp. NG35 / MG37 / MG39 / MG40 / MG43 / MG44	0.03							
Proteus mirabilis	0.03							
Ralstonia sp. HOTB67	0.03							
Sphingobacteriales sp. unclassified	0.18							
Spirochetes								

	A2 sample		A2 CMM culture					
	py	Sanger	10d	120d- py	120d-Sanger	150d	310d	480d
Treponema denticola	0.06							
Treponema lecithinolyticum	0.06							
Treponema maltophilum	0.24							
Treponema socranskii ss. *socranskii* / ss 04	0.21			0.10				
Synergistetes								
Fretibacterium sp. HOT360 / HOT453	1.90	2.2						
Fretibacterium fastidiosum	0.34							

Table 26. Composition of the endodontic sample C and derived CMM culture.

	C sample		C CMM culture												
	py	Sanger	10d	20d	30d	50d-py	50d-Sanger	70d	70d-mucin	110d	110d-mucin	140d-py	140d-Sanger	140d-mucin-py	140d-mucin-Sanger
Actinobacteria															
Actinomyces israelii	0.18														
Actinomyces naeslundii						0.12									
Actinomyces neuii ss. *anitratus*											3.4				2.2
Actinomyces odontolyticus				4.6		0.12				1.1					
Actinomyces sp. HOT170						0.23								0.24	
Actinomyces sp. HOT175			1.4												
Actinomyces sp. HOTB78 / HOTE33 / HOTF78 / HOT169	0.09														
Olsenella sp. HOT809												3.09			
Bacteroidetes															
Capnocytophaga sp. HOT380 / HOTE54	1.06														
Capnocytophaga sp. unclassified	0.09	14.6													

	C sample		C CMM culture												
	py	Sanger	10d	20d	30d	50d-py	50d-Sanger	70d	70d-mucin	110d	110d-mucin	140d-py	140d-Sanger	140d-mucin-py	140d-mucin-Sanger
Prevotella denticola / HOTG57 / HOTG70	0.09														
Prevotella oralis	0.09											0.36			
Prevotella oris	0.09														
Prevotella sp. HOT300	0.09														
Firmicutes															
Anaeroglobus geminatus	0.09														
Clostridiales sp. HOTC47											5.7	13.09		25.46	23.3
Eubacterium infirmum													6.32		
Eubacterium yurii	13.84	6.3	23.2	16.9	1.1	8.41		12.5	38.6	5.4	5.7	19.27		10.60	10.0
Filifactor alocis	0.09														
Finegoldia sp. HOTD24											3.4			5.97	3.3
Granulicatella adiacens			1.4	3.1		0.35		2.1	0.0		1.1	0.18	2.11	1.71	1.1
Mogibacterium diversum / vescum /	3.19	6.3				0.70				2.2	1.1	1.27		1.46	1.1
Oribacterium sinus	0.09														
Parvimonas micra / HOT393		1.0										40.36	72.63		
Peptoniphilus indolicus												1.27	14.74	1.22	22.2

	C sample		C CMM culture												
	py	Sanger	10d	20d	30d	50d-py	50d-Sanger	70d	70d-mucin	110d	110d-mucin	140d-py	140d-Sanger	140d-mucin-py	140d-mucin-Sanger
Peptostreptococcus stomatis	56.25	35.4	5.8	6.2	1.1	7.59	1.4			1.1	11.5	2.36	1.05	4.51	1.1
Pseudoramibacter alactolyticus												16.73	1.05		
Selenomonas sp. HOTF21	0.09														
Streptococcus anginosus	0.09														
Streptococcus gordonii /HOTH24	0.09										8.0		2.11	2.19	2.2
Streptococcus mitis bv 2 / HOTC56												0.18			
Streptococcus sanguinis						1.64						0.18			
Streptococcus sp. HOT058				6.2	5.4	13.67	5.7	16.7	13.6	2.2	6.9	0.18		1.10	1.1
Streptococcus sp. HOT064			1.4												
Streptococcus sp. HOT431												0.18			
Veillonella dispar / *parvula* / HOTG30	0.27		46.4	53.8	92.4	43.11	90.0	64.6	47.7	77.2	51.7			22.29	24.4

	C sample		C CMM culture												
	py	Sanger	10d	20d	30d	50d-py	50d-Sanger	70d	70d-mucin	110d	110d-mucin	140d-py	140d-Sanger	140d-mucin-py	140d-mucin-Sanger
Fusobacteria															
Fusobacterium naviforme	0.71					0.70									
Fusobacterium nucleatum ss. animalis	13.49	25.0	20.3	9.2		22.31				10.9	1.1			18.64	6.7
Fusobacterium nucleatum ss. vincentii	0.27	1.0				0.23	2.9	4.2							
Proteobacteria															
Proteus mirabilis	0.18														
Spirochetes															
Treponema maltophilum	6.03	2.1													1.1
Treponema sp. HOT258						0.82						1.27		4.63	
Treponema vincentii	3.19	8.3													
Synergistetes															
Fretibacterium fastidiosum	0.18														
TM7															
TM7 sp. clone TSS007	0.09														

Appendix 2: Composition of CBD biofilms.

Table 27. Composition of anaerobic CBD biofilms.

	BHI							BHI mucin								SHI
Samples Species	8 d	16 d	24 d	24 d py	32 d	40 d	48 d	8 d	16 d	24 d	24 d py	32 d	40 d	48 d	88 d	8 d
Actinobacteria	2.50	5.0	1.3	0.2			1.3		1.3	1.3	0.2	1.3	2.6	3.8		
Actinomyces sp. HOT169				0.1												
Actinomyces sp. HOT172												1.3				
Atopobium rimae													1.3	1.3		
Bifidobacterium dentium													1.3	2.5		
Olsenella uli	2.5										0.1					
Rothia mucilaginosa		5.0	1.3	0.1			1.3		1.3	1.3	0.1					
Bacteroidetes		5.0	10.0	11.6	5.0	1.3	23.8	1.3	3.8	23.8	23.0	12.5	10.0	23.8	1.3	
Alloprevotella rava											0.8					
Bacteroidetes sp. HOT365 / HOTG44											0.1	2.5				
Capnocytophaga leadbetteri											0.1					
Porphyromonas endodontalis							1.3			2.5	1.0		3.8	1.3		
Prevotella buccae				0.1							0.2					
Prevotella dentalis											0.1					
Prevotella oralis		3.8	5.0	7.4	5.0	1.3	17.5		2.5	16.3	15.9	5.0	2.5	13.8		
Prevotella oris		1.3		0.7						1.3	0.9			5.0		
Prevotella oulorum											0.4					
Prevotella pleuritidis			5.0	2.3							0.2	1.3				
Prevotella saccharolytica				0.1							0.5					
Prevotella sp. HOT292/300				0.9			5.0			1.3	0.2		2.5	2.5	1.3	
Prevotella sp. HOT306											0.1					

	BHI							BHI mucin								SHI
Samples Species	8 d	16 d	24 d	24 d py	32 d	40 d	48 d	8 d	16 d	24 d	24 d py	32 d	40 d	48 d	88 d	8 d
Prevotella sp. HOT315													1.3			
Prevotella sp. HOT526									1.3	2.5	2.7	3.8		1.3		
Prevotella tannerae								1.3								
Tannerella forsythia				0.1												
Firmicutes	96.3	85.0	65.0	62.5	91.3	93.8	63.8	92.5	88.8	63.8	61.9	71.3	58.8	46.3	45.0	66.3
Anaeroglobus geminatus	3.8	20.0	16.3	17.4	1.3	8.8	18.8	30.0		6.3	8.2	18.8	25.0	17.5		2.5
Burkholderiales sp. HOTA57	1.3									1.3	0.1					
Catonella morbi	6.3	1.3		2.5	1.3	1.3			5.0	1.3	3.0	1.3			2.5	
Centipeda periodontii				0.4												
Clostridiales sp. HOT093											0.1				1.3	
Dialister invisus / HOTA97			1.3	1.7		1.3	3.8	2.5		3.8	2.1	1.3		1.3		
Dialister pneumosintes				0.1	1.3	3.8	2.5	5.0		3.8	4.0	1.3	1.3			
Erysipelothrichaceae sp. HOTA18											0.1					
Erysipelotrichales sp. HOTC62											0.1					
Eubacterium nodatum	1.3					5.0										
Eubacterium saburreum				0.1												
Eubacterium yurii				0.1												
Filifactor alocis		3.8	2.5	2.1	5.0		1.3	2.5			1.1		5.0	3.8		
Granulicatella adiacens	1.3		2.5					2.5	3.8	1.3	0.2				2.5	10.0
Lachnospiraceae sp. HOTA61				0.1												
Lactobacillus paracasei				0.1												
Megasphaera micronuciformis											0.1					

	BHI							BHI mucin								SHI
Samples Species	8 d	16 d	24 d	24 d py	32 d	40 d	48 d	8 d	16 d	24 d	24 d py	32 d	40 d	48 d	88 d	8 d
Mogibacterium diversum / neglectum / vescum	1.3			0.1	3.8		1.3				0.9	1.3	1.3	1.3		
Parvimonas micra	3.8		6.3	1.2	5.0	12.5		7.5		3.8	0.8					
Peptostreptococcaceae sp. HOT081				0.7							1.6					
Peptostreptococcaceae sp. HOT091				0.1							0.2					
Peptostreptococcaceae sp. HOT369 / HOT103	3.8	1.3		0.7					2.5	1.3	0.2	1.3	1.3	1.3		
Peptostreptococcaceae sp. HOTE46										1.3	0.1	2.5	1.3		21.3	
Peptostreptococcus stomatis									7.5		1.4	6.3	2.5			
Selenomonas dianae				0.9							0.4					
Selenomonas infelix				1.5							0.8					
Selenomonas sp. HOT136	1.3				2.5		2.5					1.3				
Selenomonas sp. HOT146				0.2												
Selenomonas sp. HOT149											0.2					
Selenomonas sp. HOT479				0.1												
Selenomonas sp. HOTB30				0.1												
Selenomonas sp. HOTF21				1.5							0.4					
Selenomonas sp. HOTF72	1.3			0.4												
Shuttleworthia satelles														1.3		
Solobacterium moorei				0.4	3.8	2.5				1.3	0.3					
Sphingomonas sp. HOT003			3.8				1.3	1.3						2.5	2.5	
Streptococcus constellatus / intermedius	8.8	10.0	7.5	6.7	53.8	45.0	12.5	41.3	8.8	3.8	4.4	15.0	10.0	8.8		2.5

	BHI							BHI mucin								SHI
Samples Species	8 d	16 d	24 d	24 d py	32 d	40 d	48 d	8 d	16 d	24 d	24 d py	32 d	40 d	48 d	88 d	8 d
Streptococcus gordonii				2.4							0.1					
Streptococcus mitis			1.3	1.1						1.3	0.1					5.0
Streptococcus salivarius															11.3	
Streptococcus sp. HOT058	17.5								25.0	11.3		3.8	2.5		3.8	5.0
Streptococcus sp. HOT070									3.8	2.5	6.4					
Streptococcus sp. HOT071										1.3	1.7					
Streptococcus sp. HOTC56	2.5	2.5			2.5							1.3		1.3		41.3
Veillonella atypica	3.8	5.0									0.2					
Veillonella dispar / parvula / HOTG30	26.3	25.0	18.8	19.3	6.3	11.3	13.8		25.0	18.8	22.4	16.3	8.8	7.5		
Veillonella rogosae	6.3	7.5	3.8	0.2	5.0	2.5	5.0		3.8		0.3					
Veillonella sp. HOTG30	2.5	2.5		0.2												
Veillonellaceae sp. HOT155	3.8	6.3	1.3	0.2			1.3		3.8		0.4					
Fusobacteria	1.3	3.8	20.0	19.1	3.8	0.0	7.5	0.0	3.8	11.3	12.2	7.5	12.5	7.5	2.5	0.0
Fusobacterium nucleatum ss. animalis	1.3	3.8	20.0	17.5	3.8		7.5		2.5	11.3	12.1	7.5	12.5	7.5		
Fusobacterium nucleatum ss. polymorphum				0.2							0.1					
Fusobacterium nucleatum ss. vincentii				1.3												
Fusobacterium periodonticum				0.1					1.3						2.5	
Leptotrichia shahii											0.1					
Proteobacteria	0.0	1.3	1.3	5.6	0.0	5.0	2.5	0.0	2.5	0.0	0.5	0.0	1.3	1.3	18.8	0.0
Campylobacter curvus				0.1							0.2					
Campylobacter gracilis									2.5		0.1					

	BHI							BHI mucin								SHI
Samples Species	8 d	16 d	24 d	24 d py	32 d	40 d	48 d	8 d	16 d	24 d	24 d py	32 d	40 d	48 d	88 d	8 d
Campylobacter rectus / HOTG43			1.3	5.3		5.0	2.5				0.2		1.3	1.3	16.3	
Campylobacter showae				0.1												
Leptothrix sp. HOT025		1.3		0.3											2.5	
Spirochetes			1.3	0.4			1.3				0.4	1.3	3.8	1.3	32.5	33.8
Treponema denticola / HOT246			1.3	0.4			1.3				0.2		2.5	1.3		
Treponema lecithinolyticum											0.2	1.3	1.3		32.5	1.3
Treponema maltophilum																32.5
SR1											0.1					
SR1 sp. HOT345											0.1					
Synergistetes			1.3	0.7				6.3			1.6	6.3	11.3	16.3		
Fretibacterium fastidiosum			1.3	0.7				6.3			1.6	6.3	11.3	16.3		
TM7				0.3							0.2					
TM7 sp. HOT355				0.3							0.2					

Table 28. Composition of aerobic CBD biofilms.

Samples / Taxa	BHI_G		BHI		BMM	
		PMA		PMA		PMA
Actinobacteria						
Actinobaculum sp. HOT183						0.04
Actinomyces dentalis / HOT449	0.01					
Actinomyces gerencseriae						0.04
Actinomyces israelii						0.04
Actinomyces massiliensis						0.06
Actinomyces naeslundii / HOT171	0.05		0.01	0.01	0.03	0.18
Actinomyces odontolyticus / *meyeri*	0.01	0.05		0.11	0.04	0.07
Actinomyces sp. HOT170						0.04
Actinomyces sp. HOT175	0.01		0.02	0.01		0.06
Actinomyces sp. HOT180				0.03		
Actinomyces sp. HOT181	0.06	0.04			0.03	0.03
Actinomyces sp. HOT448						0.04
Actinomyces sp. HOTC25				0.01		0.01
Actinomyces sp. HOTD50						0.04
Actinomyces sp. HOTE63					0.01	0.01
Actinomyces sp. HOTE91	0.02					0.01
Actinomyces sp. HOTB78 / HOTE33 / HOTF78 / HOT169	0.03	0.15	0.01	0.26	0.05	0.18
Atopobium parvulum		0.03	0.05	0.03	0.06	0.12
Atopobium rimae	0.40				0.05	
Atopobium sp. HOT416				0.05		0.02
Olsenella uli						0.01
Propionibacterium acidifaciens	0.01		0.01	0.01		0.02
Propionibacterium acnes	0.01					
Propionibacterium propionicum				0.06		0.02
Propionibacterium sp. HOT194						0.02
Rothia dentiocariosa		0.01	0.01	0.26	0.01	0.22
Rothia sp. HOT188	0.01			0.06		0.09
Bacteroidetes						
Bacteroidetes sp. HOT272						0.01
Bacteroidetes sp. HOT274		0.04		0.06		0.25
Bacteroidetes sp. HOT365 / HOT281 / HOTG44			0.01			0.01
Bacteroidetes sp. HOT511				0.05		0.04
Bergeyella sp. HOT322				0.01		0.02
Capnocytophaga gingivalis / *granulosa*	0.03			0.02		0.33
Capnocytophaga sp. HOT326				0.02		0.04
Capnocytophaga sp. HOT329				0.01		0.04
Capnocytophaga sp. HOT332						0.02
Capnocytophaga sp. HOT335 / HOT412 / HOT323	0.01			0.01		0.06

Samples / Taxa	BHI_G		BHI		BMM	
		PMA		PMA		PMA
Capnocytophaga sp. HOT380 / HOTE54						0.04
Capnocytophaga sp. HOTB79						0.01
Capnocytophaga sp. HOTH18						0.01
Capnocytophaga sputigena / HOTG66				0.08		0.12
Flavobacteriales sp. uncultured	0.50	6.78	1.16	15.61	2.95	6.37
Porphyromonas catoniae / HOT279	0.22	0.45	0.42	0.47	0.13	1.96
Porphyromonas endodontalis / HOT395 / HOTG50	0.56	0.28	0.29	0.12	0.44	0.27
Porphyromonas gingivalis / HOTF92	0.02			0.13	0.02	0.05
Porphyromonas sp. HOT275 / HOT278 / HOTB43	0.03		0.03	0.04	0.03	0.10
Porphyromonas sp. HOT279 / HOT284			0.01	0.02	0.02	0.07
Prevotella baroniae						0.02
Prevotella buccae	0.27	0.26	0.14	0.07	0.59	0.18
Prevotella denticola / HOTG57 / HOTG64 / HOTG70	0.03		0.02			0.10
Prevotella intermedia	0.05			0.13	0.01	0.37
Prevotella loescheii / HOT317	0.01	0.12		0.16	0.18	0.35
Prevotella marshii	0.01					
Prevotella melaninogenica / HOT314 / HOTE10						0.07
Prevotella micans						0.04
Prevotella multisaccharivorax						0.03
Prevotella nigrescens / HOTG56	0.05			0.02	0.01	0.20
Prevotella oralis		0.12		0.26	0.32	0.17
Prevotella oris		0.05		0.01		0.11
Prevotella oulorum / HOTD69 / HOTG60	0.03			0.08		0.20
Prevotella pallens						0.07
Prevotella pleuriditis / HOT296						0.06
Prevotella salivae / HOTE13						0.02
Prevotella shahii					0.01	0.01
Prevotella sp. clone BL216					0.01	
Prevotella sp. HOT300 / HOT292						0.07
Prevotella sp. HOT302						0.01
Prevotella sp. HOT306					0.02	
Prevotella sp. HOT472				0.02		0.09
Prevotella sp. HOT473				0.07		0.05
Prevotella sp. HOT475						0.01
Prevotella sp. HOT526						0.01
Prevotella sp. HOT781	0.02	0.28	0.04	0.99	0.02	0.58
Prevotella sp. unclutured 1						0.01
Prevotella sp. unclutured 2				0.09		0.05
Prevotella tannerae	0.03	0.03			0.06	0.09
Prevotella veroralis						0.15

Samples / Taxa	BHI_G		BHI		BMM	
		PMA		PMA		PMA
Tannerella forsythia		0.01		0.05		0.15
Tannerella sp. HOT286			0.05			0.05
Tannerella sp. unclutured						0.01
Firmicutes						
Abiotrophia defectiva / HOTB44	0.37	0.64	0.40	0.43	0.64	0.87
Anaeroglobus geminatus		0.05	0.01		0.03	
Camomonadaceae sp. HOTF47						0.01
Catonella morbi	0.66	0.08	0.41	0.55	0.74	0.43
Catonella sp. HOT451						0.01
Centipeda periodontii / *Selenomonas* sp. HOT478 / HOT136	0.23	0.33	0.18	0.40	0.42	0.45
Centipeda sp. HOTB01			0.01			
Centipeda sp. HOTD18 / HOT479 / HOT149 / HOTE50	0.10	0.08	0.15	0.12	0.12	0.10
Clostridiales sp. HOT085					0.01	0.05
Clostridiales sp. HOT075						0.01
Clostridiales sp. HOT093						0.01
Corynebacterium durum / HOTA22				0.01		0.20
Corynebacterium matruchotii / HOTA46 / HOTB00				0.01	0.01	0.31
Corynebacterium sp. HOTA16	0.05					
Dialister invisus / HOTA97	0.40	0.10	0.08	0.23	0.29	0.07
Dialister pneumosintes / HOT502 / HOTD97						0.01
Eikenella corrodens	0.47	0.40	0.46	0.28	0.29	0.69
Eubacterium infirmum		0.01		0.08	0.01	0.07
Eubacterium saburreum	0.01	0.02	0.02		0.01	0.07
Eubacterium sp. unclutured 1	0.07	0.07	0.04	0.08	0.27	0.22
Eubacterium sp. unclutured 2	0.01				0.02	0.02
Eubacterium sulci						0.03
Eubacterium yurii	0.09	1.59	0.66	0.68	0.35	1.77
Filifactor alocis	0.54	0.32	0.24	0.54	0.21	0.02
Gemella bergeriae	0.19					
Gemella morbillorum / *haemolysans*	2.24	1.93	3.87	1.77	1.57	5.78
Gemella sanguinis / HOTC54	0.17	0.29	0.19	0.06	0.05	0.21
Granulicatella adiacens / *para-adiacens* / HOTC27	0.99	1.61	0.64	0.87	1.48	1.41
Lachnospiraceae sp. HOT082 / HOT107	0.03	0.01			0.04	0.15
Lachnospiraceae sp. HOT083				0.01		
Lachnospiraceae sp. HOT100		0.02				0.02
Lachnospiraceae sp. HOT419					0.01	
Lachnospiraceae sp. HOTA61 / HOTE59	0.31	0.66	0.22	0.46	0.83	0.78
Lachnospiraceae sp. HOTB32						0.10
Lachnospiraceae sp. unclutured	0.12	0.11	0.22	0.36	0.22	0.14
Lactobacillus casei / *rhamnosus*	0.70	1.70	0.48	1.46		0.51

Samples / Taxa	BHI_G		BHI		BMM	
		PMA		PMA		PMA
Lactobacillus crispatus	0.01					0.02
Lactobacillus gasseri	0.13	0.10		0.01	0.02	0.14
Lactobacillus helveticus		0.05				
Lactobacillus nagelii / *ghanensis*	0.01					0.01
Lactobacillus oris	13.51	3.45	15.33	0.10	25.88	0.24
Lactobacillus paracasei	16.98	26.53	9.63	18.84	1.18	8.17
Lactobacillus sp. HOT461	0.40	0.05	0.31			0.01
Lactobacillus vaginalis		0.05			0.02	0.05
Megasphaera micronuciformis	0.27	0.17	0.09		0.07	0.01
Mitsuokella sp. HOT521						0.01
Mobiluncus sp. unclutured	0.02		0.04	0.02	0.03	
Mogibacterium diversum / *vescum* / *neglectum*	1.02	0.22	0.22	0.15	0.56	0.05
Mogibacterium timidum						0.01
Moraxella sp. HOTB07						0.02
Moryella sp. unclutured				0.01		
Oribacterium sinus	0.01		0.04		0.01	
Oribacterium sp. HOT108	0.03					
Oribacterium sp. HOT372 / HOT078						0.01
Parvimonas micra	2.80	5.75	6.20	4.51	8.35	3.44
Parvimonas sp. HOT110	1.19	0.86	1.72	0.22	1.16	0.28
Parvimonas sp. HOTC63	0.07	0.60	0.13	0.51	0.31	0.83
Peptococcus sp. HOT167						0.01
Peptostreptococcaceae sp. HOT081	1.57	3.96	1.62	2.43	2.63	3.86
Peptostreptococcaceae sp. HOT369 / HOT103		0.01			0.02	0.01
Peptostreptococcus stomatis / HOTE46	0.04	0.76	0.03	1.48	0.07	1.25
Pseudomonas fluorescens		0.05				
Pseudomonas stutzeri	0.01					
Pseudoramibacter alactolyticus	0.09			0.07	0.04	0.05
Selenomonas artemidis / HOT137						0.06
Selenomonas dianae / *infelix* / HOT138 / HOT146 / HOTE44 / HOTF29	0.79	1.48	0.64	1.29	2.77	1.94
Selenomonas noxia / HOTG67		0.85	0.01	0.16	0.07	0.46
Selenomonas sp. HOT126			0.01		0.02	
Selenomonas sp. HOT140 / HOTE39						0.15
Selenomonas sp. HOT481	0.06	0.03	0.08	0.14	0.04	0.04
Selenomonas sp. HOTE20						0.01
Selenomonas sp. HOTE39				0.03		0.05
Selenomonas sp. HOTF30	0.02	0.03		0.04	0.04	0.14
Selenomonas sp. HOTF82	0.01				0.02	0.06
Selenomonas sp. HOTF83 / HOTH63				0.01	0.04	0.06
Selenomonas sp. HOTF85						0.01
Selenomonas sp. HOTF87	0.02	0.01		0.13	0.09	0.01

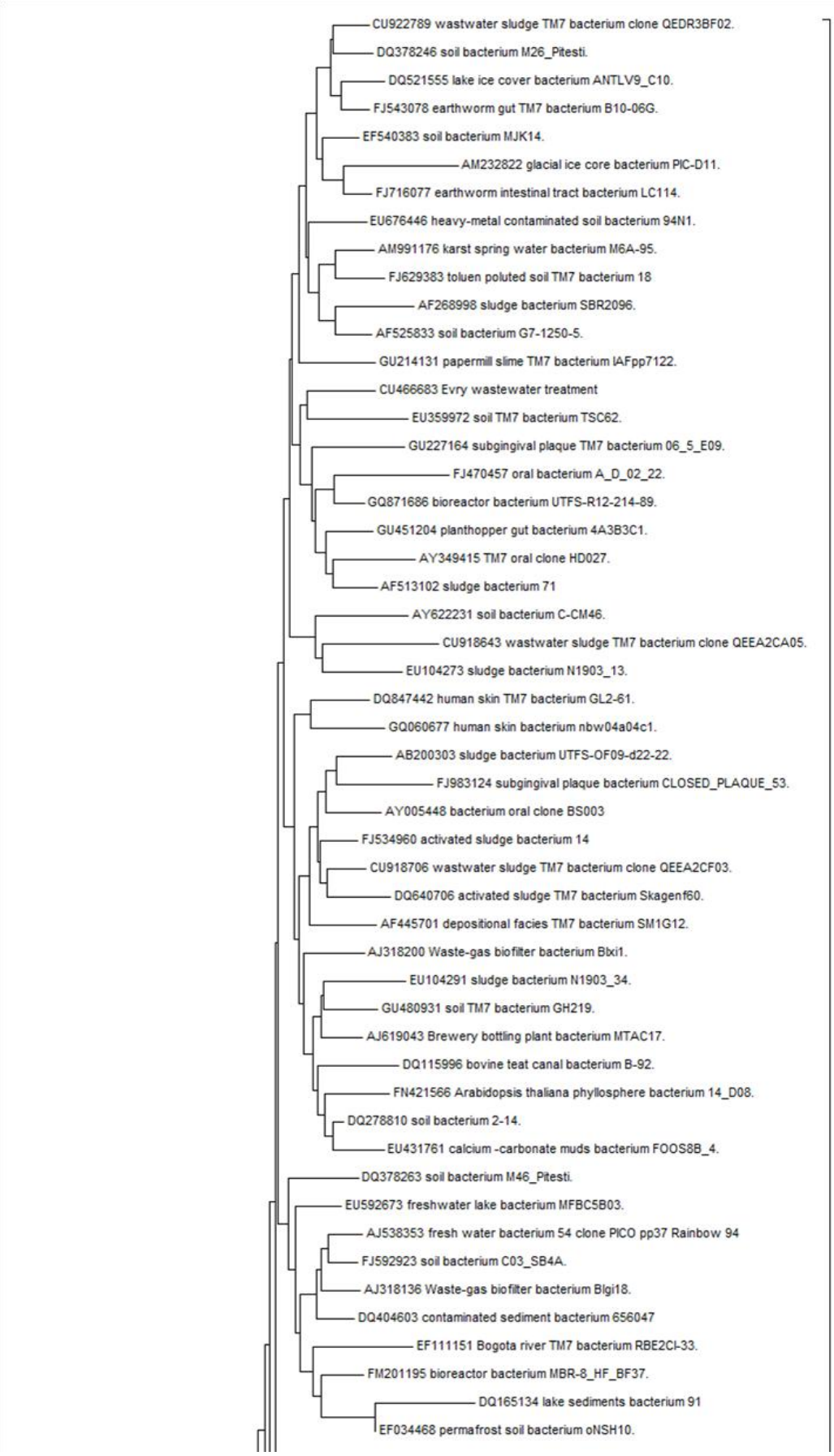
Samples / Taxa	BHI_G		BHI		BMM	
		PMA		PMA		PMA
Selenomonas sp. HOTF96		0.01			0.06	
Selenomonas sp. HOTG51						0.02
Selenomonas sp. HOTG55				0.02	0.04	0.02
Selenomonas sp. HOTH23				0.01	0.02	
Selenomonas sp. HOTH30					0.01	0.02
Selenomonas sp. HOTH32		0.02			0.04	
Selenomonas sp. HOTH66						0.09
Selenomonas sputigena / HOTE80 / HOTF22	0.16	0.04	0.06	0.18	0.23	0.13
Solobacterium moorei	0.29	0.46	0.07	0.99	0.37	0.51
Solobacterium sp. HOTA05	0.09		0.10	0.12	0.03	0.04
Staphylococcus sp. NBRC 13889	19.37	0.11	8.07	0.10	0.82	0.07
Streptococcus anginosus						0.11
Streptococcus constellatus / *intermedius* / HOTE12	2.64	5.53	3.25	3.58	4.35	8.39
Streptococcus cristatus	0.19	0.04	0.26	0.07	0.03	0.21
Streptococcus gordonii / HOTH21 / HOTH24	0.69	0.63	0.46	0.09	0.38	0.30
Streptococcus infantis	0.02					
Streptococcus mitis bv 2	0.05	0.03		0.01		0.11
Streptococcus mitis / HOTA95	0.07		0.04	0.08		0.27
Streptococcus mutans	0.01	0.10	0.01		0.02	0.06
Streptococcus oralis						0.01
Streptococcus parasanguinis II	0.01					
Streptococcus salivarius / *vestibuaris* / HOTC65	0.02					0.01
Streptococcus sanguinis	0.26	0.01	0.43			0.79
Streptococcus sp. HOT058	0.06		0.02	0.07		0.05
Streptococcus sp. HOT064 / HOTE41	1.51	0.10	0.44	0.11	0.03	0.10
Streptococcus sp. HOT066 / HOT061	0.01		0.06		0.02	0.05
Streptococcus sp. HOT070	0.08	0.03	0.01	0.06		0.09
Streptococcus sp. HOT071			0.04		0.04	0.15
Streptococcus sp. HOTB66					0.05	
Streptococcus sp. HOTC04	0.19		0.12	0.01	0.03	
Streptococcus sp. HOTC14						0.07
Streptococcus sp. unclutured	0.01					
Veillonella atypica / *dispar* / *parvula* / HOTE53 / HOTG30	3.23	6.06	3.29	2.50	4.70	5.53
Veillonella sp. HOT158						0.03
Veillonellaceae sp. HOT155		0.01			0.12	0.02
Veillonellaceae sp. HOT150		0.05				0.05
Xanthomonadaceae sp. unclutured			0.02			
Fusobacteria						
Fusobacteria sp. HOT210	0.01					
Fusobacteria sp. HOTA71						0.01
Fusobacterium nucleatum ss. *animalis*	0.09	0.31	0.12	0.72	0.80	0.41

Samples / Taxa	BHI_G		BHI		BMM	
		PMA		PMA		PMA
Fusobacterium nucleatum ss. *nucleatum* /HOT203/HOTA11	0.26	0.12	0.11	0.30	0.32	0.11
Fusobacterium nucleatum ss. *polymorphum*	2.28	0.55	2.66	0.87	2.49	2.22
Fusobacterium nucleatum ss. *vincentii* / *naviforme* / HOT210 / HOT220 / HOT370 / HOTD95	0.36	0.69	1.33	0.56	0.96	1.15
Fusobacterium periodonticum	0.97	0.12	0.72	0.09	1.26	0.36
Fusobacterium sp. HOTH27						0.04
Leptotrichia buccalis / HOT225			0.01	0.22		0.33
Leptotrichia hofstadii						0.23
Leptotrichia sp. HOT212	0.01					0.01
Leptotrichia sp. HOT213				0.07		0.04
Leptotrichia sp. HOT215						0.17
Leptotrichia sp. HOT392/HOT217				0.02		0.11
Leptotrichia sp. HOT417						0.05
Leptotrichia sp. HOT498						0.04
Leptotrichia sp. HOT847						0.02
Leptotrichia sp. HOTA45						0.03
Leptotrichia sp. oral clone 19-33						0.04
Leptotrichia sp. unclutured				0.04		
Leptotrichia trevisanii						0.01
Leptotrichia wadei				0.09		0.12
Proteobacteria						
Acinetobacter baumannii / HOTC57				0.12	0.02	0.58
Aggregatibacter paraphrophilus / *aphrophilus*	0.02	0.61	0.27	1.60	0.55	1.81
Aggregatibacter segnis / HOT458 / HOT512 / HOT513 / HOT762 / HOTG24	0.09	0.49	0.12	2.08	0.75	0.66
Aggregatibacter sp. HOTG01						0.01
Campylobacter concisus					0.02	0.01
Campylobacter curvus						0.05
Campylobacter gracilis / HOTE67 / HOTG35	0.03			0.01		0.11
Campylobacter rectus / HOTG43	0.07	1.27	0.69	1.22	2.11	0.95
Campylobacter showae / E65	4.72	4.86	8.41	9.98	7.55	9.71
Cardiobacterium hominis					0.01	0.14
Cardiobacterium valvulum						0.04
Haemophilus parainfluenzae	0.17	0.20	0.04	0.16	0.04	0.04
Haemophilus pittmaniae	0.02	0.28	0.04	0.01	0.18	0.19
Haemophilus sp. HOT036	0.02					
Haemophilus sp. HOTD10 / 035						0.01
Johnsonella ignava				0.04		0.01
Johnsonella sp. HOT166						0.01
Kingella denitrificans / HOTD55 / HOTE51				0.05		0.04
Kingella oralis						0.16

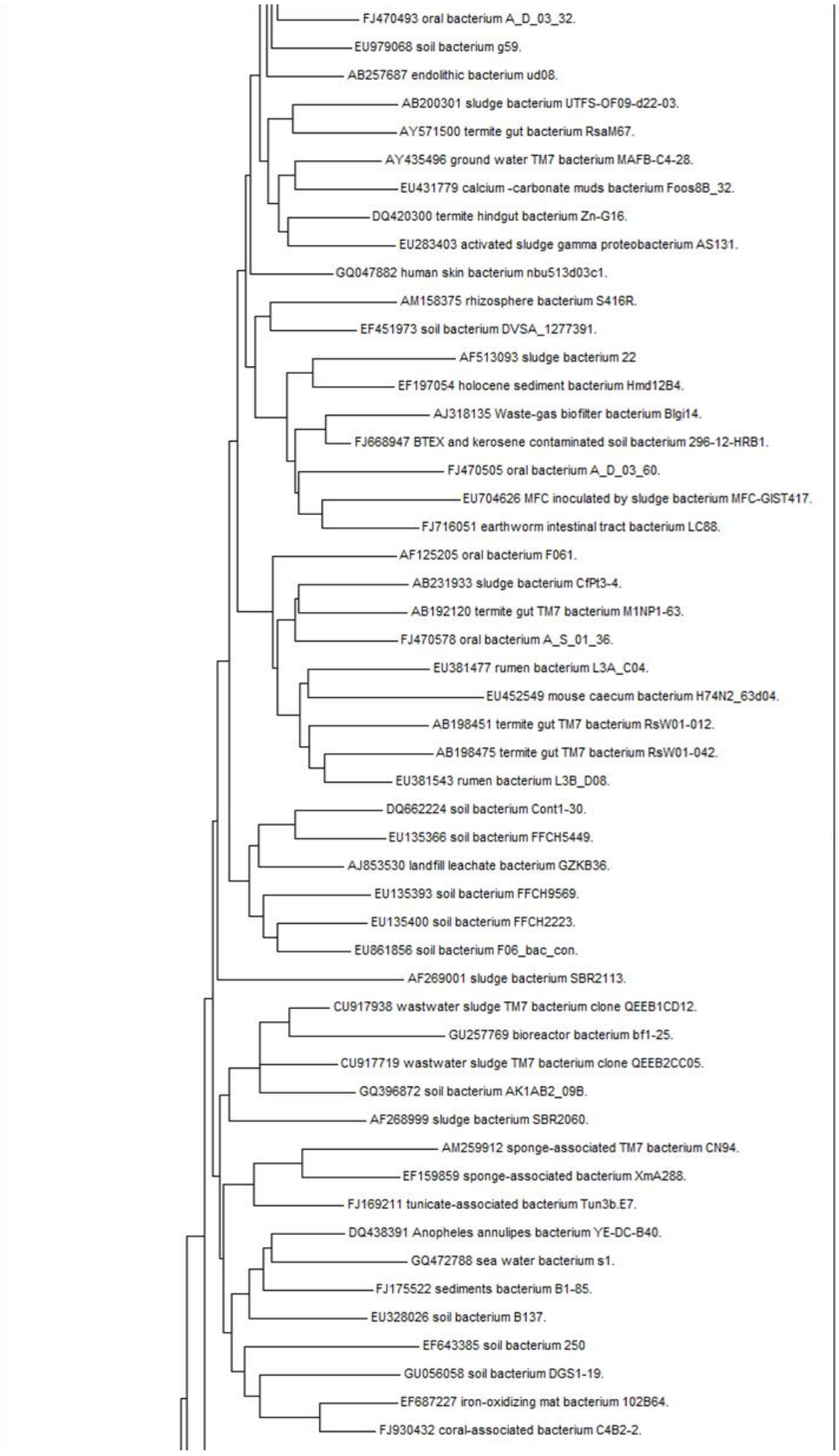
Samples / Taxa	BHI_G		BHI		BMM	
		PMA		PMA		PMA
Kingella sp. HOT012						0.01
Kingella sp. HOTD49						0.06
Lautropia mirabilis	0.01	0.11		0.41	0.08	0.51
Lautropia sp. HOTA94				0.09	0.01	0.14
Neisseria bacilliformis	0.01	0.01		0.02		0.05
Neisseria elongata	1.60	9.86	3.17	10.45	4.47	8.04
Neisseria flava / *mucosa* / *pharyngis* / *sicca*	9.60	1.43	18.37	2.11	9.71	2.10
Neisseria flavescens / *subflava*	1.27	1.64	0.30	1.46	0.36	0.68
Neisseria sp. HOT009 / HOT014 / HOT015 / HOT016 / HOTD56	0.01			0.08	0.06	0.05
Neisseria sp. HOT018						0.04
Neisseria sp. HOTD61				0.04		
Spirochetes						
Treponema denticola / HOTG47		0.08		0.01	0.04	0.06
Treponema socranskii ss 04 / *buccale*						0.02
Treponema sp. HOT237						0.01
Treponema sp. HOT769						0.05
Treponema sp. HOTG39 / HOT254						0.01
SR1						
SR1 sp. HOT345				0.02		
SR1 sp. unclutured				0.02		
Synergistetes						
Fretibacterium fastidiosum		0.35	0.17	0.57	0.41	
TM7						
TM7 phylum sp. oral clone 13-10						0.01
TM7 sp. HOT346						0.01
TM7 sp. HOT348						0.02
TM7 sp. HOT349						0.05
TM7 sp. HOT350				0.01		
TM7 sp. HOT351						0.01
TM7 sp. HOT352	0.01					
TM7 sp. HOT355	0.05	0.05	0.05	0.25	0.10	0.04
TM7 sp. HOT437 / HOT356				0.01	0.03	0.02
TM7 sp. unclutured 1				0.09		
TM7 sp. unclutured 2						0.04
TM7 sp. unclutured 3						0.01

Appendix 3. Phylogenetic tree of the TM7

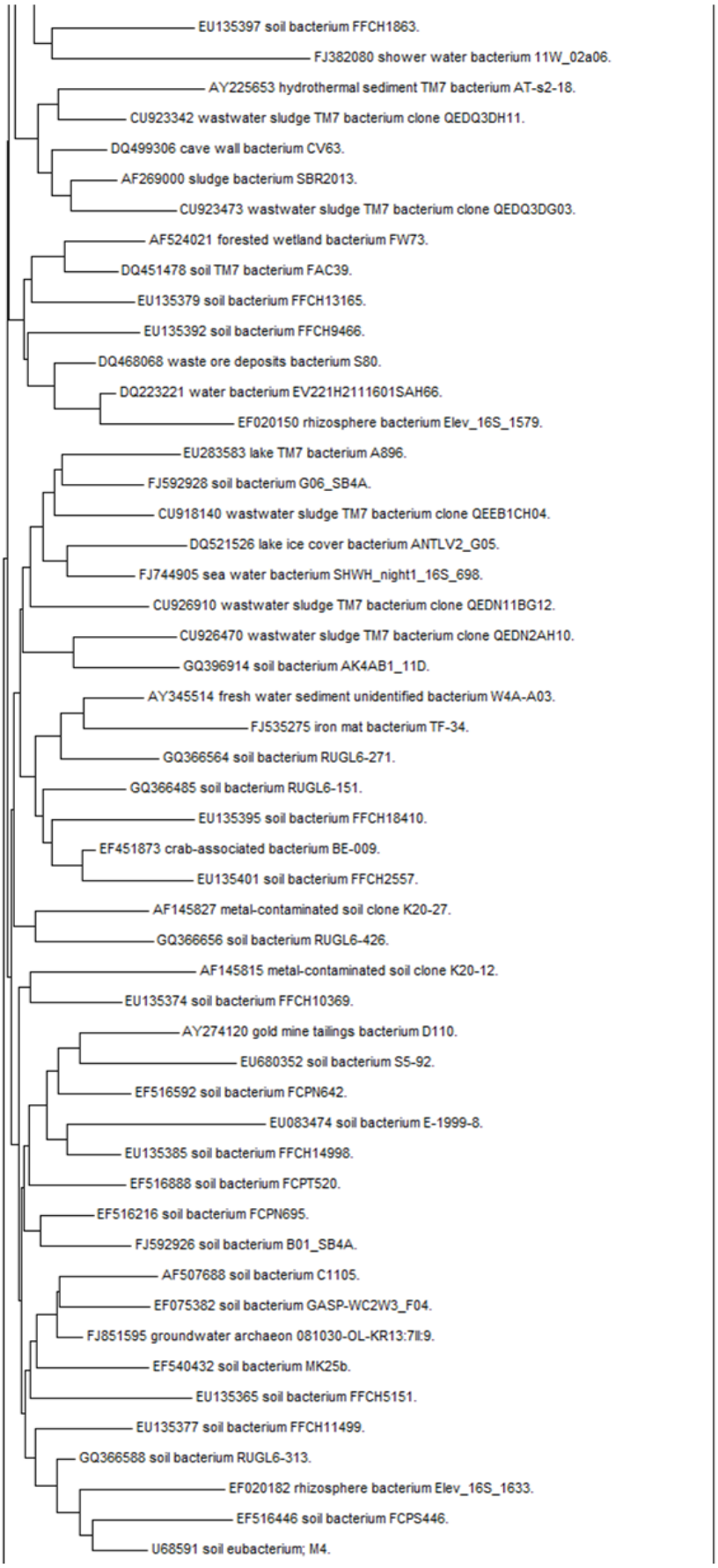
Candidate Division



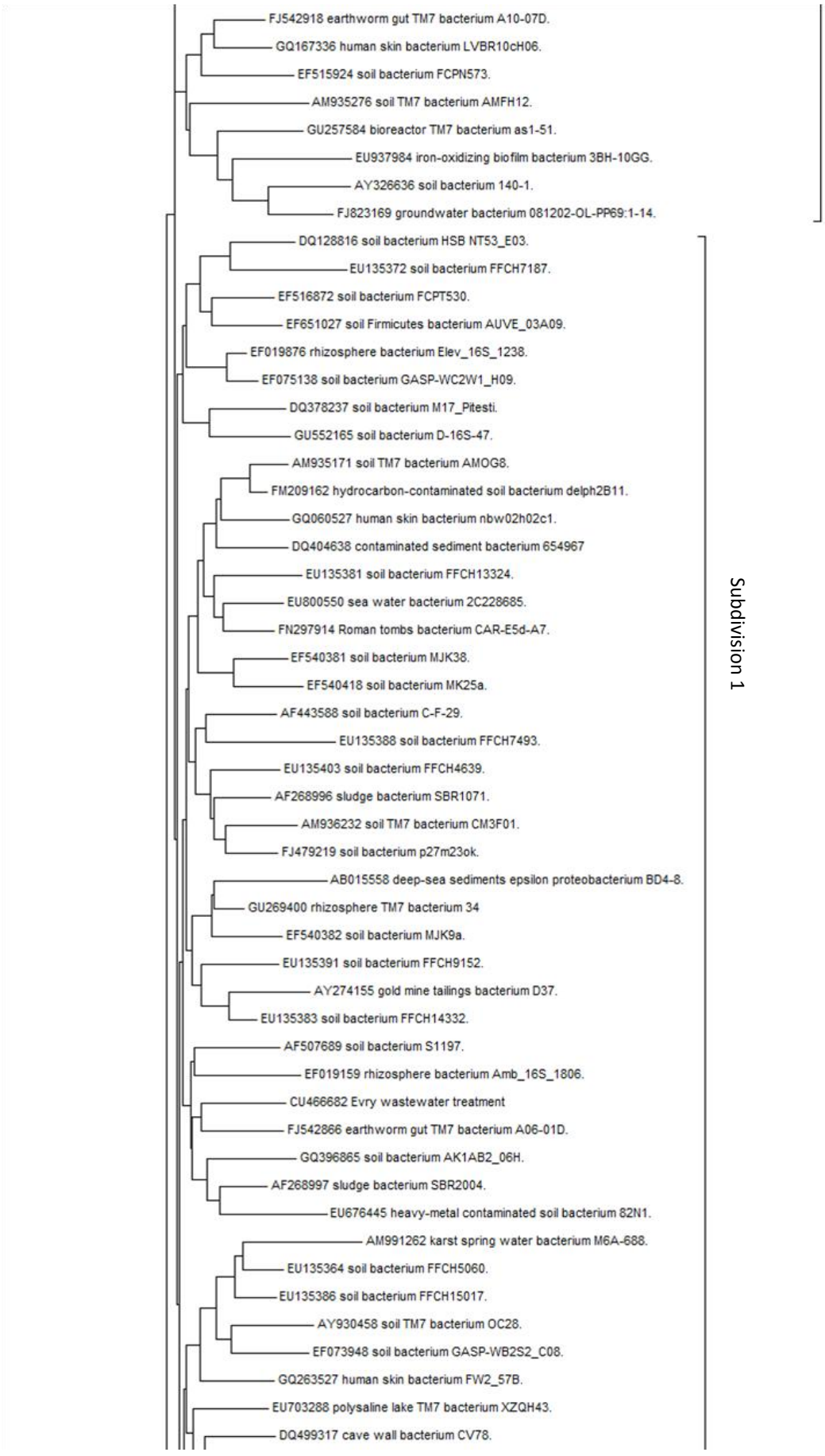
Subdivision 2

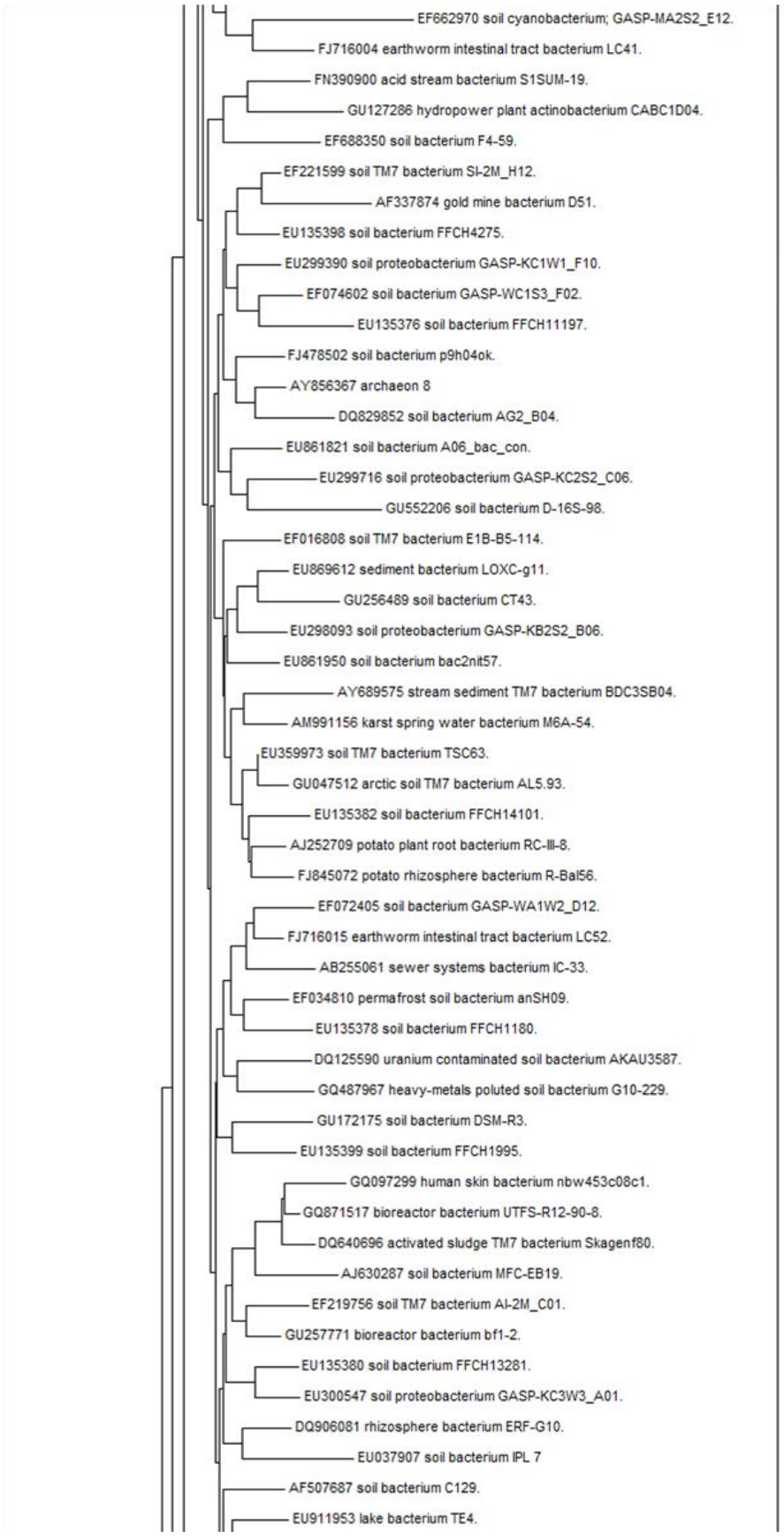


Subdivision 2



Subdivision 2


FJ542918 earthworm gut TM7 bacterium A10-07D.
GQ167336 human skin bacterium LVBR10cH06.
EF515924 soil bacterium FCPN573.
AM935276 soil TM7 bacterium AMFH12.
GU257584 bioreactor TM7 bacterium as1-51.
EU937984 iron-oxidizing biofilm bacterium 3BH-10GG.
AY326636 soil bacterium 140-1.
FJ823169 groundwater bacterium 081202-OL-PP69:1-14.
DQ128816 soil bacterium HSB NT53_E03.
EU135372 soil bacterium FFCH7187.
EF516872 soil bacterium FCPT530.
EF651027 soil Firmicutes bacterium AUVE_03A09.
EF019876 rhizosphere bacterium Elev_16S_1238.
EF075138 soil bacterium GASP-WC2W1_H09.
DQ378237 soil bacterium M17_Pitesti.
GU552165 soil bacterium D-16S-47.
AM935171 soil TM7 bacterium AMOG8.
FM209162 hydrocarbon-contaminated soil bacterium delph2B11.
GQ060527 human skin bacterium nbw02h02c1.
DQ404638 contaminated sediment bacterium 654967
EU135381 soil bacterium FFCH13324.
EU800550 sea water bacterium 2C228685.
FN297914 Roman tombs bacterium CAR-E5d-A7.
EF540381 soil bacterium MJK38.
EF540418 soil bacterium MK25a.
AF443588 soil bacterium C-F-29.
EU135388 soil bacterium FFCH7493.
EU135403 soil bacterium FFCH4639.
AF268996 sludge bacterium SBR1071.
AM936232 soil TM7 bacterium CM3F01.
FJ479219 soil bacterium p27m23ok.
AB015558 deep-sea sediments epsilon proteobacterium BD4-8.
GU269400 rhizosphere TM7 bacterium 34
EF540382 soil bacterium MJK9a.
EU135391 soil bacterium FFCH9152.
AY274155 gold mine tailings bacterium D37.
EU135383 soil bacterium FFCH14332.
AF507689 soil bacterium S1197.
EF019159 rhizosphere bacterium Amb_16S_1806.
CU466682 Evry wastewater treatment
FJ542866 earthworm gut TM7 bacterium A06-01D.
GQ396865 soil bacterium AK1AB2_06H.
AF268997 sludge bacterium SBR2004.
EU676445 heavy-metal contaminated soil bacterium 82N1.
AM991262 karst spring water bacterium M6A-688.
EU135364 soil bacterium FFCH5060.
EU135386 soil bacterium FFCH15017.
AY930458 soil TM7 bacterium OC28.
EF073948 soil bacterium GASP-WB2S2_C08.
GQ263527 human skin bacterium FW2_57B.
EU703288 polysaline lake TM7 bacterium XZQH43.
DQ499317 cave wall bacterium CV78.
Subdivision 1



Subdivision 1

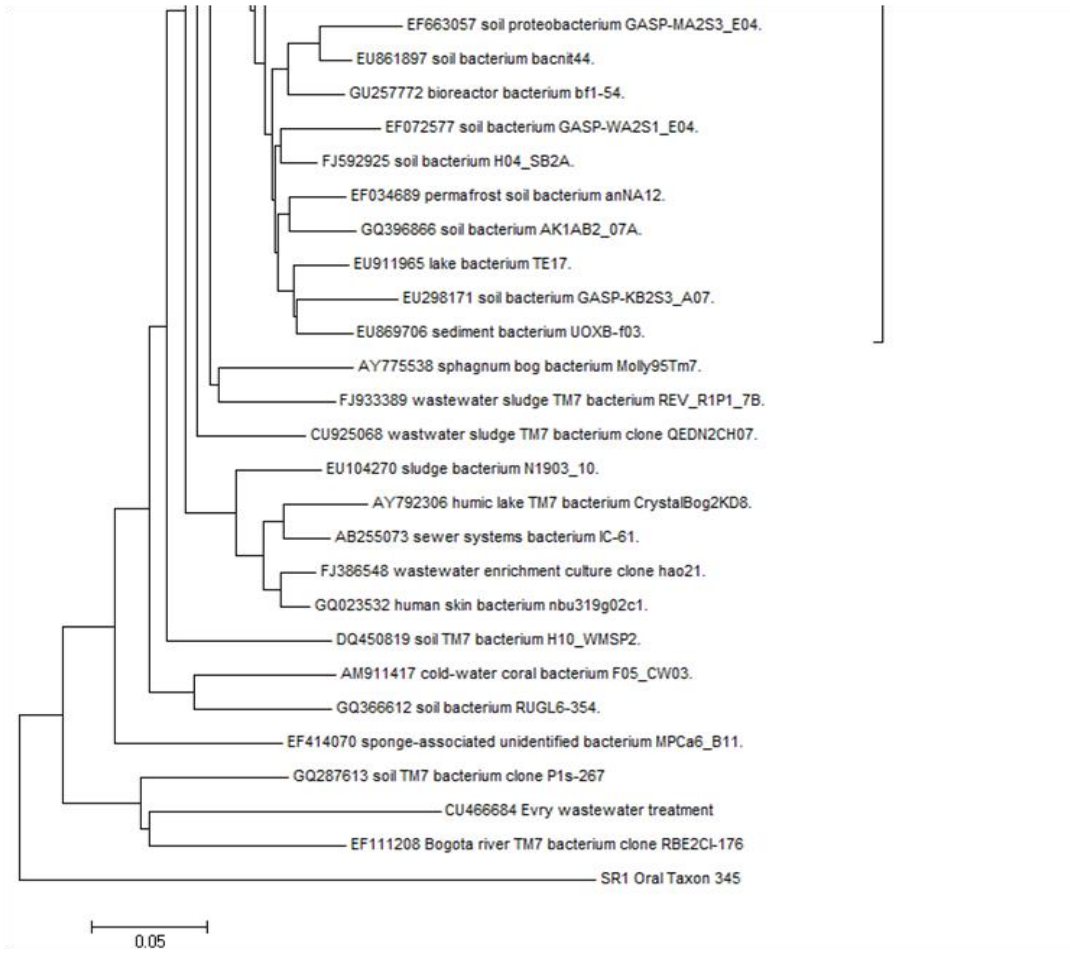


Figure 36. Phylogenetic tree of the TM7 Candidate Division.