Microbiological analysis of root canal infections using high throughput sequencing on the Illumina MiSeq platform

Hussain E H M Alenezi

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The candidate confirms that the work submitted is his own and that appropriate credit has been given where reference has been made to the work of others.

Dedication

To my father, mother, wife, children, brothers and sisters.

Acknowledgements

It would not have been possible to write this doctoral thesis without the help and support of kind people around me, to only some of whom it is possible to give particular mention here.

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Abstract

Aim

To investigate the microbial diversity of primary and secondary root canal infections using high throughput sequencing on the illumina MiSeq and culture methods.

Methods

19 subjects were recruited for the study; ten primary infections and nine secondary infections. Samples were collected before chemo-mechanical preparation (S1) and prior to obturation (S2), respectively. Microbiological culture aliquots were serially diluted and inoculated onto various non selective and selective media for total anaerobic and total aerobic counts. For high throughput sequencing, DNA was extracted and the V3/V4 region of the 16SrRNA gene was amplified using the 347F/803R primers, sequenced using the Illumina MiSeq instrument. Raw data were analysed using an open-source bioinformatics pipeline called quantitative insights into microbial ecology (QIIME).

Results

Culture: Total anaerobic counts from primary infections ranged from 1.7 $X10^1$- 7.9 $X10^6$ colony forming units (cfu)/ml (mean $\log_{10}$ cfu/ml ± SD: 3.08 ± 1.51), whilst total aerobic counts ranged from 3 $X10^3$- 4.17 $X10^5$ cfu/ml (mean $\log_{10}$ cfu/ml ± SD:3.09 ± 1.72). The quantity of microorganisms recovered from secondary infections ranged from 3 $X10^2$- 4.9 $X10^3$ cfu/ml (mean $\log_{10}$ cfu/ml ± SD: 2.81 ± 0.78) and from 2.7 $X10^2$- 8 $X10^5$ (mean $\log_{10}$ cfu/ml ± SD: 2.60 ± 1.48) with regard to total anaerobic and total aerobic viable counts, respectively.

Sequencing analysis yielded partial 16S rRNA gene sequences that were taxonomically classified into 10 phyla and 143 genera. The most represented phyla in the total sample were *Firmicutes, Proteobacteria, Actinobacteria, Bacteroidetes, Synergistetes* and *Fusobacteria.*

The most dominant genera in primary S1 samples were *Streptococcus, Bacillaceae* and *Eubacterium* while *Alkalibacterium, Bacillaceae* and *TG5* dominated the secondary infections. The majority of genera occurred at low levels. The mean number (± SD) of species-level phylotypes per canal was 63 (±14.9; range 34– 80), and 69.9 (± 12.0; range 50 – 87) in primary and secondary infections (S1) samples, respectively. A great inter-individual variation in the composition of the root canal microbiota was observed.

Conclusions

The study demonstrated the extensive diversity of the bacterial communities present in root canal infections although the majority of the taxa detected were in low abundance. The study indicates that secondary infections seem more diverse than previously anticipated.

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List of abbreviations

Bp	Base pair
$Ca(OH)_2$	Calcium hydroxide
CFU	Colony forming unit
CRF	Case report file
DenTCRU	Dental Translational and Clinical Research Unit
DNA	Deoxyribonucleic acid
DREC	Dental Research Ethics Committee
EDTA	Ethylenediaminetetraacetic acid
FDA	Food and Drug Administration
GG	Greengenes
GIC	Glass ionomor cement
GP	Gutta Percha
K_2HPO_4	Potassium hydrogen phosphate
KH_2PO_4	Potassium dihydrogen phosphate
HTS	High Throughput Sequencing
LDI	Leeds Dental Institute
MHRA	Medicines and Healthcare products Regulatory Agency
NaCl	Sodium hypochlorit
NaOCl	Sodium hypochlorite
Na_2SO_4	Sodium sulfate
$(NH_4)_2SO_4$	Ammonium sulfate
NRES	National Research Ethics Service
NGS	Next Generation Sequencing
OTUs	Operational Taxonomic Units
PCR	Polymerase Chain Reaction
PCoA	Principal Coordinate analysis
QIIME	Quantitative Insights Into Microbial Ecology
RDP	Ribosomal Database Project
REC	Research Ethics Committee
RCT	Root Canal Treatment
rDNA	Ribosomal Deoxyribonucleic acid
RNA	Ribonucleic acid
rRNA	Ribosomal Ribonucleic Acid
RTF	Reduced Transport Fluid
SSU RNA	Small Subunit Ribosomal Ribonucleic acid

Chapter 1 Introduction

1.1 Justification of the study

The human body contains up to 100 trillion organisms, which approximately equates to ten times the number of our own human cells (Di Bella *et al.*, 2013). This collection of microorganisms makes up the human microbiome. The oral microbiota represents a major component of human microbiome which has an important role in human health and disease (Xu and Gunsolley, 2014).

Oral diseases, such as periodontal disease and dental caries, are some of the most common infectious diseases of humans. For example, It is reported that up to 90% of the population are affected by periodontal disease (Xu and Gunsolley, 2014).

The oral microbiome is not only associated with inflammation in the oral cavity but also with multiple systemic diseases (Hajishengallis *et al.*, 2012), such as infective endocarditis (Xu *et al.*, 2007), diabetes (Moodley *et al.*, 2013) and colon cancer (Castellarin *et al.*, 2012).

Apical periodontitis is an inflammatory disorder of the periapical tissue (Nair, 2006). The primary cause of apical periodontitis is microbial infection of the dental pulp (Kakehashi *et al.*, 1965). Apical periodontitis is a very common disease with a prevalence of 30 -80% in different populations and generally increasing with age (Chen *et al.*, 2007; Figdor and Gulabivala, 2011).

The acute form of apical disease is usually localised intraorally but occasionally the abscess may spread and result in severe complications or even mortality (Siqueira and Rôças, 2013). Chronic apical periodontitis, however, is associated with mild or even no symptoms but it can at any point progress to an acute form such as acute apical abscess. Systemic consequences of apical periodontitis have been suggested but there is no strong evidence (van der Waal *et al.*, 2015).

When the dental pulp becomes infected, endodontic therapy is usually indicated if the tooth is to be saved. The primary goal of endodontic treatment is the elimination of infection as well as prevention of future re-infection (Sjogren *et al.*, 1997). This is traditionally achieved through mechanical and chemical means followed by an adequate seal of the root canal and tooth. When the tooth becomes infected for the first time, it is commonly referred to as

primary infection. Whilst if the tooth had been treated previously but the infection persisted or reoccurred, it is known as secondary, persistent or post-treatment infection. Failure of endodontic therapy has been attributed to several factors (Siqueira, 2001) but again the primary cause remains the persistence, or re-entry, of microorganisms in the root canal (Sjogren *et al.*, 1997; Siqueira, 2001).

The success rates of root canal treatment can reach over 90% but there is wide range and it can be as low as 30% (Friedman and Mor, 2004). An epidemiological study reported that the success rate of root canal treatment performed by endodontists is 87% compared to 72% for treatment carried out by general dental practitioners. Although seemingly high, this 15% difference equates to many millions of failed treatment in the western population. In the US, for example, about 5.1million primary endodontic infections are treated every year (Hsiao *et al.*, 2012).

The last decades have witnessed huge advances in endodontics technology. The list include Cone beam CT and Dental operating microscope, flexible titanium instruments, rotary files, apex locators, irrigation delivery devices. These advances have improved our diagnostics skills, allowed us to manage more difficult cases and reduced treatment time. However, the reported success rates for root canal treatment has not been in line with these developments. A review article argued that the strong emphasis on developing technological aids in endodontics has perhaps detracted our attention from the primary problem of endodontic disease (Bergenholtz and Spangberg, 2004). Our knowledge of endodontic microbiology is evolving but there are several unresolved and/or controversial issues, for example the composition and behaviour of the root canal microbiota (Siqueira and Rocas, 2005b). Therefore, it is clear that in order to enhance our chances of improving root canal treatment success, further research to increase our understanding of the diversity and ecology of endodontic microbiology is required.

1.2 Background

1.2.1 The dental pulp

The dental pulp is an innervated vascular connective tissue enclosed in dentine which is, in turn, covered by enamel and cementum. The hard layers provide the pulp with mechanical support and protection. There is also a close relationship between the pulp and dentine as they act as a unit and, hence, are commonly referred to as pulp-dentine complex (Pashley, 1996). The vitality of the pulp-dentine complex is important for several reasons. It acts as a sensory organ and is responsible for root development, tissue regeneration and repair (Manogue *et al.*, 2005; Smith, 2002; Cooper *et al.*, 2010). In addition, the ability of the complex to exhibit various defensive mechanisms, such as immune-inflammatory responses and defensive dentine, is well recognised (Bergenholtz, 1990).

1.2.2 Microbial role in pulpal disease

Irritation of the pulp, leading to pulpal disease, may be caused by microorganisms, mechanical, thermal, electrical stimuli or radiation (Bergenholtz, 1990). However, the often cited work of Kakehashi *et al* in 1965 indicated an aetiological role of microbes (Kakehashi *et al.*, 1965). Using 15 conventional and 21 germ-free rats, Kakehashi *et al* exposed dental pulps and observed that pulpal and apical disease only developed in the presence of microbes. In fact, exposed pulps in germ-free rats showed signs of repair. In agreement with this work, other studies were carried out on monkeys and reported similar observations (Moller *et al.*, 1981; Moller *et al.*, 2004). It is now widely accepted that although pulpal and apical disease may be initiated by non-infectious agents, the presence of microbes is essential for progression and perpetuation of the disease (Bergenholtz and Spangberg, 2004; Bergenholtz, 1990; Nair, 2004).

1.2.3 Microbial pathways to the pulp

The most common route for microbial invasion is dental caries (Hargreaves *et al.*, 2011) (Figure 1.1). Microbes can also find other routes to pulpal space via non-carious tooth surface loss, traumatic or iatrogenic exposure of dentine. Periodontal disease, and/or treatment, may cause exposure of lateral or accessory canals. Idiopathic cervical resorption can provide a portal to the dentine tubules. There are cases where no apparent dentinal exposure is clinically evident. These can be attributed to enamel micro cracks, possibly resulting from trauma (Bergenholtz *et al.*, 2007). Some also suggested that anachoresis, which is an infection of pulpal tissues caused by bacteria borne in the blood stream, is a possible avenue for microbial invasion (Fouad, 2009).

In previously root filled teeth, however, the (re)infection occurs in a place where the bacteria are already, or have been, present. The most common cause is thought to be the persistence of microbes in the root canal after treatment or reinfection due to coronal leakage (Siqueira, 2001).

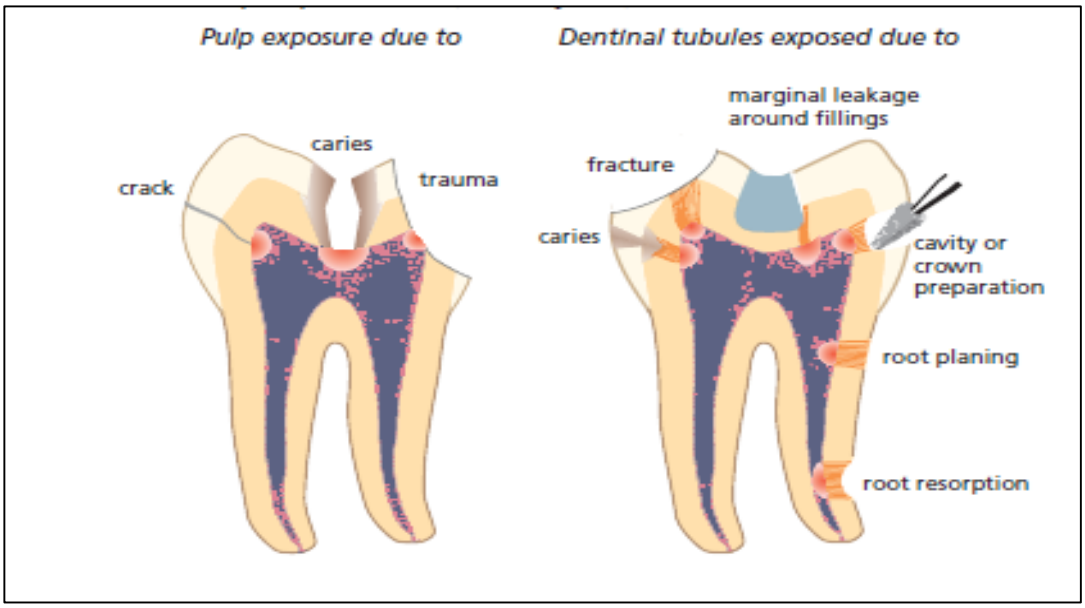

Figure 1.1: Microbial pathways to dentine and pulp (Bergenholtz *et al.*, 2007).

1.2.4 The ecology of the infected pulp

When the hard tissues are breached, commonly through caries, tooth wear, fractures or dental procedures, the dental pulp is exposed to noxious agents. The progress of the disease in the pulp is similar to other connective tissues and that includes inflammation (i.e. pulpitis) and later, if not treated, pulpal necrosis (Abbott and Yu, 2007). Within the necrotic pulp there are no defensive mechanisms and, thus, microbes colonise the pulpal space and multiply in large numbers (Bergenholtz and Spangberg, 2004).

Factors that dictate the microbial ecology in a given niche include the local pH, abundance and partial pressure of oxygen, redox potential, availability of selective nutrients, and the state of local host defences (Marsh and Devine, 2011). In canals with primary infection, there is a gradual shifting pattern in the root canal microbial composition (Sundqvist and Figdor, 2003). Pulpal exposure to the oral cavity and the availability of nutrition may favour aerobes and facultative anaerobes but at later stages the canal is dominated by obligate anaerobes. On the other hand, the harsh environment of secondary infections favours species with surviving abilities. There seems to be a high prevalence of facultative anaerobes such as enterococci and streptococci (Figdor and Sundqvist, 2007; Baumgartner, 2004).

Planktonic microorganisms (free-floating) are a pre-requisite for biofilm formation (Svensäter and Bergenholtz, 2004). A biofilm is defined as a sessile multi-cellular microbial community characterized by cells that are firmly attached to a surface and enmeshed in a matrix of extracellular polymeric substances (EPS) (Siqueira *et al.*, 2010). The first description of bacterial structure resembling biofilms in infected root canal was made by Nair (1987). Biofilms were also observed in secondary infections (Tronstad *et al.*, 1990).

1.2.5 The apical lesion

Apical periodontitis develops following pulpal necrosis and the emergence of root canal infection. It represents a host defence response to prevent root canal infection from spreading into adjacent bone marrow spaces and other remote sites. Apical periodontitis may or may not present with clinical symptoms such

as pain, tenderness and swelling. Apical bone resorption, although representing tissue destruction, occurs as part of the defensive process. When root canal treatment is initiated and bacteria are effectively eliminated, the active inflammatory lesion gradually subsides and bone regeneration usually takes place (Bergenholtz *et al.*, 2007).

1.2.6 Root canal treatment

1.2.6.1 Aim and objectives of root canal treatment

"Endodontology is concerned with the study of the form, function and health of, injuries to and diseases of the dental pulp and periradicular region, their prevention and treatment; the principle disease being apical periodontitis, caused by infection" (European Society of Endodontology, 2006).

Non-surgical root canal treatment is usually indicated when the pulp is:

- Possibly vital but (ir)reversibly inflamed, where the goal is to maintain existing periapical health and, thus, prevent apical periodontitis.
- Necrotic or infected and associated with apical periodontitis and the goal is to restore apical health.

Non-surgical root canal retreatment is indicated to treat secondary infections with failed previous root canal treatment and the goal is to restore the periradicular tissues back to health.

The standard practice of nonsurgical root canal (re)treatment involves chemo-mechanical preparation, with or without intracanal medication, root canal obturation and coronal restoration.

1.2.7 Causes of root canal treatment failure

1.2.7.1 Intra-radicular infections

The primary cause of root canal failure is the persistence, or reintroduction, of microbes in the root canal system (Siqueira, 2001). The risk of reintroduction of new microbes is dependent on asepsis, quality of obturation and coronal seal (Saunders and Saunders, 1994). Persistence of existing microbes is due to other causes; studies have demonstrated that, regardless of the technique, some parts of the root canal wall remain untouched by chemo-mechanical

preparation and therefore microbes may persist in these areas. Inadequate disinfection can also be caused by procedural errors such as instrument

separation or ledges (Siqueira, 2001). Furthermore, microbes can escape disinfection steps by residing in unreachable areas such as accessory canals, isthmuses and dentinal tubules (Haapasalo *et al.*, 2008). Failure may also be due to the presence of resistant microbes. Facultative species such as streptococci and *Enterococcus faecalis* have repeatedly been isolated and they possess the attributes to survive disinfection and harsh environmental conditions (Nair, 2006; Siqueira, 2001).

Due to the above causes, and considering the known limitations of radiographs, one might understand why a seemingly well-treated tooth can fail (Kersten *et al.*, 1987; Siqueira, 2001).

1.2.7.2 **Extra radicular infections**

Apical periodontitis is perhaps a defence barrier to keep microbes within the confines of the root canal. However, bacteria may escape this barrier and reside on the root surface (Nair, 2006). Extra radicular infections may also be caused by extruded infected dentine chips or over instrumentation. The prevalence of extra radicular infection is low (about 6-10%) and only a few species can reside in the periradicular areas. The most isolated species are *Actinomyces* and *Propionibacterium* spp. (Haapasalo *et al.*, 2008).

1.2.7.3 **Cysts**

Radicular cysts develop subsequent to apical periodontitis (Haapasalo *et al.*, 2008). Whether they heal after root canal treatment is a longstanding question. There are two types; pocket and true cysts. The former is expected to heal after conventional root canal treatment while the latter is self-sustaining and unlikely to heal (Nair, 2006).

1.2.7.4 **Foreign body reactions**

Foreign materials such as Gutta Percha (GP), sealer or paper points may become lodged in the periapical tissues and cause irritation and inflammation in the area (Haapasalo *et al.*, 2008).

1.2.7.5 **Scar tissue**

Apical lesions may heal by scar or fibrous tissue but this can be misdiagnosed radiographically as a sign of failure. Its prevalence is low (less than 7%) and it commonly occurs following surgical endodontics (Sathorn and Parashos, 2008).

1.3 Microbiological Investigations of root canal microbiota

1.3.1 Culture based studies

The pioneer work of D.W Miller started an era in which culture based methods were the gold standard (Bergenholtz *et al.*, 2007). Since then numerous culture-dependent studies have been conducted. Sundqvist *et al.* (1998) investigated 54 infected root filled teeth. In most cases each canal harboured 1-2 bacterial species and only in one case there were four species in the canal. Sundqvist's observations were in agreement with other studies (Molander *et al.*, 1998; Hancock *et al.*, 2001; Pinheiro *et al.*, 2003). More recently, Gomez *et al* (Gomes *et al.*, 2004) investigated 41 necrotic pulps (primary infections) and 19 failed root filled teeth (secondary infections). They observed that secondary infected canals contained 1-2 species while primary infections harboured up to ten species. In general, the majority of culture-based studies concluded that root canal infections consist of a few species (from 1-12 per canal) (Figdor and Sundqvist, 2007).

Cultivation based methods have been invaluable for microbiology but they suffer from a number of inherent limitations. The major drawback is the inability to cultivate the vast majority of bacterial species (Siqueira Jr and Rôças, 2005).

1.3.2 Molecular based studies

Deoxyribonucleic acid (DNA) was demonstrated as the genetic material by Oswald Theodore Avery in 1944. Its double helical strand structure composed of four bases was determined by James D. Watson and Francis Crick in 1953, leading to the central dogma of molecular biology (Fouad, 2009). The discovery of genetic material defines species and individuals, which makes the DNA sequence fundamental to the research on the structures and functions of cells and the decoding of life mysteries (Liu *et al.*, 2012).

The invention of the polymerase chain reaction (PCR) technique by Mullis was a revolution in microbiology which launched a new era of molecular identification (Mullis and Faloona, 1987). Since then an impressive array of PCR- based and other molecular techniques have been developed. There are several advantages of molecular based methods but perhaps the most important one is the ability to identify not only cultivable species, but also uncultivable taxa (Spratt, 2004). PCR is based on the *in vitro* replication of DNA through repetitive cycles of denaturing, primer annealing and extension steps.

It was inevitable then that many investigators have used PCR and other molecular methods to study endodontic diseases. Sakamoto *et al.* (2008) investigated secondary infections using 16S ribosomal RNA gene clone libraries. They reported that most teeth harboured a mixed consortium with a mean number of ten taxa per case. Another study recruited 88 subjects with primary and secondary infections (Sedgley *et al.*, 2006). They compared culture and real-time Quantitative PCR. The latter was able to detect bacteria in 100% of the cases as opposed to 55% with culture methods. More recent techniques, such as checkerboard hybridization assays, have also been exploited when, for example, Rocas and Siqueira (2008) investigated chronic apical periodontitis in 43 patients. They found that the number of taxa per canal was directly related to the size of the apical lesion. Small lesions were associated with a mean of 11 taxa per canal, while canals in teeth with larger lesions harboured a mean of 20 taxa. Overall, molecular based studies demonstrated that infected root canals harbour between 10-30 species (Siqueira and Rocas, 2009a).

1.4 Next generation sequencing technologies (NGS)

1.4.1 Overview

Next generation sequencing (NGS) technology, also known as high throughput or massively paralleling sequencing, is a blanket term that describes a number of modern technologies that are able to decipher the DNA, or RNA, with massive pace and depth (Margulies *et al.*, 2005).

The history of NGS started when Frederick Sanger developed a DNA sequencing technology based on a chain-termination method (Schuster, 2008). After years of development, Sanger sequencing, along with automated capillary electrophoresis, became the main tools for the completion of human genome project in 2001 (Collins and McKusick, 2001).

The human genome project greatly stimulated the development of powerful novel sequencing instruments to increase speed and accelerated the development of NGS (Mardis, 2011).

The NGS technologies are different from the Sanger method, which is now referred to as first generation technology, in three main aspects. First, instead of requiring bacterial cloning of DNA fragments they rely on the preparation of NGS libraries in a cell free system. Second, instead of hundreds, thousands-to-many-millions of sequencing reactions are produced in parallel. Thirdly, the sequencing output is directly detected without the need for electrophoresis (Liu *et al.*, 2012).

Human microbiome analysis is the study of microbial communities found in and on the human body. The goal of human microbiome studies is to understand the role of microbes in health and disease (van Dijk *et al.*, 2014). The advent of NGS started a revolution in metagenomic sequencing and analysis (the study of the collective genome of microorganisms from an environment). The increased throughput and decrease in costs of sequencing, coupled with additional technological advances have transformed the landscape of metagenomics (Scholz *et al.*, 2012).

1.4.2 NGS instruments

The first NGS instrument was Roche 454 by Life Sciences in 2005. Since then several instruments have been developed. Currently, as for 2013, there are four main technologies;

- The Roche 454 (pyrosequencing) technique
- The Illumina HiSeq 2000, HiSeq2500 and MiSeq platforms.
- The SOLiD system
- Ion Torrent semiconductor system.

The various NGS technologies share the same principle of massive paralleling sequencing, but there are differences in their specifics such as sequencing chemistry, read length, running time, throughput per run and reads per run (Di Bella *et al.*, 2013). The Illumina technology (Table 1.1) uses pair-ended overlapping reads which not only result in an increase of the total fragment length but also enhance the sequence quality (Kuczynski *et al.*, 2012; Lazarevic *et al.*, 2009). The Illumina MiSeq platform also have a very low error rate, compared with bench-top sequencers (Di Bella *et al.*, 2013).

Table 1.1: The Illumina sequencing instruments.

	MiSeq	HiSeq2000	HiSeq2500
Read length	50, 150, 250 or 300 bp; single read or paired end	50 or 100 bp, single read or paired end	50, 100, or 150 bp, single read or paired end
Run time	6 hrs - 3 days	3-12 days	3-12 days in standard mode, 1-5 days in rapid mode
Data generated per lane	1-25 million fragments sequenced in parallel; 0.5-15 Gbases data output	100-200 million fragments sequenced in parallel; 7.5-35 Gbases data output	100-150 million fragments sequenced in parallel; 7.5-35 Gbases data output
Uses	prokaryotic ChIP-seq, smallRNA-seq, small genome resequencing and targeted capture	RNA-Seq, large genome resequencing and targeted sequencing	de novo genome sequencing, large genome resequencing and targeted sequencing; where quick data turnaround is vital

1.5 Human microbiome investigation using NGS

1.5.1 Oral microbiome

Since NGS technologies have become available, a number of studies have been conducted to investigate and further exploit the microbiome of the oral environment. Keijser *et al* used 454 GS FLX pyrosequencing to investigate 71 saliva and 98 supragingival samples (Keijser *et al.*, 2008). They revealed a total of 28,978 unique variable (V6) tag sequences from 22 taxonomic phyla. However, the vast majority of these sequences (99.6%) belonged to one of the seven major phyla: *Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, Proteobacteria, Spirochetes*, or candidate division *TM7*. The findings suggested more diversity in plaque bacteria than those in saliva, with 267 versus 185 different genera, and 10,000 versus 5,600 OTUs at the 3% difference, respectively. Despite this, the bacterial richness estimated from rarefaction curves indicated that the richness is incomplete.

Another study used Illumina Genome Analyzer system GAII to evaluate the depth of sequencing coverage (Lazarevic *et al.*, 2009). The sequencing of saliva and oropharyngeal swabs from only three patients yielded a total of 1,373,824 sequencing read of which 330,815 were unique taxa. The study claimed to achieve much greater depth of coverage than previous oral microbiota studies. Several other studies used NGS technologies to investigate the oral microbiome in periodontal samples (Griffen *et al.*, 2012), dental caries and periodontitis (Belda-Ferre *et al.*, 2012), experimental gingivitis (Kistler *et al.*, 2013) and healthy and failing implants (Kumar *et al.*, 2012). Their findings gave new insights into the diversity of oral microbiota.

1.5.2 Endodontic microbiome

The first study to utilise NGS technologies for analysis of root canal infections was conducted by Li *et al* (Li *et al.*, 2010). Samples from seven teeth were collected with paper points and analysed using GS FLX pyrosequencing and Sanger sequencing. A total of 200,129 sequencing reads passed the quality assessment. The sequencing analysis at different taxonomic levels showed that Sanger sequencing and pyrosequencing yielded 8 *vs.* 13 phyla, 10 *vs*. 22 classes, 11 *vs*. 43 orders, 20 *vs*. 97 families, and 25 *vs*. 179 genera,

respectively. The most abundant phyla were *Bacteroidetes* (59.44%), *Firmicutes* (19.92%), *Actinobacteria* (4.79%), *Fusobacteria* (3.49%), *Proteobacteria* (3.18%), and *Spirochetes* (2.28%). The study detected six additional phyla that had not been previously reported in endodontic infections, namely: *Tenericutes*, *Deinococcus-Thermus*, *Chloroflexi*, *Cyanobacteria*, OD1 and *Acidobacteria*. The majority of genera detected were present at relatively low abundance. The study not only revealed bacterial diversity in previously inaccessible endodontic microbiota but also allowed access to low-abundance bacteria.

Siqueira *et al.* (2011) looked specifically at apical root canal microbiota using Multiplex tag encoded FLX titanium amplicon pyrosequencing. The sequencing of ten cryogenically ground apical root specimens yielded 84 genera belonging to ten phyla. The apical root region harboured a mean of 37 taxa, ranging from 13-80, which was far greater than the previously reported mean of 3 species (range 2 -8) (Siqueira *et al.*, 2007). A similar, more recent study also reported similar findings (Saber *et al.*, 2012). Another group also compared chronic and acute root canal infections using pyrosequencing (Santos *et al.*, 2011).Overall, 13 phyla and 67 different genera were detected. The results revealed that the bacterial diversity associated with acute infections is higher than chronic infections. The group also observed that there was a marked inter-individual variability in the composition of the bacterial communities. Each individual harboured a unique endodontic microbiota in terms of species richness and abundance.

More recently, a study examined extracted teeth and compared coronal and apical microbiota (Ozok *et al.*, 2012). They reported a far more complex apical diversity than previously highlighted. Recently another five studies have been published looking at various aspects of root canal infections and they indicated that the diversity is far greater than previously anticipated (Lim *et al.*, 2011; Hong *et al.*, 2013; Anderson *et al.*, 2013; Vengerfeldt *et al.*, 2014; Tzanetakis *et al.*, 2015). These are discussed in Chapter Five.

1.6 The study

1.6.1 Aim

To conduct a longitudinal study to investigate the microbiological diversity of root canal infections using high-throughput sequencing (HTS) on the Illumina MiSeq platform and culture methods.

1.6.2 Objectives

- To recruit (20) patients who are referred to Leeds Dental Institute with root canal infected teeth and require root canal treatment (pending fulfilment of inclusion criteria).
- To provide subjects with standard root canal treatment or re-treatment.
- To obtain valid pre and post-operative microbiological samples.
- To analyse samples using culture and HTS methods.
-

1.6.3 Primary question

What is the microbial composition and load of infected root canals?

1.6.4 Secondary questions

1. Does the microbiological diversity differ between primary and secondary infections?
2. Which species may persist after standard root canal treatment?

Chapter 2 Materials and Methods

2.1 Ethical approval

Prior to the commencement of the study, ethical approvals (Appendices 1-3 for the research supporting documents) were sought and obtained from the following bodies:

- National Research Ethics Service (NRES) Committee of Leeds East (REC reference number: 13/YH/0035, Appendix 4).
- Leeds Research and Development Directorate (R&D) approval was obtained from Leeds Teaching Hospitals (LTHT R&D number DT 13/ 10723, Appendix 5).

2.2 Clinical material

2.2.1 Subjects

The study population included subjects who had non-vital infected teeth with evidence of chronic apical pathology confirmed by:

- Clinical signs and symptoms, such as tenderness to percussion, soft tissue palpation and/or presence of sinus tract.
- Negative response to thermal and/or electrical pulp testing.
- Apical radiographic changes that indicative of an apical pathology in line with clinical signs and symptoms.

2.2.2 Inclusion criteria

- Teeth with primary (previously untreated) or secondary (previously root filled) root canal infections.
- Restorable teeth.
- Stable periodontal condition and absence of periodontal pockets > 4mm.

2.2.3 Exclusion criteria

- Immune deficient patients such as HIV or leukaemia.
- Pregnancy.
- Under 18 years old.
- Patients who had antibiotics in the last month.
- Teeth with severe anomalies.
- Cases where microbiological sampling may not be optimum or compromised by an ineffective coronal seal, for instance:
  1. Teeth with post(s).
  2. Teeth with root curvature of >15°.
  3. Teeth which fail to show radiographic evidence of patent canals.

2.3 Sample size

The sample size of this pilot study was determined following statistical advice by a qualified biostatistician at the Centre of Epidemiology and Biostatistics, University of Leeds. We proposed to aim for 20 subjects (or teeth) with an expected dropout rate of < 15%. This section is further discussed in Chapter Five.

2.4 Recruitment

The study population was selected from patients who had been referred for root canal treatment to the new-patient Restorative Consultant Clinics, at the Leeds Dental Institute. The first contact and initial screening of potential participants were conducted by the duty restorative consultant as part of the consultation visit. Potential participants who appeared eligible for inclusion, and were interested in the study, were imminently met by the chief investigator for further screening and for provision of detailed information regarding the aims, duration, risks and benefits of the research as well as the procedures of the root canal treatment (or retreatment) and prognosis. They were then given participant information sheets and allowed a minimum of two days to freely consider taking

part in the study. Participants who agreed, were seen in the Leeds Dental Translational and Clinical Research Unit (DenTCRU) and asked to sign a consent form before re-confirming their eligibility and commencing the study. A Case Report File (CRF) was allocated for each recruited participant with a unique number, to record relevant details, checklists and study data.

2.5 Withdrawal and exclusion

Participants were completely free to withdraw from the study at any time without providing a reason. Participants could be excluded from the study for various reasons. Possible causes of exclusion included severe protocol deviation, eligibility violation, adverse events or changes to inclusion or exclusion criteria.

2.6 Clinical research team and settings

The research was conducted in the Leeds Dental Translational and Clinical Research Unit (DenTCRU), the University of Leeds (Figure 2.1). The Unit is equipped with all materials and equipment required for the clinical research. The research team included two highly experienced dental nurses who were involved in designing research record documents, participants' recruitment, record keeping, clinical treatment and patient management.

Figure 2.1: DenTCRU unit at the Leeds Dental Institute.

2.7 Endodontic treatment and clinical procedures

The root canal (re) treatment, was delivered over three clinical visits in all cases according to the agreed protocol. The clinical treatment and materials used in the study were considered as standard with the exception of tooth surface decontamination and microbiological sample collection procedures (Figure 2.2).

All the completed cases were relatively straightforward and, hence, the protocol was followed. The next section describes the clinical stages but first, and in order to avoid repetition, the following clinical steps are mentioned here because they were common in all visits.

Each clinical session started with the following:

1. Updating the medical history.
2. Recording any changes or adverse events since the last visit.
3. Re-confirming inclusion and exclusion criteria.

After the administration of adequate local anaesthesia, the tooth was isolated with a sterile clamp (Claudius Ash, UK) and rubber dam (Coltene, Whaledent) and a caulking agent, Oraseal (Ultraden, Inc) to ensure a tight seal around the tooth.

The tooth surface decontamination procedures were as follows:

The tooth surface was polished with pumice and rinsed with sterile saline (Braun B, UK). Using a sterile cotton pellet, the tooth surface was then wiped with 3% (v/v) hydrogen peroxide (APC Pure, Manchester, UK) followed by 2.5% (v/v) sodium hypochlorite (Henry Schien, UK) as described by Siqueira *et al.* (2004) and Sakamoto *et al.* (2008), which is a protocol modified from Ng *et al.* (2003). The tooth was washed with 3 ml of sterile saline, polished with pumice and washed again with 9 ml (3 full syringes) sterile saline to remove any remaining solution. Finally, the integrity of seal around the tooth was re-checked and re-established when required.

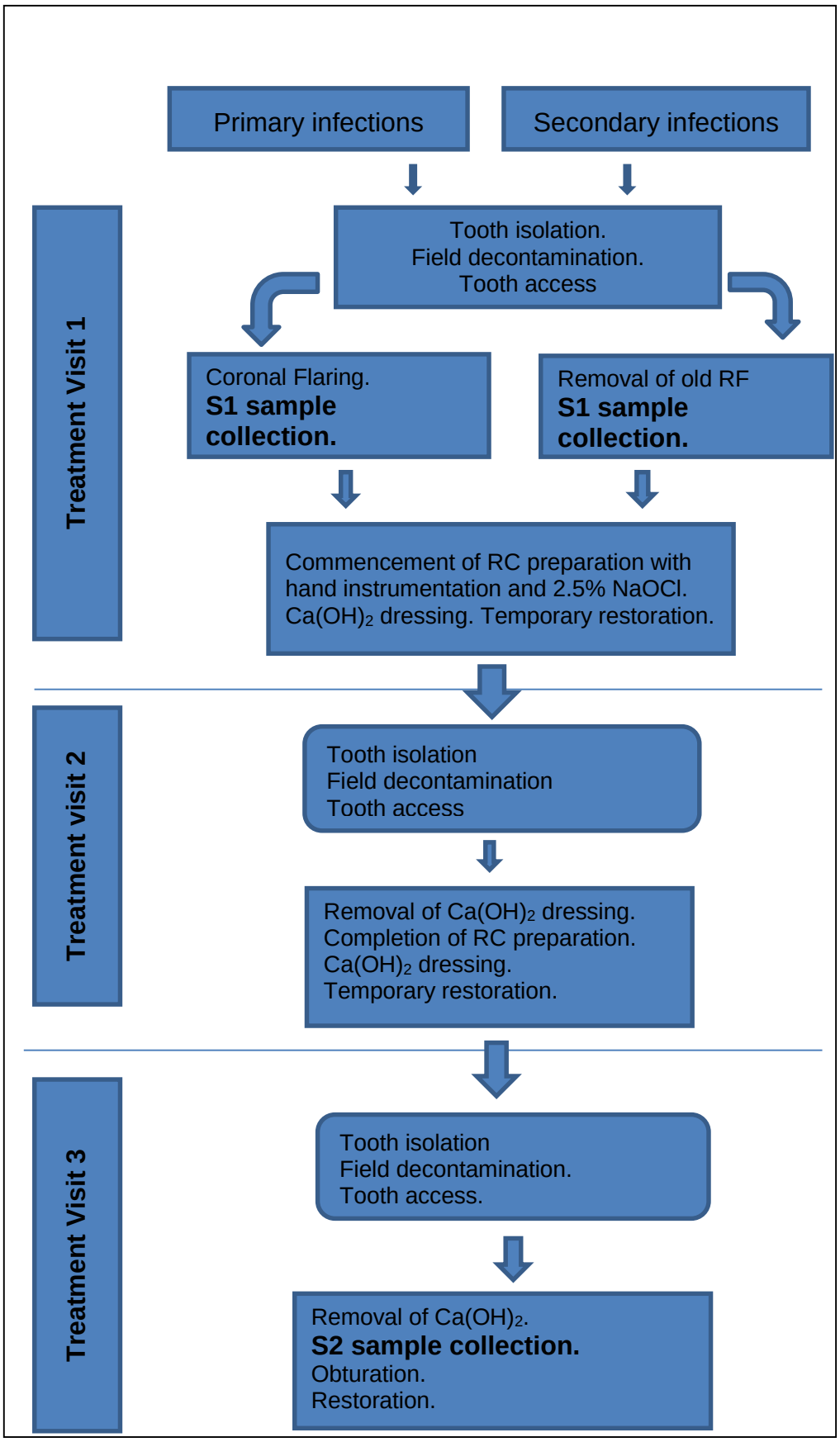

Figure 2.2: Flowchart of the clinical procedures undertaken.

2.7.1 1st Treatment visit

The objectives of this visit were:

- To gain tooth access.
- To obtain a microbiological sample (sample S1) prior to chemo-mechanical preparation.
- To commence chemo-mechanical preparation
- To adequately seal the tooth.

Access through the tooth was gained with sterile high and slow hand pieces burs. Any caries present in the access cavity was removed and the previously described decontamination procedures were repeated. The next step varied depending on whether tooth had primary or secondary infection:

2.7.1.1 Primary infections

After completing the removal of the pulp chamber roof, coronal flaring was performed with sterile SX ProTaper files (Dentsply Maillefer) and/or sterile Gate-Glidden burs (Dentsply). The canal's width and length were explored with a K-file of at least size 20. Size 20 file should move relatively freely within the canal and be within 2mm of the apex. This was verified with an electronic apex locater (ZX, Morita, Tokyo, Japan) and/or periapical radiographs (PA). If the canal was narrow or the file did not reach the working length, smaller files were used to enlarge the canal until it reached the required dimensions. To avoid disturbing the microbiota within the canal, only minimal irrigation with sterile saline was used at this stage. When the appropriate working length was accomplished, the S1 sample was collected as described in detail in Section 2.8. After obtaining the S1 sample, initial root canal preparation was initiated. Hand instrumentation with ProTaper or K-flexofiles were utilised and 2.5% (v/v) sodium hypochlorite was used as an irrigant. The canal was then dried with sterile paper points (Plandent) and dressed with calcium hydroxide [$Ca(OH)_2$] (Hypocal, Ellman). A sterile cotton pellet was placed before sealing with Glass Ionomer cement (Fuji JX, GC).

2.7.1.2 **Secondary infection**

Secondary infections are failed root-filled teeth. In the majority of the cases the old root filling was GP, except for two cases where the root filling materials were a silver point and a soft paste-like materials.

The GP and paste-like material were removed as follows; after gaining straight access to the root filling, every effort was made to remove the root filling mechanically using a combination of Gates- Glidden, ProTaper retreatment files, Hedstrom files (QED) and/or K-files (QED). This proved sufficient in the majority of cases. However, in two cases a solvent (EndoSolv) was required to soften the gutta-percha and complete the removal of the root filling. This was vital to avoid jeopardizing the treatment outcome. To minimise any possible further damage to the microbiota of the canal, the only irrigant used at this stage was sterile saline. The complete removal of the root filling was confirmed with a periapical radiograph. Following this, the S1 sample was obtained as described in Section 2.8. After obtaining the S1 sample, initial canal disinfection and preparation was initiated. Hand instrumentation with ProTaper or K-files were utilised and 2.5% (v/v) sodium hypochlorite was used as an irrigant. The canal was then dried with sterile paper points and dressed with $Ca(OH)_2$. A sterile cotton pellet was placed and the tooth was sealed with Glass Ionomer cement. The silver points and separated instrument case were a straightforward case. The silver point was relatively loose and was grasped and removed with Removal forceps. Following this, the S1 sample was obtained and stored for further processing.

2.7.2 2nd Treatment visit

The objectives were:

- To re-access the tooth.
- To complete canal preparation and disinfection procedures.
- To reseal the tooth.

After the administration of the local anaesthesia and tooth isolation as described in the first treatment visit, the canal was re-accessed with sterile burs as described previously. Using hand files and irrigation, the $Ca(OH)_2$ dressing

was removed from the canal. Mechanical preparation of the canal was completed with hand instrumentation using ProTaper files and/or K-files. The researcher was free to choose either file type and the preparation technique as judged appropriate for a particular case. Copious amount of 2.5% (v/v) sodium hypochlorite was used throughout the preparation. The exact amount of the irrigant solution varied according to the case, which depended mainly on the size of the canal and the amount of preparation needed. As a general rule, the researcher used at least a full 3 ml syringe every other file use. The final irrigant was sterile saline solution. The canal was then dried with sterile paper points. Non-setting $Ca(OH)_2$ was then placed in the canal with a long intracanal syringe to the full working length. A sterile cotton pellet was then placed and the tooth was sealed with glass ionomer cement.

2.7.3 3rd Treatment visit

The objectives were:

- To re-access the tooth.
- To remove the dressing.
- To obtain the S2 sample immediately prior to obturation.
- To obturate the canal.
- To restore the tooth.

After the usual checks, the canal was re-accessed with burs as described in the first visit. Using hand files and irrigation, the $Ca(OH)_2$ dressing medication was removed from the canal. The canal was then copiously irrigated with sterile saline to remove any remnant of $Ca(OH)_2$ and then dried with sterile paper points. Master cone periapical radiographs were obtained to confirm the length. Just prior to obturation, the S2 sample was obtained as described in Section 2.8. To complete the obturation of the tooth, the master cone was coated with Zinc Oxide based sealer (Tubliseal, Kerr) and the obturation was completed with GP (Dentsply). The cold lateral compaction technique was utilised in all cases. The GP was then cut at the cemento-enamel junction, sealed with VitroBond (3M, ESPE) and the tooth was restored with glass ionomer cement. A post-operative radiograph was obtained. The patient was then fully informed about possible treatment outcome and future reviews. At this point their participation in the clinical treatment was completed. In most cases patients

were referred back to their referring clinician for permanent restoration and future reviews.

2.8 Microbiological sample collection

Root canal biofilm samples were collected following the protocol described by Moller (Moller, 1966). In total, two samples were collected from each subject:

- (S1) was collected prior to chemo-mechanical preparation.
- (S2) was collected immediately prior to obturation.

The sample collection procedures were as follows:

The canal was filled with about 0.5-2 ml of sterile saline. A new sterile surgical glove was worn before sampling and a sterile file (Dentsply, UK) of at least size 20 was introduced into the canal and moved with gentle filing motion to disrupt the biofilm. The file was then placed in the sample collection tube (Bijou) which contained 1.5 ml of reduced transfer fluid (RTF, containing; 0.45 g of K_2HPO_4, 0.45g of KH_2PO_4, 0.90 of NaCl, 0.1875 g of $(NH_4)_2SO_4$, 0.40 g of Na_2CO_3, 0.20 g of Dithiothreitol, 10 ml of 0.1 M EDTA and 1000 ml of distilled water). A sterile paper point was then inserted in the canal to the full working length to absorb the canal contents and then transferred to the collection tube. This was repeated until all fluid and biofilm were absorbed. In multi rooted teeth, the sample was collected from the canal with the apical pathology.

Upon collection, the sample was immediately placed in a jar with an anaerobic gas generating sachet and immediately transferred to the oral microbiology laboratory. Upon arrival, the collection tube was vortexed for 30 seconds and then placed in the anaerobic workstation for further laboratory analyses.

2.9 Laboratory procedures and experiments

Laboratory procedures flowchart (Figure 2.4).

2.9.1 Optimisation experiment 1

2.9.1.1 Sampling technique and number of paper points

Optimisation of the sampling technique was performed, by estimating the number of paper points required for sample collection to obtain the maximum amount of microbial material.

Two extracted single rooted lower right canines were accessed by sterile burs and placed in two sterile bijou containers. The canal in each tooth was inoculated with 2 ml of freshly obtained saliva from the researcher and Brain Heart Infusion (BHI) was used as growth medium. This was incubated in a walk-in incubator at 37^{o}C for 5 days. After the incubation period, two different sample collection methods were performed. The first sampling method was as described in Section 2.8 , while in the second method only 3 paper points were used. The DNA was extracted using the QIAGEN DNA mini kit as described in Section 2.9.6. The main conclusion from this experiment was that the first sampling method yielded significantly more DNA than the second and, hence, it was used in this study.

2.9.2 Initial microbiological sample preparation

Microbiological sample preparation steps were completed in the anaerobic work station (Concept 1000 INVIVO2, RUSKINN) under strict aseptic conditions using sterile instruments (Figure 2.3). As mentioned above, each sample was placed in 1.5 ml of RTF in a Bijou container. The sample was then divided into two aliquots; the first aliquot comprised 1.2 ml of the sample pipetted into a 1.5 ml microcentrifuge tube and this was designated for DNA processing while the remaining 300 µl was utilised for culturing.

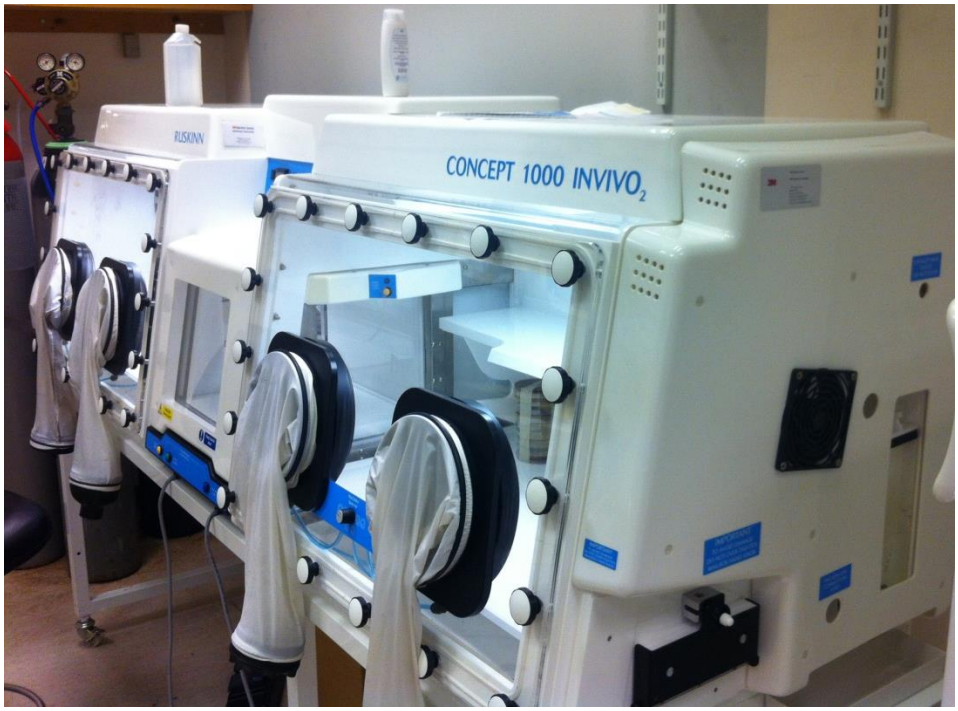

Figure 2.3: Anaerobic work station (Concept 1000 INVIVO2, RUSKINN).

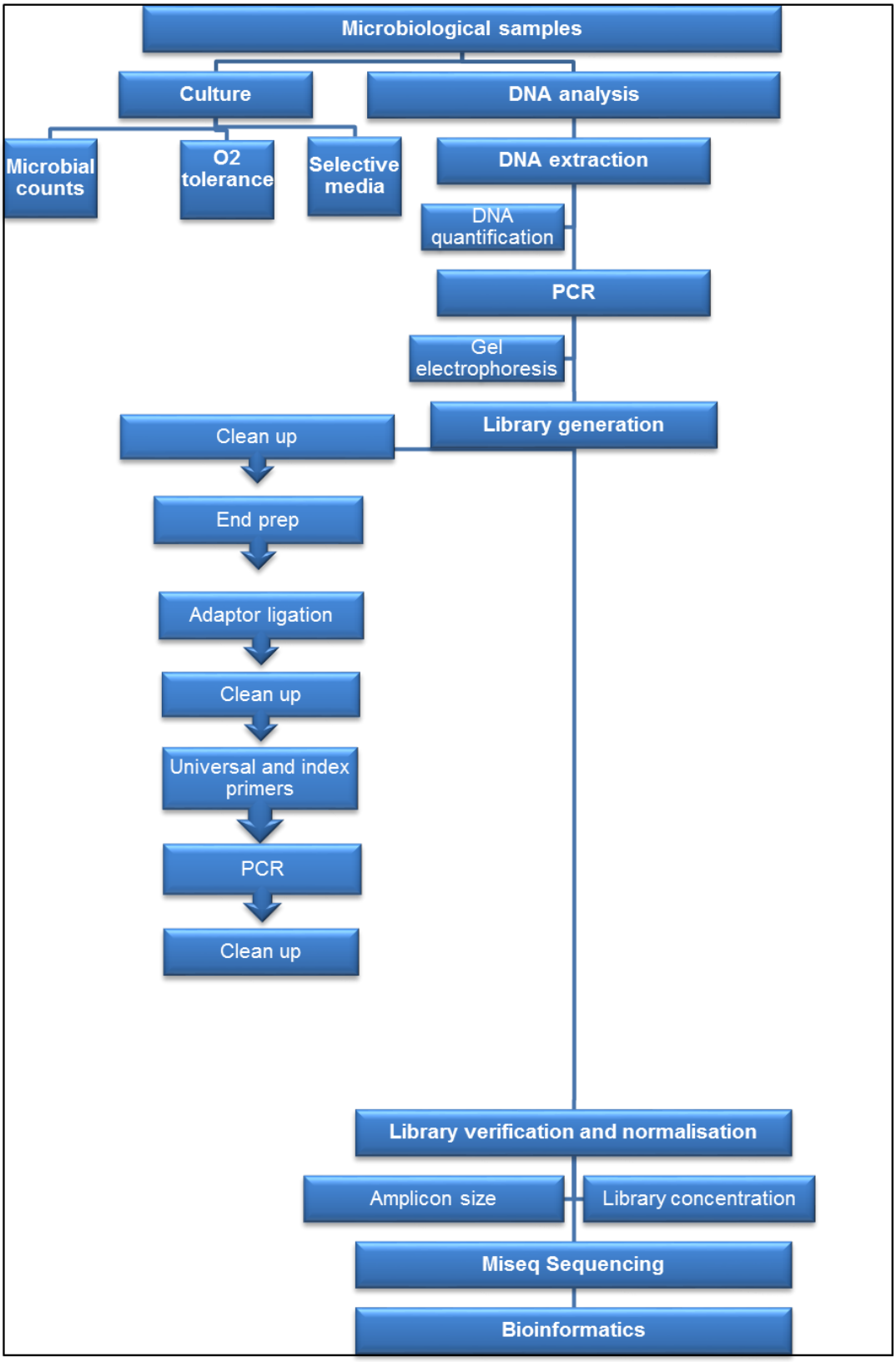

Figure 2.4: Flowchart of the laboratory procedures

2.9.3 Microbiological culture experiments

Due to the low volume of samples aliquoted for carrying out the culturing (300µl), it was decided to utilise the whole volume for serial dilution and not to include a neat sample in the analyses. Initially, for the first few microbiological samples, a 10-fold serial stepwise dilution was performed to dilute each sample from 10^{-1} to 10^{-5}. However, after observing that there was too little or no microbial growth on all plates with higher dilutions, it was decided to use 10^{-3} as the highest dilution. The procedures were as follows. Three clean 7 ml polystyrene bijou containers were used and labelled as 10^{-1}, 10^{-2} and10^{-3} . 2700 µl sterile RTF was added to each container. The 300 µl sample was transferred to the 10^{-1} dilution and then thoroughly mixed by shaking and pipetting. This was repeated for the other two dilutions.

After completing the serial dilutions, 100 µl was plated, and spread using disposable spreaders, on various selective and nonselective media, in triplicate and the incubated as described in Table 2.1.

Table 2.1: **The media selected for the study and the incubation conditions.**

Total count or group	Medium	Incubation Anaerobic/Aerobic	Temp.	Incubation time
Total anaerobic	**CBA[a] anaerobic**	Anaerobic	37 ºC	7 days
Total aerobic	**CBA aerobic**	Aerobic	37 ºC	7 days
***Actinomyes* spp.**	**CFAT[b]**	Anaerobic	37 ºC	7 days
***Bifidobacterium* spp.**	**Mupirocin-based selective agar**	Anaerobic	37 ºC	7 days
***Lactobacillus* spp.**	**Rogosa agar**	Aerobic	37 ºC	7 days
Yeast	**Sabouraud agar**	Aerobic	37 ºC	7 days
Mutans streptococci	**TYCSB agar[c]**	Anaerobic	37 ºC	7 days

[a] CBA- Colombia Blood Agar.
[b] CFAT- Cadmium Sulfate Fluoride Acridine Trypticase Agar.
[c] -TYCSB- Tryptone Yeast Extract Cystine with Sucrose and Bacitracin Agar.

2.9.4 Culture data analyses

2.9.4.1 Variables collected

Microbial counts for pre- and post-operative samples were expressed as the number of colony forming units (cfu)/ml. Descriptive statistics such as mean and standard deviation were used to summarise the data.

2.9.4.2 Statistical analyses

The data collected from each case were entered into Microsoft Office Excel 2010 spreadsheets. Differences between primary and secondary samples and between S1 and S2 samples were statistically tested. Logarithmic transformation was applied to the original observations (cfu/ml) to make the distribution more symmetric. A paired t-test was used to evaluate the difference between S1 and S2 samples collected from the same individual. An independent two sample t-test was used to test the difference between primary and secondary infection samples. Unequal variance between two samples was assumed. All tests were performed using IBM SPSS Statistics Version 21.

2.9.5 Sample DNA preparation for NGS

2.9.5.1 Optimisation experiment 2

2.9.5.1.1 Comparisons of DNA extraction kits:

Freshly collected saliva samples were tested using two different DNA extraction kits; QIAamp DNA mini kit (Qiagen) and UltaClean Microbial DNA isolation kit (MOBIO Laboratories). The kits were used according to the manufacturer's protocols. The main result indicated a higher DNA yield was obtained from the saliva samples using the QIAamp, DNA mini kit (Qiagen) and hence it was used for study.

2.9.6 DNA extraction procedures

The second aliquot contained 1.2 ml of the microbiological sample and this was designated for DNA extraction. DNA was extracted using the QIAamp DNA Mini Kit (Qiagen), and following the manufacturer's protocol. The extraction procedures were as follows; the sample was centrifuged for 10 minutes at 7500 rpm to isolate the bacterial pellet and this was re-suspended in 180 µl Buffer ATL. To lyse the bacterial cells in the sample, 20 µl proteinase K was added, mixed by vortexing for 30 seconds and incubated for 3 hours at 56^{o}C. Vortexing was repeated for 30 seconds every hour as recommended by the manufacturer. At the end of the incubation, a brief centrifugation was performed to remove the drops from the lid. 200 µl Buffer AL was then added to the sample, mixed by vortexing and incubated for 10 minutes at 70°C to produce a homogeneous solution. Next, 200 µl absolute ethanol was added and, after vortexing the sample for 15 seconds, the whole mixture was transferred to the QIAamp Mini spin column (in 2 ml collection tube). This was then centrifuged for 1 minute at 8000 rpm. The collection tube containing the infiltrate was then discarded and replaced with a new 2 ml collection tube. Following this, 500 µl Buffer AW1 was added and the sample centrifuged at 8000 rpm for 1 minute. After discarding the collection tube containing the infiltrate and replacing it with a new 2 ml collection tube, 500 µl Buffer AW2 was added and the sample centrifuged at full speed (14000 rpm) for 3 minutes. Next, the collection tube was discarded but this time replaced with a 1.5 ml microcentrifuge tube. Finally, 200 µl Buffer AE was added to the sample, incubated at room temperature for 1 minute and then centrifuged for 1 minute at 8000 rpm. The spin column was discarded and the 1.5 ml microcentrifuge tube containing the sample DNA in a 200 µl volume was stored at – 20°C.

2.9.7 Measurement of extracted DNA concentration in the samples

Measurement of the DNA concentration in each sample was conducted using the Quant-iT PicoGreen Kit (Invitrogen, Paisley, UK). The protocol described below was sufficient for five samples and the standard DNA, in duplicate.

2.9.7.1 **Preparation of the assay buffer**

6000 µl 1X TE buffer was prepared from 20X TE buffer by adding 300 µl of 20X TE to 5700 µl of DNase free water. This working solution was intended for the reagent, standard DNA and the micro-well plate preparation.

2.9.7.2 **Preparation of reagent**

A 200 fold dilution of the PicoGreen reagent was prepared in a Bijou container by adding 10 µl of reagent to 1990 µl of TE buffer from Section 2.9.7.1. The prepared reagent was maintained away from direct light.

2.9.7.3 **Preparation of the standard DNA**

The DNA concentration was expected to be low and, hence, the low-range standard curve was selected. The low range protocol included two steps;

2.9.7.3.1 **Step 1**; a 50X fold dilution of the 2 µg/ml of stock DNA was prepared by adding 1 µl of the stock DNA to 49µl TE buffer.

2.9.7.3.2 **Step 2**; from this, 10 µl was pipetted and added to 390 µl of TE buffer, in a 1.5 microcentrifuge, to prepare 40X fold dilution to yield 50 ng/ml of stock DNA.

The standard DNA was then added to a microwell plate according to Table 2.2, below.

Table 2.2: Low range standard DNA distribution.

Vol of TE (µl)	Vol of 50 ng/ml stock DNA (µl)	Final DNA conc. in PicoGreen assay
0	100	25 ng/ml
50	50	12.5 ng/ml
90	10	2.5 ng/ml
99	1	250 pg/ml (0.25 ng/ml)
100	0	0

2.9.7.4 **Preparation of micro-well**

The micro well plate was labelled in duplicate to receive the samples containing the extracted DNA (Figure 2.5, A). The well was prepared by first pipetting 99 µl of TE into each well and then adding 1 µl of each sample in the corresponding well. Finally, 100 µl of the reagent prepared from Section 2.9.7.2, was added to each well. The micro-plate was then ready for analysis using the spectrofluorometer (Figure 2.5, B).

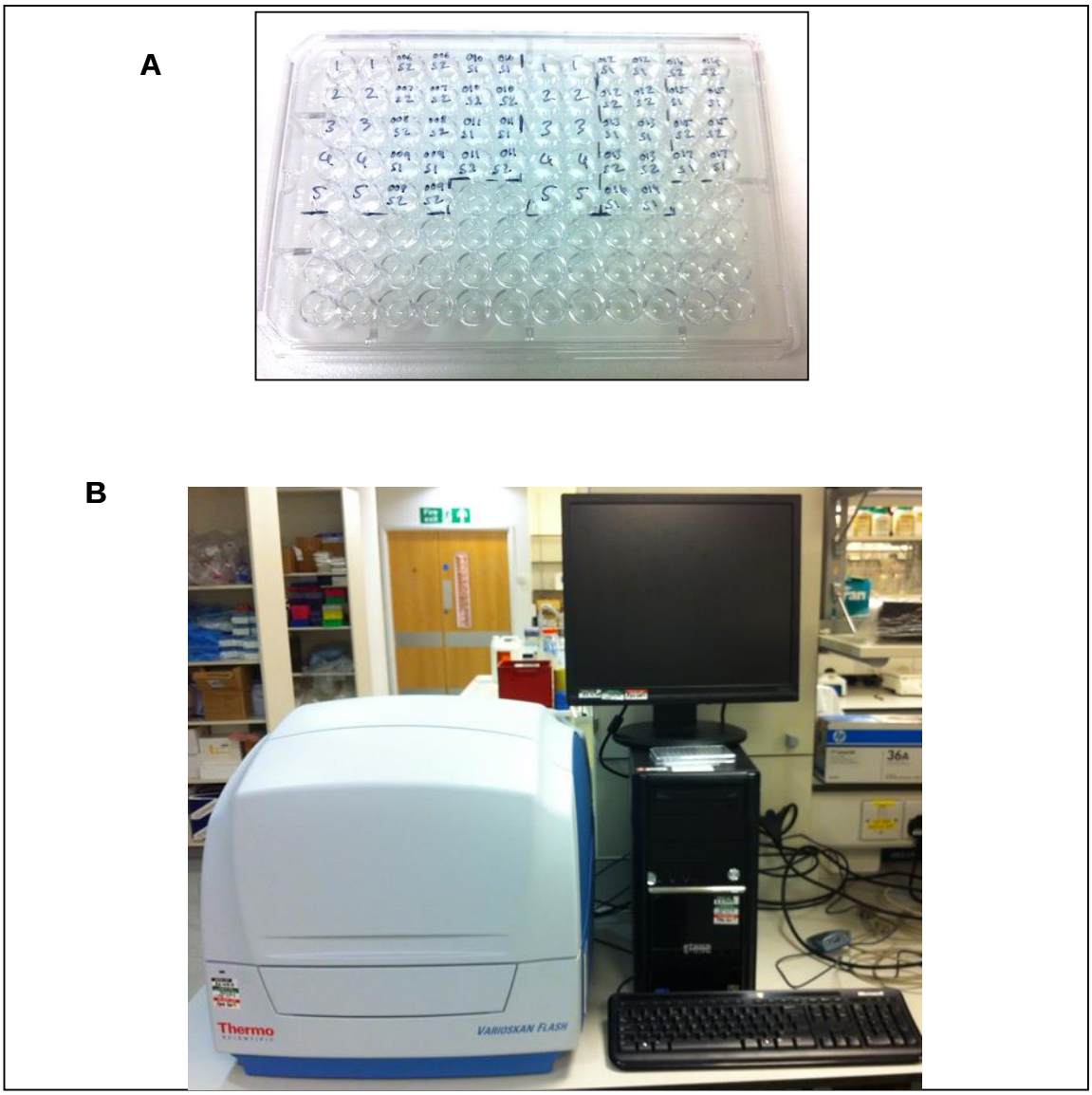

Figure 2.5: A: Well plate example. The first 2 lanes occupied with the standard DNA. The sample DNA is placed in duplicate. B: The spectrofluorometer (Varioscan flash, Thermo).

2.9.8 NGS library generation

The following protocol describes the steps carried out to amplify the targeted 16SrRNA gene V3-V4 regions of the bacteria present in each of the collected samples, and processes required to prepare the purified DNA fragments for next generation sequencing.

2.9.8.1 **Optimisation experiment 3**

2.9.8.1.1 Evaluation of various primer combinations

The aim was to compare 347F/ 803R primers to the V4F/V4R primer combination using the Master Green TAq kit. Extracted DNA samples from saliva were amplified using the Master Green TAq Kit and the two primer combinations. The PCR products were checked using agarose gel electrophoresis which showed that more PCR products were obtained using 347F/803R primers and, hence, these were used in the study.

2.9.8.2 **Optimisation experiment 4**

2.9.8.2.1 Optimisation of PCR conditions

The aim was to compare two different melting temperatures (Tms) for the 347F/ 803R primers using the Q5 High Fidelity DNA polymerase kit. Extracted DNA samples from saliva were amplified under two different Tms (60 and 63^{o}C). The PCR products were checked using agarose gel electrophoresis. No significant difference was observed between the two primer Tms.

2.9.9 Polymerase Chain reaction (PCR)

2.9.9.1 **Amplification of the extracted DNA**

The 16S rRNA gene is about 1542 basepairs (bp) long and contains nine hypervariable (V) regions interspersed within conserved regions. For the present study, the third (V3) and fourth (V4) hypervariable regions were

targeted for amplification using the Q5 High Fidelity DNA polymerase kit (New England BioLabs Inc., Life Technologies Inc. US) according to the manufacturer's protocol. The forward 347F and reverse 803R (Eurogentec, Belgium) universal bacterial primers were used for the process (Table 2.3).

Table 2.3: The forward and reverse primer sequences used for PCR.

Primer	Sequence	Bases
347F	5'-GGA-GGC-AGC-AGT-RRG-GAA-T-3'	19
803R	5'-CTA-CCR-GGG-TAT-CTA-ATC-C-3'	19

After thawing all the reagents on ice, they were gently vortexed and briefly centrifuged. The components of the reaction master mixture were first prepared, without the template DNA, in a 2 ml collection tube (Table 2.4). The master mixture was then distributed as aliquots of 23 µl in individual 0.5 ml PCR tubes. 2 µl of the templates DNAs were added to the individual PCR tubes to complete a total volume of 25 µl of PCR reaction mixture as recommended by the manufacturer.

Table 2.4: Master mix agents and their volumes for each PCR tube.

Component	Volume (µl)
5X Q5 reaction buffer	5
10 mM dNTPs	0.5
10 mM 347F primer	1.25
10 mM 803R primer	1.25
template DNA	2
High fidelity DNA polymerase	0.25
5X Q5 enhancer	5
Nuclease free water	9.75
Total	25

The individual PCR tubes were gently centrifuged to collect all components in the bottom of the tube and were then loaded onto the Prime thermal cycler (Techne, Bibby Scientific, UK) using the PCR conditions described in Table 2.5, below.

Table 2.5: The thermocycling conditions for the PCR.

Step		Temperature	Time
Initial Denaturation		98 ºC	30 seconds
35 cycles:	**Denaturation** **Annealing** **Extension**	98 ºC 60 ºC 72 ºC	10 seconds 30 seconds 30 seconds
Final extension:		72 ºC	2 minutes
Hold:		10 ºC	

2.9.10 DNA Gel electrophoresis

The presence of PCR products was checked using agarose gel electrophoresis. The agarose gel was prepared by adding 0.42 g of agarose (Severn Biotech limited, Worcester, UK) to 60 ml of 1X TAE Buffer (Tris-acetate-EDTA) (Sigma, Gillingham, UK) and then heating the solution in a microwave in several short intervals until the agarose was completely dissolved. 1 µl of GelRed DNA stain (Biotium, UK) was then added to the solution which, after a thorough mixing, was poured into a gel casting tray. One or two, gel comb(s) was placed in the tray to generate the wells and the solution was allowed to set at room temperature for at least one hour.

After the gel was set, sufficient TAE buffer was added to cover the tray and the wells. The samples were then prepared by thoroughly mixing 2.5 µl of the PCR product with 1 µl of Bromophenol Blue dye using a pipetter on a sterile paraffin film sheet. After mixing and loading the samples onto the gel, it was run using the Power Pac 300 (RioRad, USA) at 100 V, 400 mA for about 20 minutes. The gel was visualised using the Bio-Rad (ChemiDoc MP) Ultraviolet image system.

2.9.11 PCR product sample Clean up

MicroCLEAN (Microzone Ltd, UK) was used to purify the PCR product samples. 23 µl of the microCLEAN solution was added to the PCR product and thoroughly mixed by pipetting. The solution was centrifuged at 13000 rpm for 5 minutes and then the supernatant was carefully removed. The solution was centrifuged again for 2 minutes and the supernatant was removed. The DNA pellet was resuspended in 55.5 µl of nuclease-free water (Ambiol).

2.9.12 Addition of adaptors and indexes

2.9.12.1 NEBNext End preparation

After thawing all reagents on ice, 55.5 µl of the DNA sample produced from Section 2.9.11, was mixed with 3.0 µl End Prep Enzyme mix and 6.5 µl of End Repair Reaction Buffer (10X) to yield a total volume of 65 µl. After mixing by pipetting and a quick spin to collect the liquid from the side of the tube, the mixture tube was placed in the Prime thermal cycler (Techne, Bibby Scientific, UK) using the program Table 2.6, below.

Table 2.6: Thermal cycle settings for the End preparation.

Time	Temperature ^{o}C
30 minutes	20
30 minutes	65
Hold	4

2.9.12.2 Adaptor ligation

Next, 15 µl Blunt/TA ligase master mix, 2.5 µl NEBNext Adaptor for Illumina and 1 µl ligation enhancer were directly added to the end Prep reaction mixture. After thorough mixing by pipetting and a brief centrifugation, the mixture was incubated at 20 ^{o}C for 15 minutes in the Prime thermal cycler (Techne, Bibby Scientific, UK). 3 µl of USER Enzyme was added to the ligation mixture and mixed before another 15 minutes incubation at 37 ^{o}C in the Prime thermal cycler.

2.9.12.3 Clean up of adaptor ligated DNA (without size selection)

AMPure XP beads (Beckerman Coulter, Inc) were first placed at room temperature and resuspended by vortexing. 86.5 µl of the beads were added to the ligation mixture, mixed by pipetting and vortexing before incubation for 5 minutes at room temperature. Following a quick centrifugation, the tube was placed on a magnetic stand to separate the beads from the supernatant and left for about 5 minutes until the solution was clear. The supernatant was then carefully discarded without disturbing the beads that contained the DNA targets. The beads were washed, while on the magnetic stand, by adding 200 µl of 80% (v/v) freshly prepared ethanol (Sigma, UK) and then incubated for 30 seconds before carefully removing the supernatant. After repeating this for a total of three times, the beads were further air dried for 10 minutes. To elute the DNA target from the beads, 28 µl of 0.1X TE was added, mixed by pipetting, quickly centrifuged and then placed on the magnetic stand until the solution was clear. Finally, 23 µl of the solution is transferred to a new PCR tube for amplification.

2.9.12.4 PCR amplification

The 23 µl of the adaptor ligated DNA mixture produced in the last clean up step was mixed with:

- 25 µl of NEBNext High Fidelity 2XPCR master mix.
- 1 µl of universal PCR primer, and
- 1 µl of Primer Index 1-38 (one index for each sample). (Table 2.7 for adaptor and universal primer sequence) (Appendix 6 for a list of all 38 primer indexes).

Table 2.7: NEBNext adaptor and universal primer sequences.

Component	Sequence
NEBNext adaptor	5’ -/5Phos/GAT-CGG-AAG-AGC-ACA-CGT-CTG-AAC-TCC-AGT-C/ideoxyU/A-CAC-CAC-TCT-TTC-CCT-ACA-CGA-CGC-TCT-TCC-GAT-C*T-3’
NEBNext Universal primer	5’-AAT-GAT-ACG-GCG-ACC-GAG-ATC-TAC-ACT-CTT-TCC-CTA-CAC-GAC-GCT-CTT-CCG-CTT-CCG-ATC*-T-3’

After mixing by pipetting and a brief centrifuge, the mixture tubes were then placed in the Prime thermal cycler for PCR amplification using the following the conditions presented in Table 2.8, below.

Table 2.8: PCR conditions.

Step	Temperature ^{o}C	Time	Cycles
Initial denaturation	98	30 seconds	1
Denaturation **Annealing** **Extension**	98 65 72	10 seconds 30 seconds 30 seconds	15
Final extension	72	5 minutes	1
Hold	4		

2.9.12.5 Clean up of PCR amplification

AMPure XP beads (Beckerman Coulter, Inc) were first placed at room temperature and resuspended by vortexing. 50 µl of the beads were added to the PCR reaction mixture, mixed by pipetting and vortexing before incubation for 5 minutes at room temperature. Following a quick centrifugation, the tube was placed on a magnetic stand to separate the beads from the supernatant and left for about 5 minutes until the solution was clear. The supernatant was then carefully discarded without disturbing the beads that contain the DNA targets. The beads were washed, while on the magnetic stand, by adding 200 µl of 80% (v/v) freshly prepared ethanol (Sigma Aldrich, UK) and then incubated for 30 seconds before carefully removing the supernatant. The beads were further air dried for 10 minutes while the PCR plate was on the magnetic stand and lid open. To elute the DNA target from the beads, 33 µl of 0.1X TE (Life technologies, UK) was added, mixed by pipetting, quickly centrifuged and then placed on the magnetic stand until the solution was clear. Finally, 28 µl of the supernatant was transferred to a new PCR tube and stored at -20 ^{o}C for next stage.

2.9.13 Assessment of amplicon sizes

The Agilent D1000 ScreenTape system (Agilent technologies Inc., Germany) is a tape-based platform for a simple, fast and reliable electrophoresis to verify the amplicon sizes of each sample from the final library preparation (Figure 2.6)

The system consists of three elements;

- 2200 TapeStation system.
- D100 ScreenTape with D1000 Reagents (D1000 ladder, D1000 Sample buffer), and
- Agilent 2200 TapeStation software.

This laboratory procedure was entirely performed by the NGS facility at the University of Leeds, St James Hospital. 1 µl from each DNA sample was utilised, without dilution, to prepare for this analysis. Upon completion of the procedure, the results of the analyses were collected and stored on a USB drive.

Using the 347F/803R primers, and after the addition of primers and adapters, the expected library DNA amplicon size should exceed 490bp which is recommended by Illumina in order to achieve an overlap of approximately 50bp.

Figure 2.6: TapeStation instrument (Agilent technologies Inc.)

2.9.14 Libraries quantification and normalization

The Qubit Kit Assays (Invitrogen, Life Technologies), a fluorometric-based quantification method that uses DNA (RNA or protein) binding dyes, was utilised to quantify the libraries. Before the start of the process, the DNA samples and standards were thawed on ice (at about 4 ^{o}C) whereas the Qubit buffer and reagent were stored at room temperature. The working solution was prepared by diluting the Qubit reagent 1:200 in Qubit buffer. This was first prepared as a master mix sufficient for a total of 76 samples (38 samples in duplicate) as well as for two standard tubes. The individual assay tubes were prepared in a clear 0.5 PCR tubes according to Table 2.9, below.

Table 2.9: Volumes for preparing individual assays for Qubit.

Volume	Standard assay tubes	Sample assay tubes
Vol of working solution to add	190 µl	199 µl
Vol of standard to add	10 µl	0
Vol of DNA sample to add	0	1 µl
Total vol in each assay tube	200 µl	200 µl

After this, the tubes were briefly vortexed and then incubated for 2 minutes at room temperature. Finally the tubes were inserted in the Qubit 2.0 Fluorometer to obtain the concentration readings in mg/ml.

The Qubit readings were utilised for library normalisation. Using the average of the two readings, the volume required to obtain 100 ng was calculated according to the following equation and example presented in Table 2.10, below.

Average of conc. (n) ng/µl = 100 ng/ volume (X) µl

Table 2.10: An example for calculating the final volume from each sample.

Sample	1st reading	2nd reading	Average
001S1	47.2 ng/µl	31.2 ng/µl	39.2 ng/µl

(X) µl = 100 / 39.2 = 2.6 µl

The final multiplexed indexed library was pooled by added the calculated volumes from each sample into a single 2.0 ml collection tube. This was transferred to the NGS facility (Clinical Science Building, St James' hospital campus, University of Leeds).

2.10 MiSeq final sample preparation, loading and running

This part was conducted by the NGS facility's staff. The collection tube containing the multiplexed pooled library was transferred to the NGS facility to be run with 2x 300 bp pairs. A sample sheet containing sample codes, universal primers, adaptors and unique indexes sequences had been completed in an excel sheet and transferred to the NGS facility as requested.

After the MiSeq running (Figure 2.7), the raw data files, generated as Fast files were electronically dispatched to the chief investigator for further analyses.

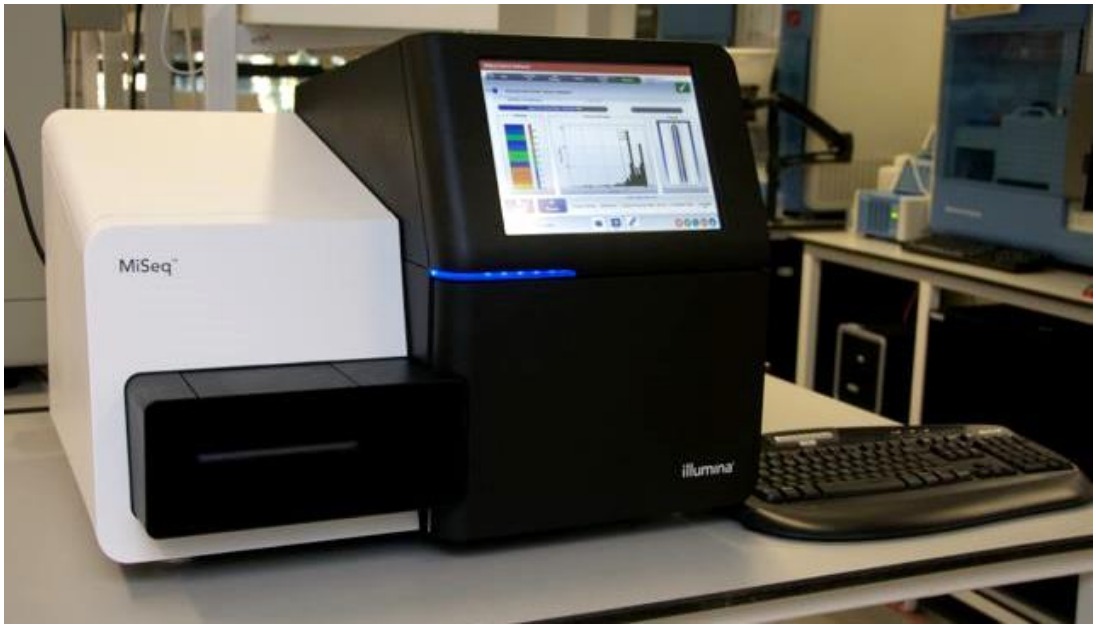

Figure 2.7: MiSeq benchtop platform.

2.11 Sequencing analyses and data processing

Data handling was carried out using the Advanced Research Computing (ARC) available at the University of Leeds, which basically consists of a constellation of High Performance based servers and storage. Access to and files transfer to ARC was facilitated using MobaXTerm v7.6 (Figure 2.8) and FileZilla v3.10.3 X server software (Figure 2.9

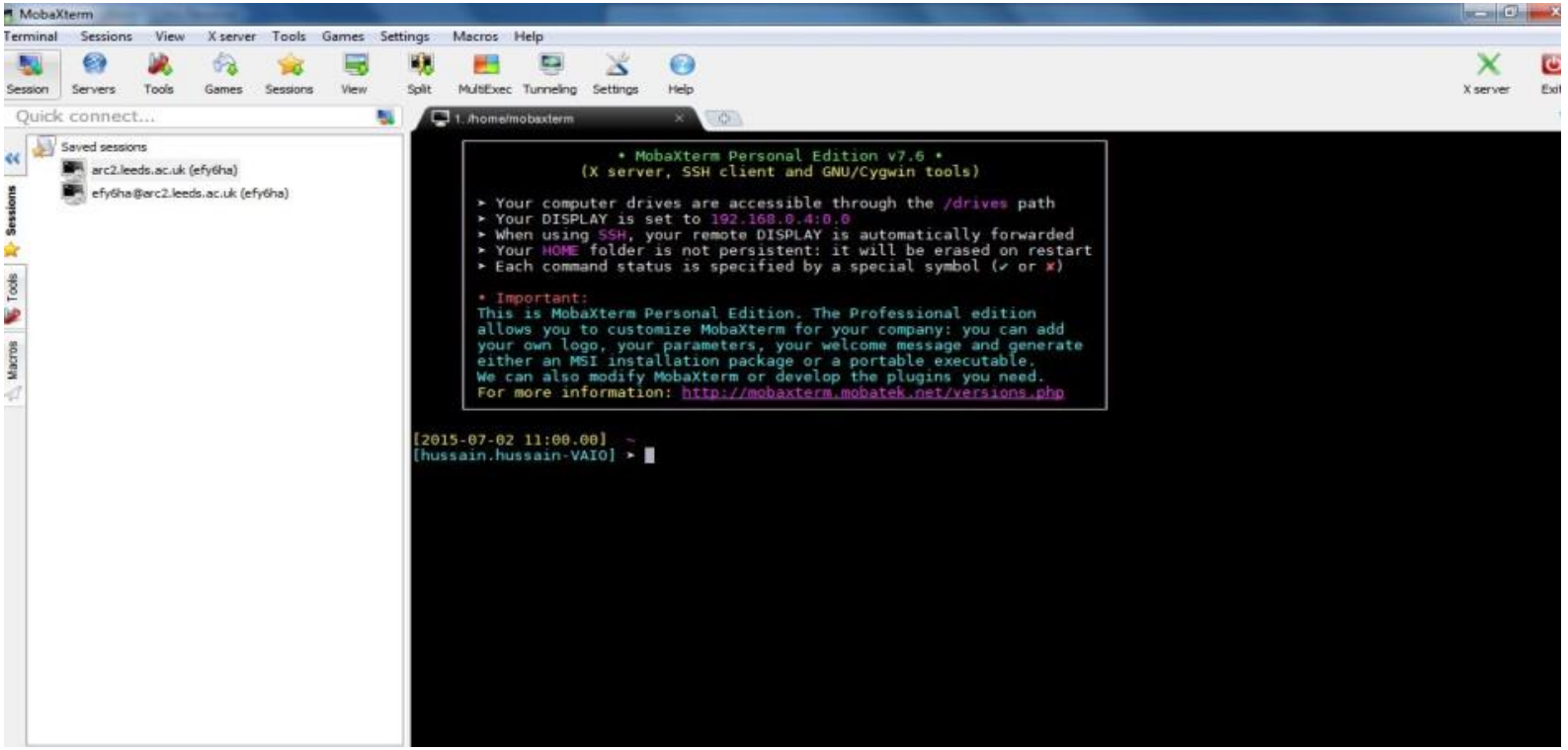

Figure 2.8: Screenshot of the MobaXTerm application.

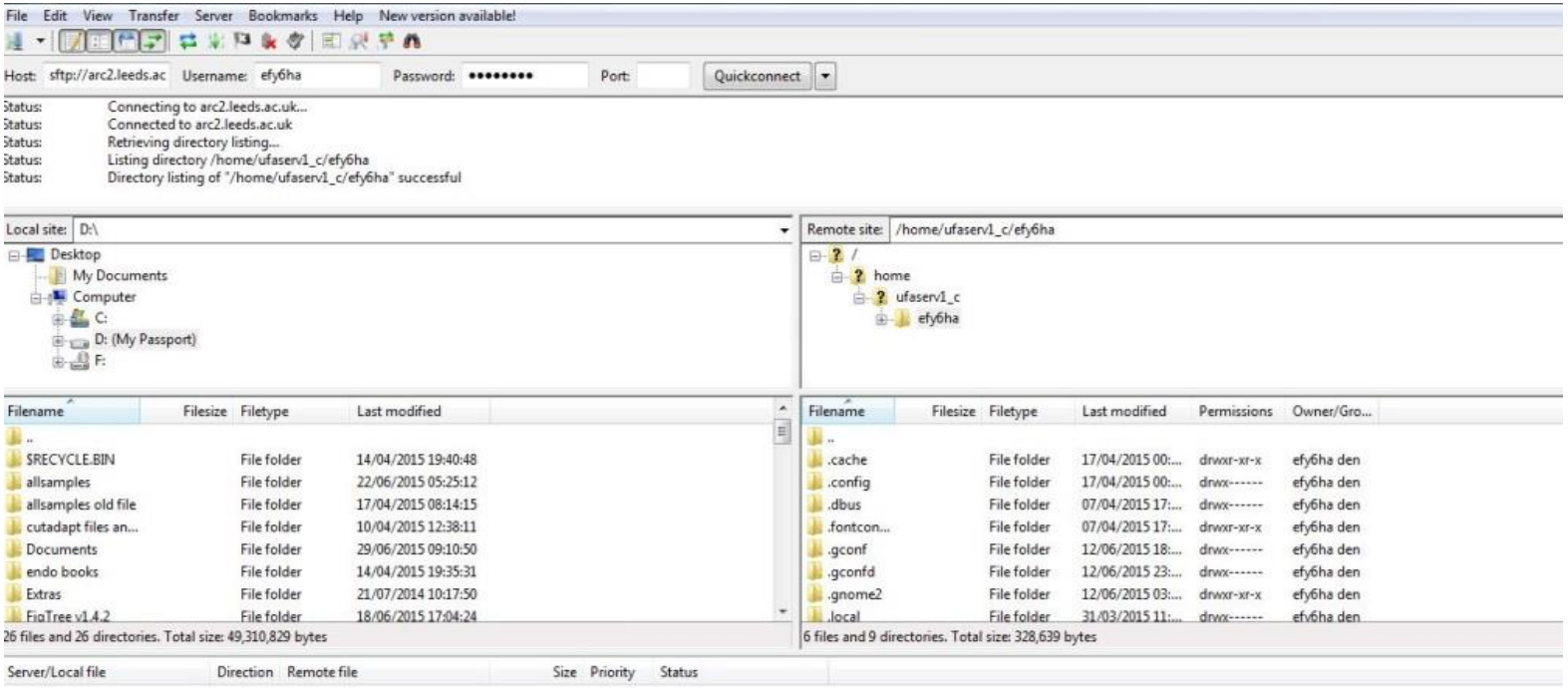

Figure 2.9: Screenshot of the FileZilla application.

The raw sequencing data generated by the MiSeq instrument were stored as Fastq files (with quality score encoded with a single ASCII). Final data processing and analyses were carried out using Quantitative Insights Into Microbial Ecology (QIIME), which is an open-source bioinformatics pipeline for performing microbiome analysis from raw DNA sequencing data. Prior to using QIIME, the fastq files were pre-processed as follows; the linker primer sequences were removed from the reads and then the reads were re-joined again. These steps were carried out using cutadapt v1.8 and fastq-join computer programs. The fastq files were then converted to fasta files using a script (computer commands) constructed using Python v2.7.10 (Figure 2.10).

QIIME was then used to process the sequences into OTUs. This was done in several steps according to Table 2.11, below.

Table 2.11: Steps for data processing using QIIME.

1. Pick OTUs	The sequences clustered into (OTUs) Using the default uclust matching, (0.97 sequence similarity).
2. Pick representative	To be used for taxonomic identification of the OTU and phylogenetic alignment
3.Assign taxonomy	To compare OTUs against a Greengenes (GG) database
4. Make OTU table	
5. Align sequences	De novo and assignment using PyNAST
6. Filter alignment command	
7. Build phylogenetic tree command	

allsamples_denovo... | allsamples_diversit... | allsamples_diversit... | test.sh | test0012.sh | test0021.sh

```
#$ -l h_rt=24:00:00
#$ -cwd
#$ -V
#$ -l h_vmem=4G
module load qiime/1.8.0
make_otu_heatmap_html.py -i allsamples/otu_table.biom -o allsamples/OTU_Heatmap/
summarize_taxa_through_plots.py -i allsamples/otu_table.biom -o allsamples/wf_taxa_summary -m mapping3.txt
summarize_taxa_through_plots.py -i allsamples/otu_table.biom -o allsamples/infection_wf_taxa_summary -m mapping3.txt -c infection
summarize_taxa_through_plots.py -i allsamples/otu_table.biom -o allsamples/sample_wf_taxa_summary -m mapping3.txt -c sample
summarize_taxa_through_plots.py -i allsamples/otu_table.biom -o allsamples/infection_sample_wf_taxa_summary -m mapping3.txt -c infection-sample
alpha_diversity.py -i allsamples/otu_table.biom -m chao1,PD_whole_tree -o adiv_chao1_pd.txt -t allsamples/rep_set.tre
alpha_rarefaction.py -i allsamples/otu_table.biom -m mapping3.txt -o allsamples/wf_arare -t allsamples/rep_set.tre
compare_alpha_diversity.py -i PD_whole_tree.txt -m mapping3.txt -c sample,infection -d 100 -o allsamples/sample_infection_PD100
beta_diversity_through_plots.py -i allsamples/otu_table.biom -o allsamples/wf_bdiv_even88757 -m mapping3.txt -t allsamples/rep_set.tre -e 88757
make_2d_plots.py -i allsamples/wf_bdiv_even88757/unweighted_unifrac_pc.txt -o allsamples/wf_bdiv_even88757/unweighted_unifrac_2d -m mapping3.txt -k white -p
 allsamples/wf_bdiv_even88757/prefs.txt
make_distance_histograms.py -d allsamples/wf_bdiv_even88757/unweighted_unifrac_dm.txt -m mapping3.txt -o allsamples/wf_bdiv_even88757/distance_histograms -p
 allsamples/wf_bdiv_even88757/prefs.txt
jackknifed_beta_diversity.py -i allsamples/otu_table.biom -o allsamples/jack -e 100 -t allsamples/rep_set.tre -m mapping3.txt
make_bootstrapped_tree.py -m allsamples/jack/unweighted_unifrac/upgma_cmp/master_tree.tre -s allsamples/jack/unweighted_unifrac/upgma_cmp/jackknife_support.txt -o
 allsamples/jack/unweighted_unifrac/upgma_cmp/jackknife_named_nodes.pdf
evince allsamples/jack/unweighted_unifrac/upgma_cmp/jackknife_named_nodes.pdf
```

Figure 2.10: An example of data analysis script generated using Python.

The data produced were then used to obtain the following analyses:

- Heat map was generated using OUT table.
- Assessment of community diversity within a sample (alpha diversity) or between a collection of samples (beta diversity).
- Jackknifing analysis to measure robustness of the sequencing effort. This was produced as jackknifed weighted 3d PCoA plots and jackknifed trees.
- Shared OTUs to assess similarities between primary and secondary samples.

Chapter 3 Results

3.1 Demographic data

Details of demographic and clinical data are shown in Table 3.1.Twenty two teeth, from 19 participants, were recruited for the study. Three teeth from three participants were excluded from the study, two of which were due to subsequent violation of exclusion criteria and the third was due to failed screening.

Sixteen participants (19 teeth) completed the study. The participants were 12 females and four males with a mean age of 43 years (range 20-67 y, SD= 12.71). Ten of the 19 teeth were diagnosed with primary infection as opposed to nine teeth with secondary infection. The 19 teeth included 12 incisors, three premolars and four molars. The maxilla was represented by 14 teeth while five teeth were present in the mandible.

Regarding clinical symptoms at baseline, 11 teeth had initially presented with mild pain or discomfort while the rest were pain free. Reduction of pain was reported by patients during visits in most cases and at the final visit, all showed very mild or no symptoms. Soft tissue changes were observed in eight cases, five of which were swellings and the rest were sinus tracks.

With regards to the radiographic radiolucency of the recruited teeth, all teeth included in the study showed radiographic changes at the start of the treatment. This was recorded into three categories as;

- Only widening of periodontal ligament (and/ or loss of lamina dura);
- Less than 10 mm in diameter of apical radiolucency ; or
- More than 10 mm diameter of apical radiolucency.

Of those recruited, 14 patients presented a radiolucent lesion of < 10 mm in diameter of apical radiolucency, three cases presented with cyst-like lesions of >10 mm in diameter and two presented with only widening of periodontal ligament.

Table 3.1: Demographic and clinical data of teeth included in the study.

						Soft tissue		Radiolucency		
Participants	**Age (years)**	**Gender[a]**	**Tooth[b]/Canal[c]**	**Infection**	**Discomfort/Pain**	**Sinus**	**swelling**	**Only Widening of PL[d]**	**<10mm**	**>10mm**
SUB-001	25	F	LR6/D	Primary	Yes	No	Yes	No	Yes	No
SUB-002	67	F	UR4/P	Secondary	No	No	No	No	Yes	No
SUB-003	34	F	LR7/D	Primary	Yes	No	Yes	No	Yes	No
SUB-006	28	F	UR1	Primary	No	No	No	No	Yes	No
SUB-007	49	F	LR6/D	Secondary	Yes	Yes	No	No	Yes	No
SUB-008	20	M	UL1	Primary	No	No	No	No	Yes	No
SUB-009	43	M	UR2	Secondary	Yes	No	Yes	No	No	Yes
SUB-010	57	F	LL5	Secondary	No	No	No	No	Yes	No
SUB-011	36	F	LL1	Secondary	No	No	No	No	Yes	No
SUB-012	35	F	UR4/P	Secondary	Yes	Yes	No	No	Yes	No
SUB-013	41	M	UR1	Primary	Yes	Yes	Yes	No	No	Yes
SUB-014	65	F	UR1	Primary	No	Yes	No	No	Yes	No
SUB-015	43	M	UR1	Secondary	Yes	No	Yes	No	No	Yes
SUB-017	26	F	UR1	Secondary	Yes	No	No	No	Yes	No
SUB-018	52	M	UR6/D	Primary	Yes	No	No	Yes	No	No
SUB-019	47	F	UL2	Primary	Yes	No	No	No	Yes	No
SUB-020	47	F	UL1	Primary	Yes	No	No	Yes	No	No
SUB-021	50	F	UL2	Secondary	No	No	No	No	Yes	No
SUB-022	50	F	UR2	Primary	No	No	No	No	Yes	No

a: F – female; M – male. **b**: U-upper, L-lower, R-right, L-left. **c**: D-distal, P-palatal. **d**: PL-periodontal ligaments

3.2 Microbial Culture

3.2.1 Assessment of the presence of viable microorganisms in all samples

Microbial growth (Table 3.2) was observed in over half of the samples (21/38: 55.3%). Samples obtained before root canal chemo-mechanical preparation procedures (S1) demonstrated the presence of viable microorganisms in 78.9% (15/19), while samples collected after the completion of root canal chemo-mechanical preparation and medication, immediately prior to root canal obturation, (S2) demonstrated viable growth in only 31.6% (6/19) of the cases.

A closer evaluation of infection type, i.e. primary infection or secondary infection, showed that growth was detected in 80.0% (8/10) and 77.8% (7/9) of S1 samples from primary and secondary infections, respectively.

S2 samples from primary and secondary infections showed viable growth in only 50.0 % (5/10) and 11.0% (1/9), respectively.

Table 3.2: Presence of viable microorganisms in samples from primary and secondary infected teeth.

	All samples			Primary		Secondary	
	Total	**S1**	**S2**	**S1**	**S2**	**S1**	**S2**
Number in which growth detected/ Total	21/38	15/19	6/19	8/10	5/10	7/9	1/9
Percentage in which growth detected (%)	55.3	78.95	31.58	80.0	50.0	77.78	11.11

3.2.2 Total microbial loads in S1 samples from teeth with primary and secondary infections

Total anaerobic counts from primary infections ranged from 1.7 $X10^1$- 7.9 $X10^6$ cfu/ml (mean $\log_{10}$ cfu/ml ± SD: 5.9 ± 1.9), whilst total aerobic counts ranged from 3 $X10^3$- 4.2 $X10^5$ cfu/ml (mean $\log_{10}$ cfu/ml ± SD: 4.7 ± 1.5) (Table 3.3 and Figure 3.1).

The quantity of microorganisms recovered from secondary infections ranged from 3 $X10^2$- 5 $X10^3$ cfu/ml (mean $\log_{10}$ cfu/ml ± SD: 3.3 ± 0.8) and from 2.7 $X10^2$- 8 $X10^5$ (mean $\log_{10}$ cfu/ml ± SD: 5 ± 1.3) with regard to total anaerobic and total aerobic viable counts, respectively (Table 3.3 and Figure 3.1).

The differences between the (normalised) mean logarithm values in primary and secondary S1 samples were statistically tested using unpaired *t*-test (for independent samples). The differences between total anaerobic microbial counts were statistically significant (p=0.012) whereas the differences in total aerobic counts were not significant (p=0.789).

Table 3.3: Total anaerobic and aerobic microbial counts (cfu/ml) in primary and secondary S1 samples.

Sample	Microbial count	Number in which growth detected/total (%)	CFU/ml range		Mean Count cfu/ml
			Minimum	Maximum	
Primary S1	**Total anaerobic**	7/10 (70.0)	1.7 X 10^1	7.9 X 10^6	8.3 X 10^5
	Total aerobic	7/10 (70.0)	3.1 X 10^3	4.1 X 10^5	5.4 X 10^4
Secondary S1	**Total anaerobic**	7/9 (77.8)	3.0 X 10^2	4.9 X 10^3	1.8 X 10^3
	Total aerobic	6/9 (66.7)	2.7 X 10^2	8.0 X 10^5	8.9 X 10^4

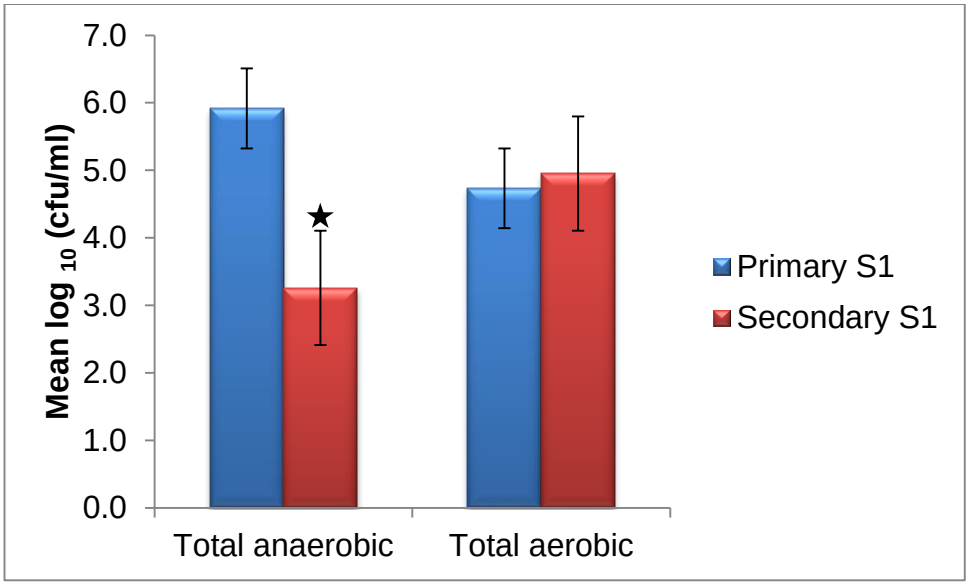

Figure 3.1: Total anaerobic and aerobic viable counts (mean log $_{10}$ cfu/ml ± SD) for primary and secondary infections S1 samples. * p<0.05 compared with Primary S1.

3.2.3 Growth of specific groups of microorganisms

Examination of selective media from the ten primary infection S1 samples (Table 3.4) revealed that the most frequently detected genera or groups of organisms were *Actinomyces* spp. (50%), mutans streptococci (40%) and *Bifidobacterium* spp. (20%) while *Lactobacillus* spp. and yeasts were only observed in one instance.

Table 3.4: Microbial counts (cfu/ml) of specific groups/genera detected in primary infection S1 samples.

Group	Number in which detected/Total (%)	CFU/ml range		Mean Count CFU/ml
		Minimum	Maximum	
***Actinomyces* spp.**	5/10 (50.0)	1.2 X 10^3	2.0 X 10^4	4.0 X 10^3
***Bifidobacterium* spp.**	2/10 (20.0)	2. X 10^3	6.3 X 10^6	6.3 X 10^5
***Lactobacillus* spp.**	1/10 (10.0)	3.5 X 10^3	3.5 X 10^3	3.9 X 10^2
Yeast	1/10 (10.0)	3.0 X 10^2	3.0 X 10^2	7.8 X 101
Mutans streptococci	4/10 (40.0)	1.3 X 10^2	1.3 X 10^4	2.1 X 10^3

On examination of selective media from secondary infection S1 samples, *Actinomyces* spp. were the most prevalent species as it was isolated from five out of the total nine secondary cases (55.0%) (Table 3.5). Mutans streptococci and *Bifidobacterium* spp. were much less prevalent than in primary cases as each were only isolated once. Yeasts were not detected in the secondary infection samples. Figure 3.2 summarises mean $\log_{10}$ cfu/ml ± SD counts of the various groups/genera for both primary and secondary S1 samples.

As mentioned above, *Actinomyces* spp. was the most frequently isolated in both primary and secondary infection S1 samples with a range of 1.9 $X10^3$- 2 $X10^4$ cfu/ml (mean $\log_{10}$ cfu/m ± SD l: 3.6 ± 1.1) and from 6.7 $X10^1$- 1.2 $X10^3$ cfu/ml (mean $\log_{10}$ cfu/ml ± SD: 2.5 ± 0.6), respectively. The difference was not found to be statistically significant (p =0.096). It was not possible to statistically evaluate the other groups because some values, or the sums from equations, were equal or near zero.

Table 3.5: Microbial counts (cfu/ml) of specific groups/genera detected in secondary infection S1 samples.

Group	Number in which detected/Total (%)	CFU/ml range		Mean Count CFU/ml
		Minimum	Maximum	
***Actinomyces* spp.**	5/9 (55.6)	6.7 X 10^1	1.2 X 10^3	3.3 X 10^2
***Bifidobacterium* spp.**	1/9 (11.0)	1.7 X 10^2	1.7 X 0^2	6.3 X 10^1
***Lactobacillus* spp.**	1/9 (11.0)	6.7 X 10^1	6.7 X 10^1	5.2 X 10^1
Yeast	ND[a]	ND	ND	ND
Mutans streptococci	1/9 (11.0)	1.0 X 10^2	1.0 X 10^2	5.6 X 10^1

a. ND – not detected

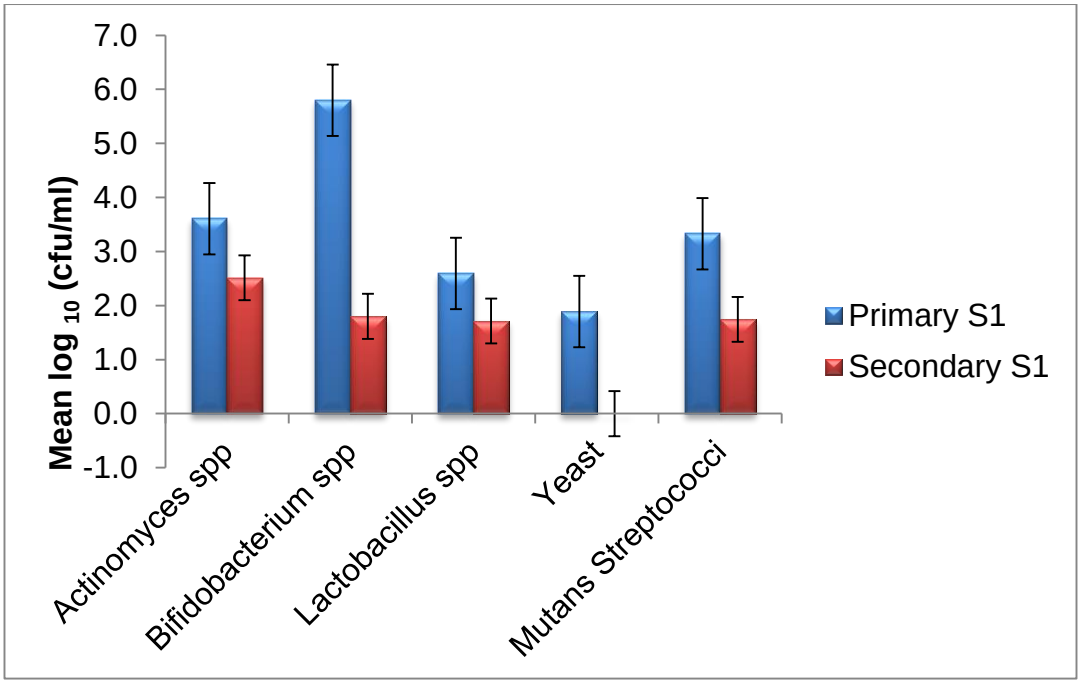

Figure 3.2: Viable counts (mean log $_{10}$ cfu/ml ± SD) for the selected groups/genera detected in primary and secondary S1 samples. (NB groups with no column indicate that organisms were below the detection limit of the assay).

3.2.4 Effect of root canal chemo-mechanical preparation

3.2.4.1 Comparison of microbial counts in all S1 and S2 samples

The total anaerobic counts from S1 samples ranged from 1.7 $X10^1$- 7.9 $X10^6$ cfu/ml (mean log_{10} cfu/ml ± SD: 5.6 ± 1.5), whilst total aerobic counts ranged from 2.7 $X10^2$- 8.0 $X10^5$ cfu/ml (mean log_{10} cfu/ml ± SD: 4.9 ± 1.4) (Table 3.6, Table 3.7, Figure 3.3).

There was a marked reduction in the quantity of microorganisms in S2 samples as they ranged from 1.3 $X10^2$- 9.5 $X10^3$ cfu/ml (mean log_{10} cfu/ml ± SD: 2.7 ± 0.7) and from 2.33 $X10^2$- 8.0 $X10^5$ (mean log_{10} cfu/ml ± SD: 5 ± 1.3) with regard to total anaerobic and total aerobic viable counts, respectively.

The effects of root canal chemo-mechanical preparation in reducing the total anaerobic and total aerobic microbial loads were statistically significant (p= 0.0001 and 0.004 respectively).

With regards to the selected groups, *Actinomyces* spp. were present in over half of the 19 cases (53%) with a mean log_{10} cfu/ml ± SD of 3.36 ± 0.91 whilst mutans streptococci and *Bifidobacterium* spp. were present in five and three cases with mean log_{10} cfu/ml ± SD values of : 3.06 ± 0.75 and 5.52 ± 1.21, respectively. The root canal treatment reduced the prevalence to only two cases for each group and the mean log_{10} cfu/ml values were also reduced. The reductions in the mean log_{10} cfu/ml values were statistically significant for *Actinomyces* spp. and *Bifidobacterium* spp. (p= 0.014 & 0.046) but it was not significant for mutans streptococci (p=0.053).

Figure 3.3 compares the mean log_{10} cfu/ml values for S1 samples to those from S2 and demonstrates the notable reduction in microbial counts in all groups.

Table 3.6: Microbial counts (cfu/ml) for all S1 samples (primary S1 and secondary S1 combined).

Total microbial count or group	Number in which growth detected/total (%)	CFU/ml range		Mean Count CFU/ml
		Minimum	Maximum	
Total anaerobic	14/19 (73.6%)	1.7×10^1	7.9×10^6	4.4×10^5
Total aerobic	13/19 (68.4)	2.7×10^2	8.0×10^5	7.1×10^4
***Actinomyces* spp.**	10/19 (52.6)	6.7×10^1	2.0×10^4	2.3×10^3
***Bifidobacterium* spp.**	3/19 (15.8)	1.7×10^2	6.3×10^6	3.3×10^5
***Lactobacillus* spp.**	2/5 (10.5)	6.7×10^1	3.5×10^3	2.3×10^2
Yeast	1/19 (5.3)	3.0×10^2	3.0×10^2	6.3×10^1
Mutans streptococci	5/19 (26.3)	1.0×10^2	1.3×10^4	1.2×10^3

Table 3.7: Microbial counts (cfu/ml) for all S2 samples (primary S2 and secondary S2 combined).

Total microbial count or group	Number in which growth detected/total (%)	CFU/ml range		Mean Count CFU/ml
		Minimum	Maximum	
Total anaerobic	5/19 (26.3)	1.3×10^2	9.5×10^3	5.2×10^2
Total aerobic	5/19 (26.3)	2.3×10^2	1.3×10^4	8.2×10^2
***Actinomyces* spp.**	2/19 (10.5)	6.7×10^1	1.1×10^3	6.4×10^1
***Bifidobacterium* spp.**	2/19 (10.5)	1.0×10^2	2.0×10^3	1.6×10^2
***Lactobacillus* spp.**	ND[a]	ND	ND	ND
Yeast	ND	ND	ND	ND
Mutans streptococci	2/19 (10.5)	3.3×10^2	8.7×10^2	8.9×10^1

a- ND – not detected

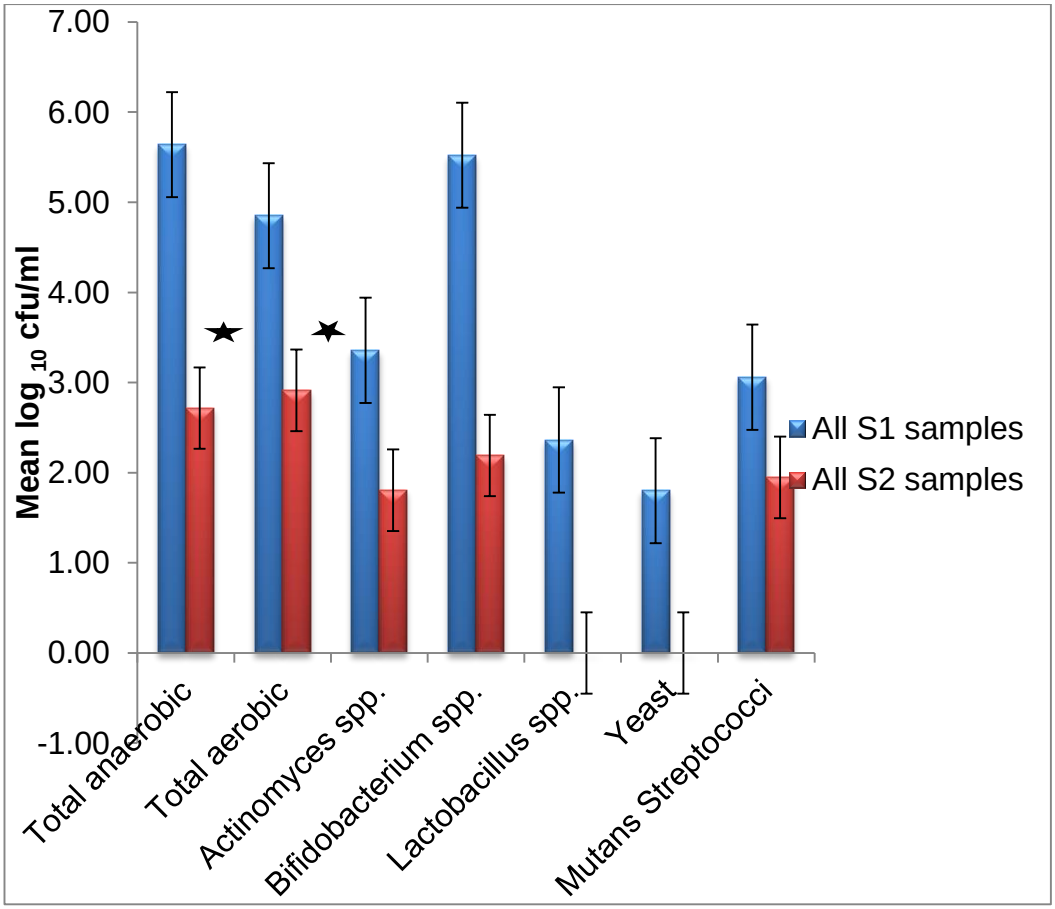

Figure 3.3: Microbial viable counts (mean log $_{10}$ cfu/ml ± SD) for all S1 and S2 samples. * p<0.05 compared with S1. (NB groups with no column indicate that organisms were below the detection limit of the assay).

3.2.4.2 **Primary infections**

The effect of root canal preparation in primary infections followed almost the same trend as the combined overall results described above (Table 3.8, Table 3.9 and Figure 3.4). The prevalence was reduced from 70% for both total anaerobic and aerobic growth to 50 % and 40%, respectively. The total anaerobic mean $\log_{10}$ cfu/ml ± SD in S1 samples was 5.9 ± 1.9 and this was reduced to a mean $\log_{10}$ cfu/ml ± SD value of 3 ± 0.9 in S2 samples. This reduction was statistically significant (p=0.007). For the total aerobic counts,

the mean log_{10} cfu/ml ± SD were decreased from 4.7 ± 1.5 to 3.2 ± 1.0 for S1 and S2 samples, respectively. However, this reduction was not statistically significant (*p*=0.076).

In the other selected groups, the reduction in *Actinomyces* spp. counts had a *p* value of 0.043 which is statistically significant. In contrast, the reductions in mutans streptococci and *Bifidobacterium* spp. were not statistically significant as the *p* values were 0.116 and 0.218, respectively.

Table 3.8: Total anaerobic, aerobic and the selected groups counts (cfu/ml) for primary S1 samples.

Total microbial count or group	Number in which growth detected/Total (%)	CFU/ml range		Mean Count CFU/ml
		Minimum	Maximum	
Total anaerobic	7/10 (70)	1.7 X10^1	7.9 X 10^6	8.3 X 10^5
Total aerobic	7/10 (70)	3.1 X 10^3	4.2 X 10^5	5.4 X 10^4
***Actinomyces* spp.**	5/10 (50)	1.2 X 10^3	2.0 X 10^4	4.0 X 10^3
***Bifidobacterium* spp.**	2/10 (20)	2.4 X 10^3	6.3 X 10^6	6.3 X 10^5
***Lactobacillus* spp.**	1/10 (10)	3.5 X 10^3	3.5 X 10^3	3.9 X 10^2
Yeast	1/10 (10)	3.0 X 10^2	3.0 X 10^2	7.8 X 10^1
Mutans streptococci	4/10 (10)	1.3 X 10^2	1.3 X 10^4	2.1 X 10^3

Table 3.9: Total anaerobic, aerobic and the selected groups counts (cfu/ml) for primary S2 samples.

Total microbial count or group	Number in which detected/Total (%)	CFU/ml range		Mean Count CFU/ml
		Minimum	Maximum	
Total anaerobic	5/10 (50)	1.3 X 10^2	9.5 X 10^3	9.5 X 10^2
Total aerobic	4/10 (40)	1.9 X 10^3	1.3 X 10^4	1.5 X 10^3
***Actinomyces* spp.**	2/10 (20)	6.7 X 10^1	1.1 X 10^3	7.7 X 10^1
***Bifidobacterium* spp.**	2/10 (20)	1.0 X 10^2	2.0 X 10^3	2.5 X 10^2
***Lactobacillus* spp.**	ND	ND	ND	ND
Yeast	ND	ND	ND	ND
Mutans streptococci	2/10 (20)	3.3 X 10^2	8.7 X 10^2	1.2 X 10^2

3.2.4.3 **Secondary infections**

There was a reduction in all groups in secondary infection samples both in terms of prevalence and microbial loads (Table 3.10, Table 3.11, Figure 3.4). In fact only one case showed growth above the detection limit in S2 samples. It was not possible to statistically evaluate the other groups because some values, or the sums from equations, were equal or near zero.

Table 3.10: Total anaerobic, aerobic and the selected groups counts (cfu/ml) for secondary S1 samples.

Total microbial count or group	Number in which growth detected/total (%)	CFU/ml range		Mean Count CFU/ml
		Minimum	Maximum	
Total anaerobic	7/9 (77.8)	3.0×10^2	5.0×10^3	1.8×10^3
Total aerobic	6/9 (66.7)	2.7×10^2	8.0×10^5	9.0×10^4
***Actinomyces* spp.**	5/9 (55.6)	6.7×10^1	1.2×10^3	3.3×10^2
***Bifidobacterium* spp.**	1/9 (11.0)	1.7×10^2	1.7×10^2	6.3×10^1
***Lactobacillus* spp.**	1/9 (11.0)	6.7×10^1	6.7×10^1	5.2×10^1
Yeast	ND	ND	ND	ND
Mutans streptococci	1/9 (11.0)	1.0×10^2	1.0×10^2	5.6×10^1

Table 3.11: Total anaerobic, aerobic and the selected groups counts (cfu/ml) for secondary S2 samples.

Total microbial count or group	Number in which detected/Total (%)	CFU/ml range		Mean Count CFU/ml
		Minimum	Maximum	
Total anaerobic	ND[a]	ND	ND	ND
Total aerobic	1/9 (11.0)	2.3×10^2	2.3×10^2	7.0×10^1
***Actinomyces* spp.**	ND	ND	ND	ND
***Bifidobacterium* spp.**	ND	ND	ND	ND
***Lactobacillus* spp.**	ND	ND	ND	ND
Yeast	ND	ND	ND	ND
Mutans streptococci	ND	ND	ND	ND

a- ND-not detected.

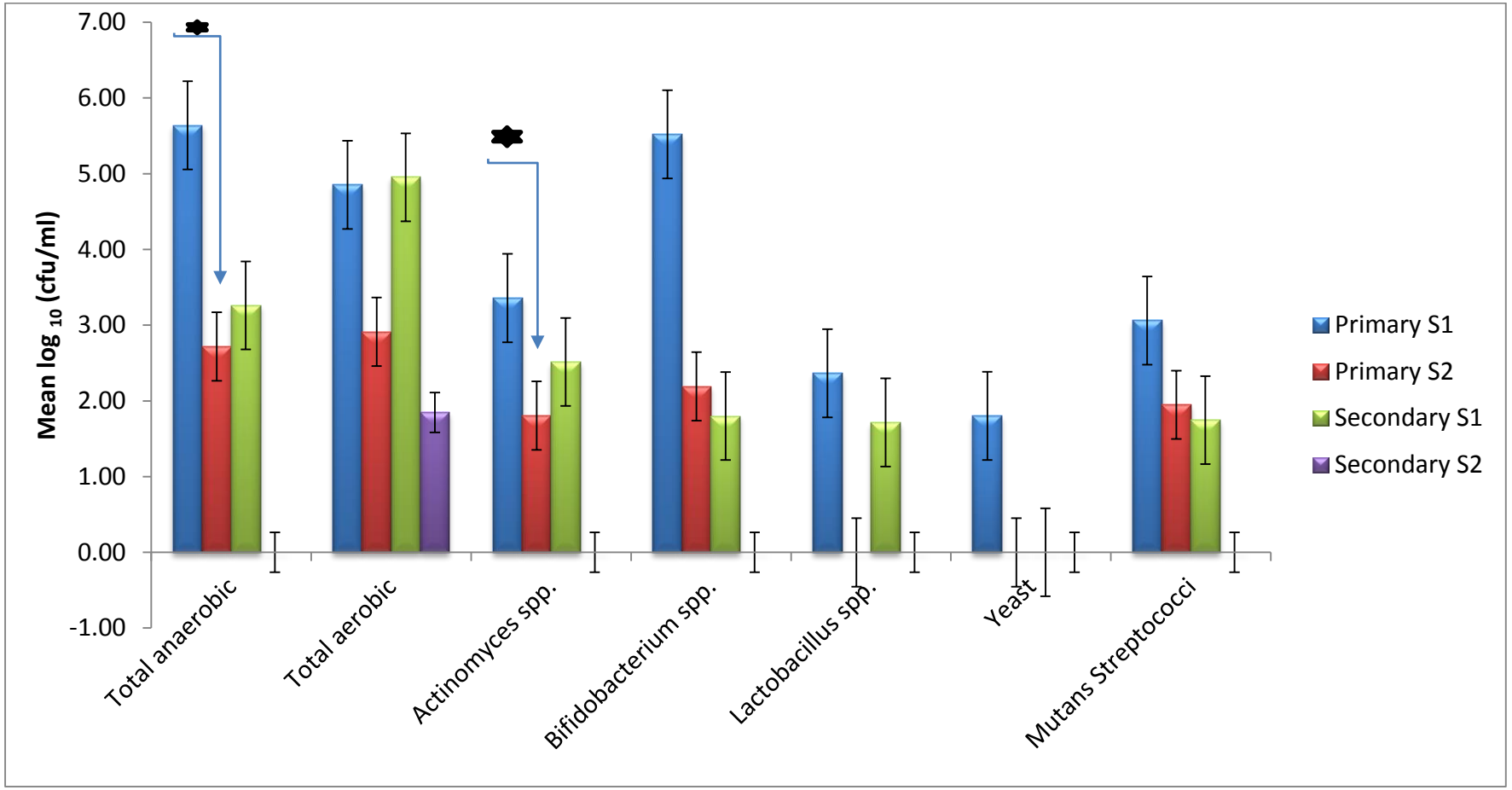

Figure 3.4 : Microbial viable counts (mean log_{10} cfu/ml ± SD) for all different samples categories. * $p<0.05$ compared with Primary S1. (NB groups with no column indicate that organisms were below the detection limit of the assay).

3.3 DNA sample preparation and library results

3.3.1 Extracted DNA concentrations

The DNA extracts (total of 38 samples comprising 19 S1 and 19 S2 samples), were quantified using the Quant-IT^{TM} PicoGreen extraction kit, as described in Section 2.9.7 (Table 3.12). In a final volume of 200 µl, the mean amount of DNA was 56.8ng (range= 1.2- 478.3, SD=94.3). S1 samples yielded more DNA than S2 samples with a mean of 88.7 ng (3.6- 478.3, 123.9) compared to 24.9 ng (1.2- 118.9, 27.6), respectively. The highest DNA concentration was observed in Primary S1 samples with a mean of 92.42 (3.6- 478.3, 138.0). DNA concentration details from individual cases are included in Table 3.13.

Table 3.12: Summary of the amount of extracted DNA in ng for all sample categories. (The total volume for each sample was 200 µl).

	Primary S1	Primary S2	Secondary S1	Secondary S2
Mean:	92.4	15.0	84.6	35.9
Standard deviation:	138.0	14.8	114.4	34.8
Maximum:	478.3	47.3	375.6	118.9
Minimum:	3.6	1.2	14.8	4.7

3.3.2 PCR and electrophoresis

In order to check that the polymerase chain reaction (PCR) procedures (Section 2.9.9) was successful, agarose gel electrophoresis (Section 2.9.10) was performed. The gel was visualised using the Bio-Rad Ultraviolet image system and the images indicate that vast majority of samples had been successfully amplified (Figure 3.5).

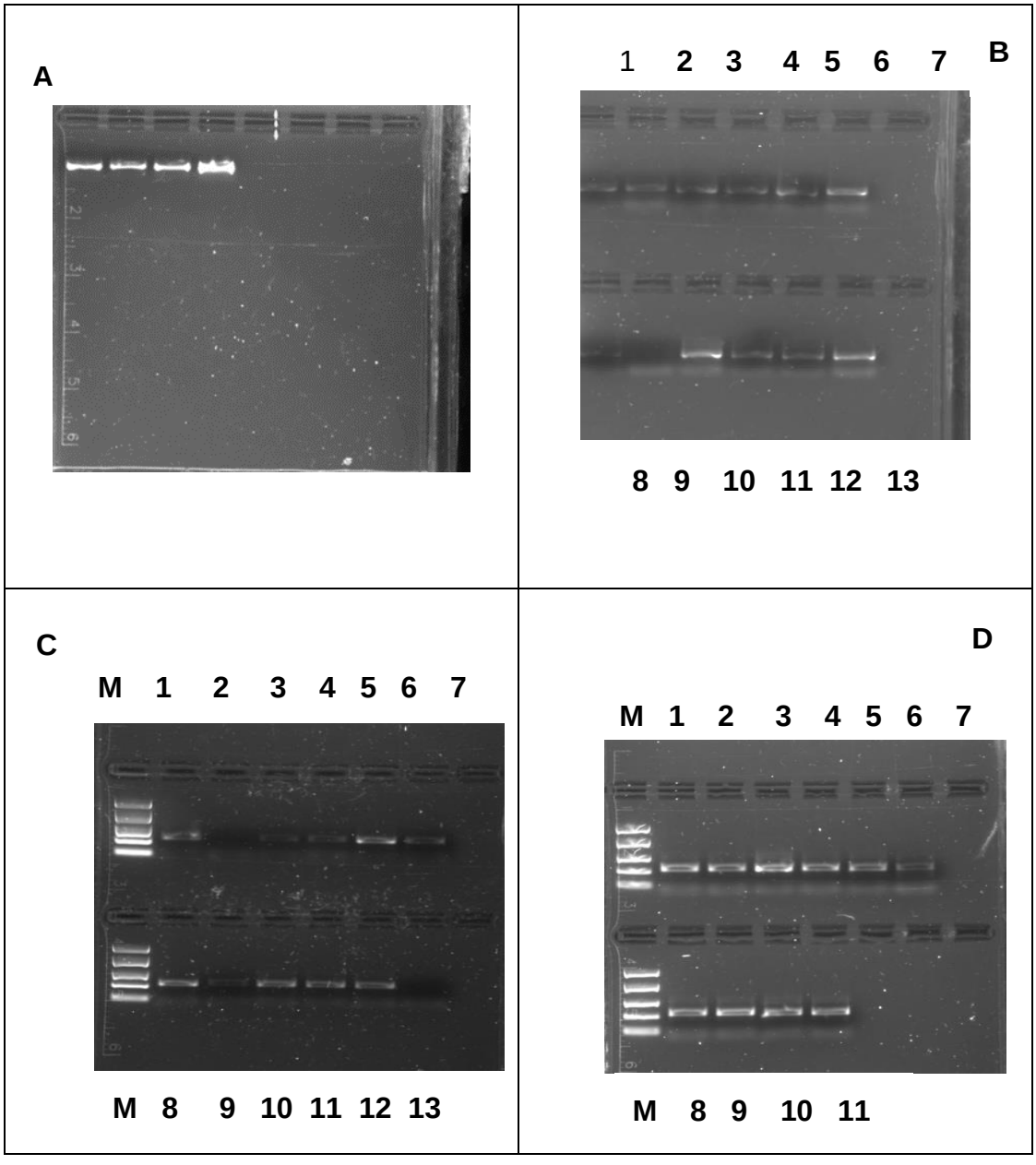

Figure 3.5: Agarose gel electrophoresis for amplified DNAs. A: the bottom row relates to the 4 DNA samples that migrated from the well (top row). In the other figures 2 rows were used. In C&D the marker (M) occupies the 1st column. Note that in B (lane 9) and C (lanes 2 and 13) refer to failed samples that were later discarded.

3.3.3 Library size verification:

To achieve the recommended overlap in the pair-end reads, as described in Chapter Two and discussed in Chapter Five, the expected size of the library DNA amplicon should ideally exceed 490 bp including the linker primers and adaptors. This was verified using TapeStation™. The results (Table 3.13) showed that most libraries achieved the recommended size with a mean of 549 bp (SD=103.2). Figure 3.6 shows the images from the TapeStation and Table 3.13 details library amplicon sizes for each sample. From the table note that library amplicon size generated in samples 011S2 and 017S2 were substantially short (126,127, respectively) and were discarded.

3.3.4 Amplicon concentration:

Amplicon concentrations were measured using Qubit™ as described in Section2.9.14. Table 3.13 shows amplicon concentrations for each sample. The mean library concentration was 31.1 ng/µl (SD=20).

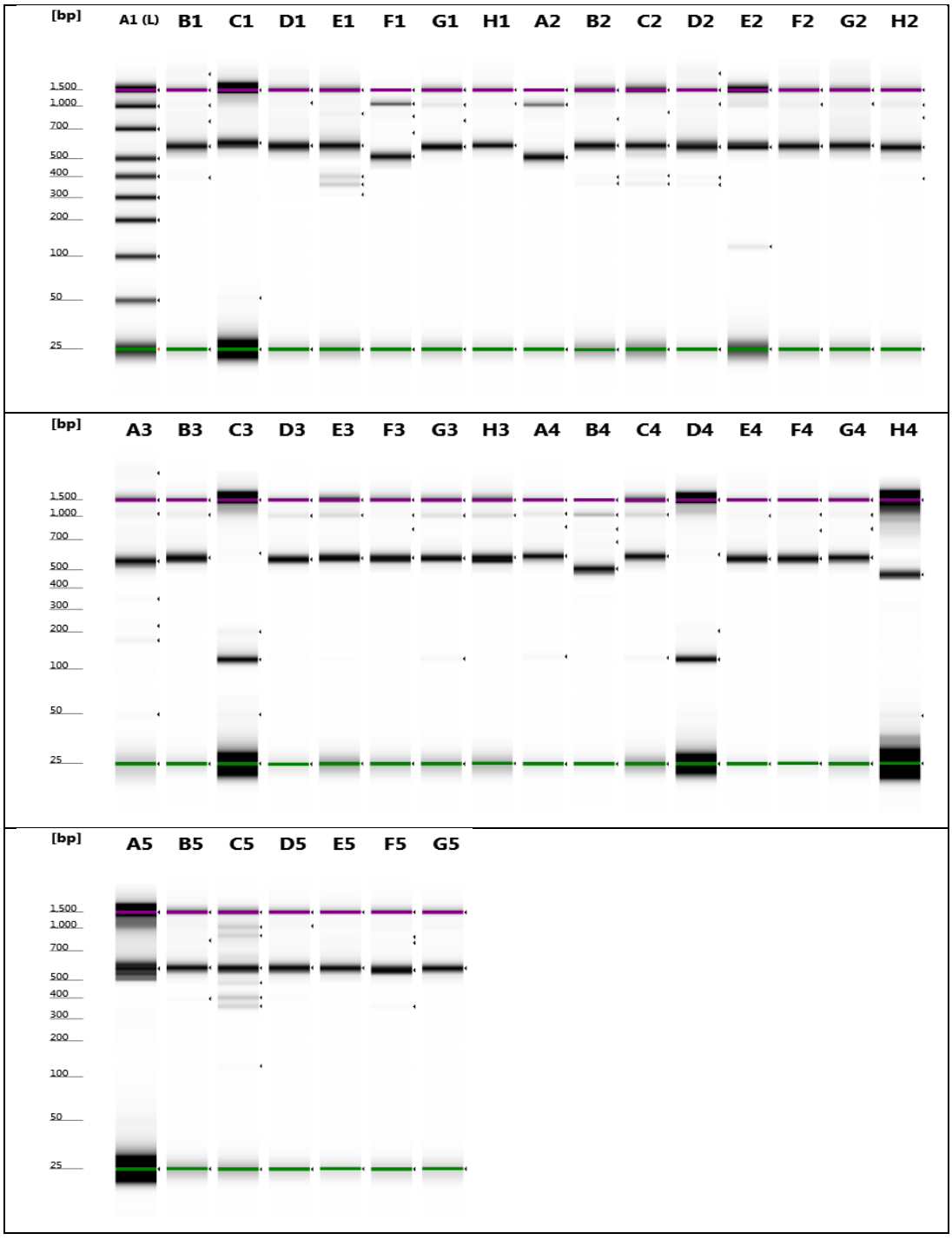

Figure 3.6: The images from TapeStation library size measurements for all samples. The A1 (L) well is occupied by the ladder and the samples are in the other wells. Note that the samples in wells C3 & D4 were substantially short in size and were later excluded.

Table 3.13: Data summary for the DNA extracts, library size measurement and library concentration in tubes prior normalisation. Note that samples shown in red bold italic relate to those excluded at various stages. Table continues overpage.

Sample ID	Extracted DNA concentration ng/200ml	Final library size in bp	Library concentration in tube ng/µl:
001 S1	49.28	583	39.2
001 S2	20.10	607	2.66
002 S1	121.05	588	33.9
002 S2	118.89	588	38.7
003 S1	74.16	514	34.3
003 S2	34.38	580	14.65
006 S1	43.07	589	37.1
006 S2	1.17	509	55.3
007 S1	375.60	590	18.5
007 S2	4.67	589	18.6
008 S1	90.94	580	88
008 S2	7.89	577	7.83
009 S1	85.07	584	52.9
009 S2	27.84	588	88.8
010 S1	39.62	576	55.4
010 S2	20.27	558	39.8
011 S1	35.72	581	35.5
011 S2	***46.69***	***126***	***14.9***
012 S1	14.78	571	37.2
012 S2	9.99	576	20.6
013 S1	478.30	579	32.5
013 S2	6.11	578	27.7
014 S1	64.43	582	26.8
014 S2	7.29	592	17.75
015 S1	44.92	506	41.8
015S2	39.39	591	10.8
017 S1	***22.43***	***127***	***0***
017S2	***45.08***	***572***	***51.3***
018S1	24.87	574	40.1
018S2	5.83	581	36.1
019S1	***3.63***	***473***	***2.14***
019S2	***6.83***	***579***	***3.21***
020 S1	30.28	585	18
020 S2	47.33	584	17.1

Sample ID	Extracted DNA concentration ng/200ml	Final library size in bp	Library concentration in tube ng/µl:
021 S1	21.86	586	29.4
021 S2	10.40	583	35
022 S1	65.27	576	36.3
022 S2	13.31	582	20.85

3.4 MiSeq sequencing data

3.4.1 Total sequence information

Before proceeding to the final data processing and analyses, preparation of the raw MiSeq data files was required in order to allow them to be properly handled by QIIME, with the aid of various software packages and programmes. These pre-processing steps included removing primer linker sequences, re-joining the reads and converting the file fastq to fasta file format. These sequences data were then further processed and analysed using QIIME, following the instructions provided in the QIIME website tutorials (QIIME).

In total, five clinical samples (011 S2, 017 S1&S2, 019 S1& 019S2) were discarded at various stages due to inadequate amplification, short library amplicon size and/or poor raw sequencing quality. The remaining 33 samples passed the quality control process by the MiSeq filtering and QIIME pipelines.

After filtering, a total count of 7,804,376 OTUs were assigned by QIIME using the Greengenes database(QIIME), based on a default uclust matching (0.97 similarity) with a number of observation of 34,957 (table density, fraction of non-zero values=0.209). The average OTUs count per sample was 236,496 (± 84,653) with a range of 88,757 – 541,982. (Table 3.14).

Also during OTUs *de novo* picking, uclust constructs a phylogenetic tree based on similarities and differences in *de novo* picked OTUs. The phylogenetic tree was constructed as a Newick formatted tree file (.tre) and was visualised using FigTree v1.4.2 program (QIIME). This tree was further used by QIIME for other analyses.

Table 3.14: OTUs count per sample. Note the number of OTUs in the S2 sample of subject 003 (Bold italic) is substantially more than its corresponding S1 sample. This appears to be an outlier.

	Sample ID	No of OTUs (S1)	No of OTUs (S2)
1	001	118722	101094
2	002	234077	140622
3	003	162666	***541982***
4	006	232654	255910
5	007	149610	264071
6	008	234512	88757
7	009	199041	152637
8	010	216061	199589
9	011	254896	Discarded
10	012	247238	225749
11	013	244332	233844
12	014	368848	188708
13	015	291464	273234
14	017	Discarded	Discarded
15	018	331011	272242
16	019	Discarded	Discarded
17	020	305616	184330
18	021	292914	223177
19	022	239934	334834

3.4.2 Taxonomical identification and abundance

3.4.2.1 Abundance

A total of ten bacterial phyla were assigned by the Greengenes database (Figure 3.7). At lower classification levels, 21 different bacterial classes, 35 orders, 87 families and 143 genera were represented in the root canal samples. About 5% of the sequences could not be assigned to phyla level.

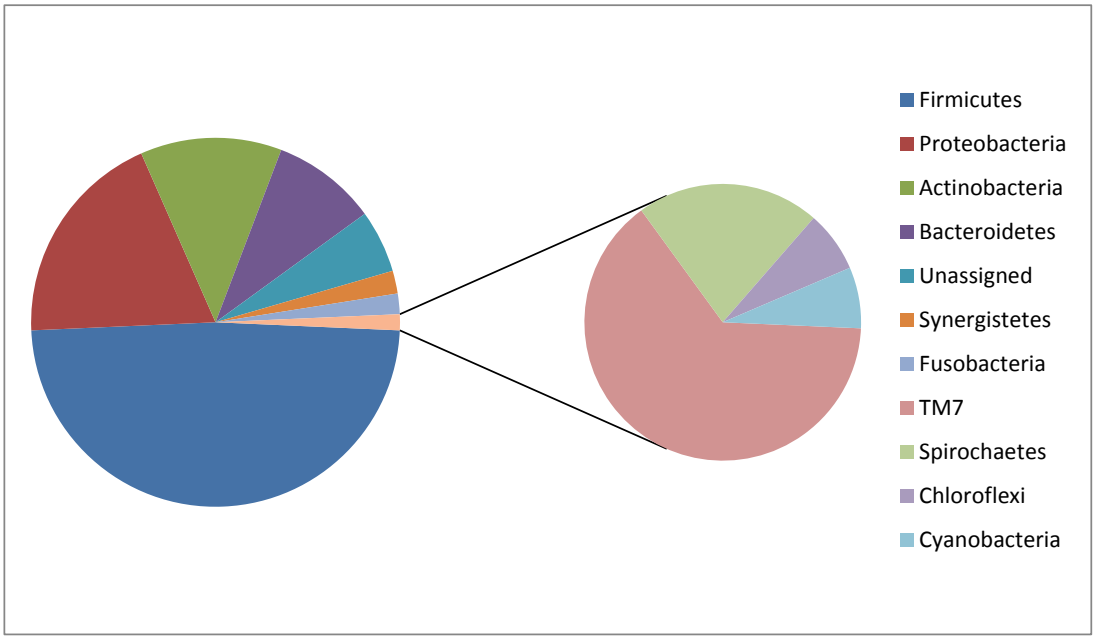

Figure 3.7: Overall abundance at phyla level in all samples.

Overall, the most abundant phyla were *Firmicutes* (48.6%), *Proteobacteria* (19.1%), *Actinobacteria* (12.4%), *Bacteroidetes* (9.2%), and *Synergistetes* (2.0%), *Fusobacteria* (1.8%) (Table 3.15). Each of the other 4 phyla (*Spirochetes*, *Cyanobacteria*, *Chloroflexi* and *TM7*) corresponded to less than 1% of the overall sequences.

The three most abundant phyla (*Firmicutes*, *Proteobacteria* and *Actinobacteria*) were in the same order of abundance and had similar percentages in primary and secondary infections. *Bacteroidetes* were present in about 10.7% in primary infection compared to 7.6% in secondary infections. The percentage of *Synergistetes* (3.4%) and *TM7* (1.2%) in secondary infections were relatively higher than in primary infections (0.6%, and 0.6%) respectively (Table 3.15 and Figure 9). Details according to sample and infection type are represented in Table 3.16.

Table 3.15: Abundance of phyla according to infection type presented as percentage.

Taxonomy- Phyla level	Total abundance	Primary	Secondary
Firmicutes	48.6%	49.6%	47.5%
Proteobacteria	19.1%	17.1%	21.2%
Actinobacteria	12.4%	13.4%	11.4%
Bacteroidetes	9.2%	10.7%	7.6%
Unassigned	5.5%	5.3%	5.8%
Synergistetes	2.0%	0.6%	3.4%
Fusobacteria	1.8%	1.9%	1.6%
TM7	0.9%	0.6%	1.2%
Spirochetes	0.3%	0.5%	0.1%
Chloroflexi	0.1%	0.1%	0.1%
Cyanobacteria	0.1%	0.0%	0.1%

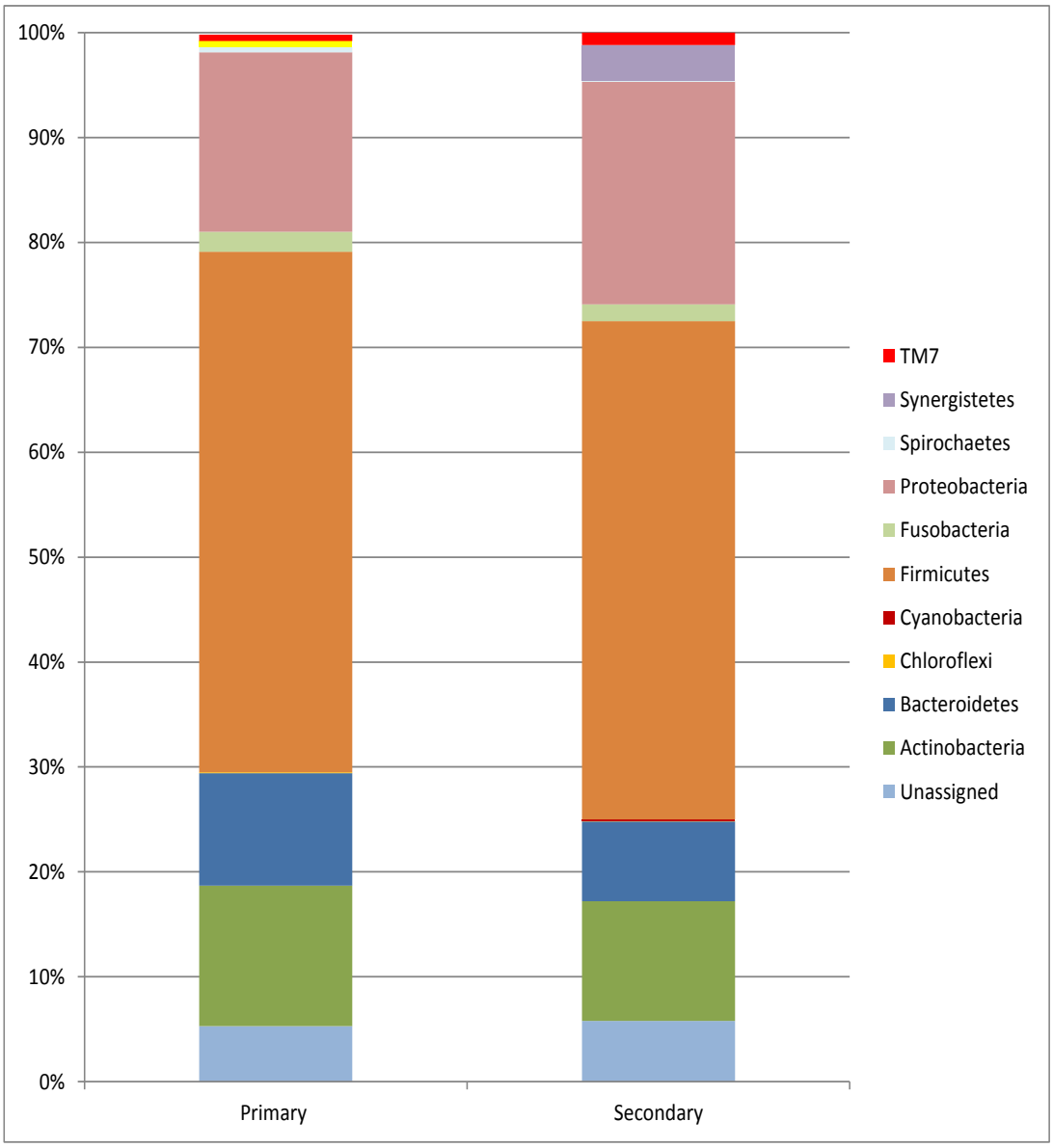

Figure 3.8: Phyla abundance according to the type of infection.

Table 3.16: Bacterial abundance at phyla level in all categories presented as percentages.

	Taxonomy-Phyla level	Primary S1	Primary S2	Secondary S1	Secondary S2
1	*Firmicutes*	50.5%	49.0%	46.2%	49.2%
2	*Actinobacteria*	14.4%	12.6%	11.9%	10.8%
3	*Bacteroidetes*	13.9%	8.3%	7.8%	7.3%
4	*Proteobacteria*	12.9%	20.3%	17.8%	25.5%
4	Unassigned	5.2%	5.3%	5.8%	5.7%
6	*Fusobacteria*	2.4%	1.6%	2.9%	0.0%
5	*TM7*	0.4%	0.8%	1.1%	1.3%
8	*Chloroflexi*	0.2%	0.0%	0.1%	0.0%
9	*Cyanobacteria*	0.0%	0.1%	0.2%	0.1%
10	*Spirochetes*	0.0%	1.0%	0.2%	0.0%
11	*Synergistetes*	0.0%	1.0%	6.0%	0.0%

With regards to genera, 143 different genera were found in the chronic root canal samples. Table 3.17 displays the most abundant genera and the full list of all 143 genera is in Appendix 7. Of these, only 26 were found at an abundance of >1% in the overall abundance. 60 % of the top ten genera belonged to the *Firmicutes* phyla.

Overall, the most abundant (all 33 samples included) were *Alkalibacterium* (7.8%), *Bacillaceae* (7.7%), *Actinotalea* (5.5%), *Paracoccus* (5.2%) and *Anaerobacillus* (4.8%). Interestingly, all of these were more abundant in Primary S2 and Secondary S2 samples than in their respective S1 samples. Other genera present in the top 30 included *Streptococcus, Enterococcus, Veillonella, Flavobacterium, Eubacterium, Selenomonas, Pseudomonas, Fusobacterium, Actinomyces, Prevotella and Bacillus* (Table 3.17 and Figure 3.9).

Table 3.17: The most abundant genera.

Genera or family level:	Overall abundance
Firmicutes;Alkalibacterium	7.8%
Firmicutes;Bacillaceae;other	7.7%
Actinobacteria;Actinotalea	5.5%
Proteobacteria;Paracoccus	5.2%
Firmicutes;Anaerobacillus	4.8%
Proteobacteria;Rhodobacteraceae;other	4.7%
Firmicutes;Lachnospiraceae;other	3.4%
Firmicutes;Streptococcus	3.0%
Firmicutes;Geosporobacter_Thermotalea	2.5%
Actinobacteria;Rothia	1.9%
Synergistetes;TG5	1.8%
Firmicutes;Enterococcaceae;g__Enterococcus	1.6%
Firmicutes;Veillonella	1.6%
Bacteroidetes;Porphyromonadaceae;Paludibacter	1.5%
Bacteroidetes;Cyclobacteriaceae;other	1.5%
Bacteroidetes;Flavobacterium	1.5%
Firmicutes;Pseudoramibacter_Eubacterium	1.5%
Firmicutes;Selenomonas	1.5%
Proteobacteria;g__Pseudomonas	1.5%
Firmicutes;Fusibacter	1.4%
Fusobacteria;Fusobacterium	1.4%
Firmicutes;Lactobacillales;other	1.3%
Firmicutes;Clostridiales;other	1.3%
Proteobacteria;Alkalimonas	1.2%
Actinobacteria;Actinomyces	1.1%
Bacteroidetes;Prevotella	1.1%
Firmicutes;Bacillus	1.1%

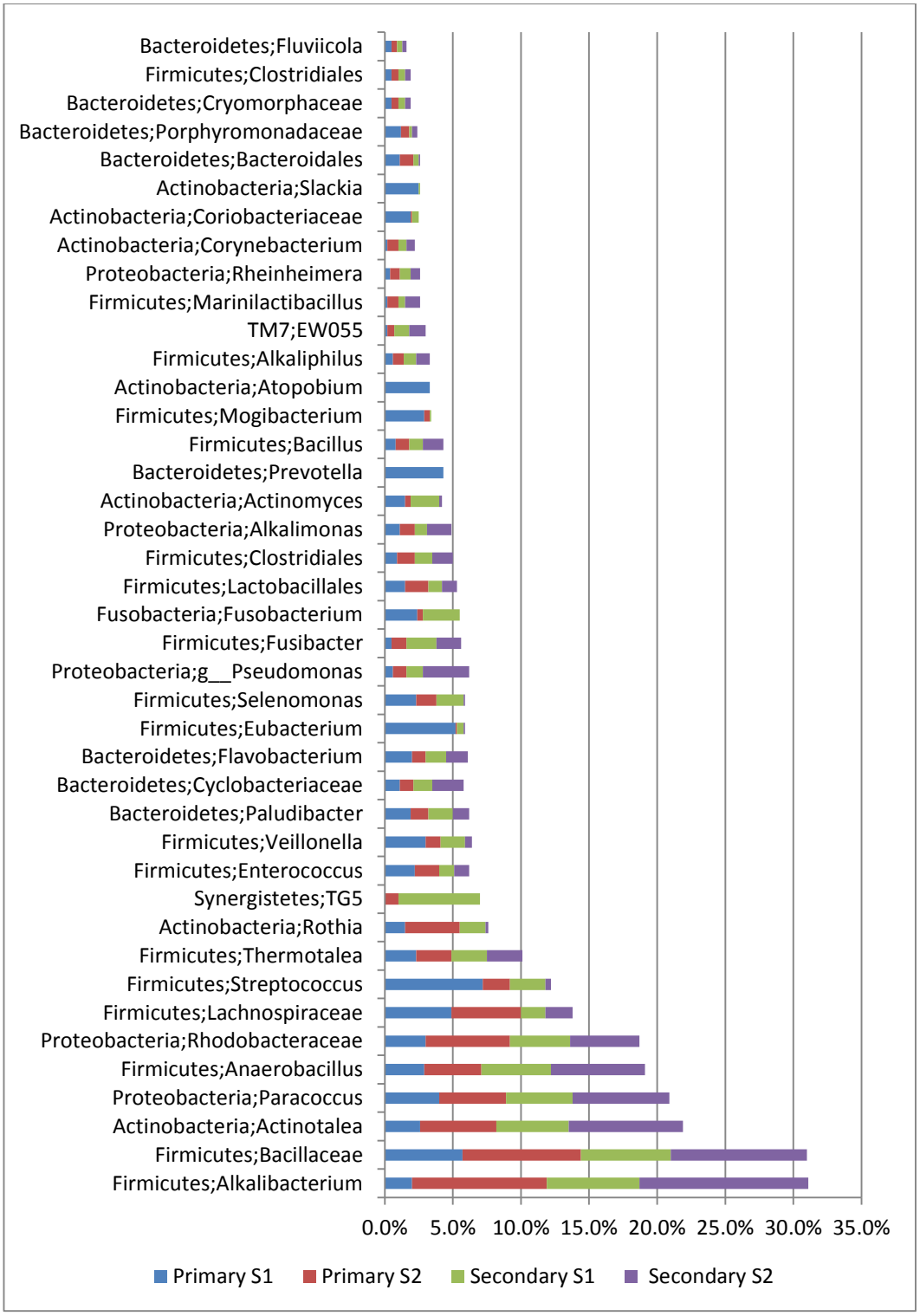

Figure 3.9: The most abundant genera or family level according to infection type (primary or secondary) and sample (S1 or S2).

Examination of only primary S1 samples (Table 3.19) revealed that the most dominant genera were *Streptococcus* (7.2%), *Bacillaceae* (5.7%) and *Eubacterium* (5.2%). Other genera such as *Prevotella* (4.3%), *Fusobacterium* (2.4%), *Enterococcus* (2.2%) and *Actinomyces* (1.5%) also appeared in the top 20 most abundant genera.

In secondary S1 samples (Table 3.19), *Alkalibacterium* (6.8%), *Bacillaceae* (6.6%) and *TG5* (6.0%) dominated. Interestingly, the former two genera were also the most dominant genera in Primary S2 and Secondary S2 samples. This may indicate survival properties of these genera. Other selected genera such as *Fusobacterium*, *Streptococcus*, *Actinomyces* and *Enterococcus* also appeared in the top 20 most dominant genera in secondary infections.

With regards to the number of species level OTUs in each canal (with at least 10 taxa per canal) (Table 3.18), the mean number (± SD) was 63 (±14.9) with range from 34 – 80 and 69.9 (± 12.0) with range from 50 - 87 in primary and secondary infections (S1) samples, respectively.

Table 3.18: Species-level OTUs per canal.

	Primary S1	**Primary S2**	**Secondary S1**	**Secondary S2**
Mean	63	57.4	69.9	66
Standard deviation	14.9	15.2	12.0	9.1
Maximum	80	80	87	77
Minimum	34	28	50	50

An examination of the heat map also indicated that there was a notable degree of inter-individual variation in both taxonomic composition and load (Figure 3.10).

Table 3.19: The most abundant genera in S1 samples only.

Genera or family level:	PriS1	*Genera or family level:*	SecS1
Firmicutes;Streptococcus	7.2%	***Firmicutes;Alkalibacterium***	6.8%
Firmicutes;Bacillaceae	5.7%	***Firmicutes;Bacillaceae***	6.6%
Firmicutes;Eubacterium	5.2%	***Synergistetes;TG5***	6.0%
Firmicutes;Lachnospiraceae;	4.9%	***Actinobacteria;Actinotalea***	5.3%
Bacteroidetes;Prevotella	4.3%	***Firmicutes;Anaerobacillus***	5.1%
Proteobacteria;Paracoccus	4.0%	***Proteobacteria;Paracoccus***	4.9%
Actinobacteria;Atopobium	3.3%	***Proteobacteria;Rhodobacteraceae***	4.4%
Proteobacteria;Rhodobacteraceae	3.0%	***Fusobacteria;Fusobacterium***	2.7%
Firmicutes;Veillonella	3.0%	***Firmicutes;Streptococcus***	2.6%
Firmicutes;Anaerobacillus	2.9%	***Firmicutes:;Thermotalea***	2.6%
Firmicutes;Mogibacterium	2.9%	***Firmicutes;Fusibacter***	2.2%
Actinobacteria;Actinotalea	2.6%	***Actinobacteria;Actinomyces***	2.1%
Actinobacteria;Slackia	2.5%	***Firmicutes;Selenomonas***	2.0%
Fusobacteria;Fusobacterium	2.4%	***Actinobacteria;Rothia***	1.9%
Firmicutes;Thermotalea	2.3%	***Firmicutes;Lachnospiraceae***	1.8%
Firmicutes;Selenomonas	2.3%	***Firmicutes;Veillonella***	1.8%
Firmicutes;Enterococcus	2.2%	***Bacteroidetes;Paludibacter***	1.8%
Firmicutes;Alkalibacterium	2.0%	***Bacteroidetes;Flavobacterium***	1.5%
Bacteroidetes;Flavobacterium	2.0%	***Bacteroidetes;Cyclobacteriaceae***	1.4%
Bacteroidetes;Paludibacter	1.9%	***Firmicutes;Clostridiales***	1.3%
Actinobacteria;Coriobacteriaceae	1.9%	***Proteobacteria;Pseudomonas***	1.2%
Actinobacteria;Rothia	1.5%	***Firmicutes;Mogibacteriaceae***	1.2%
Firmicutes;Lactobacillales;other	1.5%	***Firmicutes;Enterococcus***	1.1%
Actinobacteria;Actinomyces	1.5%	***TM7;EW055***	1.1%
Bacteroidetes;Porphyromonadaceae	1.2%	***Firmicutes;Lactobacillales***	1.0%
Bacteroidetes;Cyclobacteriaceae	1.1%	***Firmicutes;Bacillus***	1.0%
Proteobacteria;Alkalimonas	1.1%	***Proteobacteria;Alkalimonas***	0.9%
Bacteroidetes;Bacteroidales	1.1%	***Firmicutes;Alkaliphilus***	0.9%
Firmicutes;Clostridiales;other	0.9%	***Proteobacteria;Rheinheimera***	0.8%
Firmicutes;Bacillus	0.8%	***Proteobacteria;Neisseria***	0.8%
Firmicutes;Lactobacillus	0.8%	***Firmicutes;Megasphaera***	0.8%

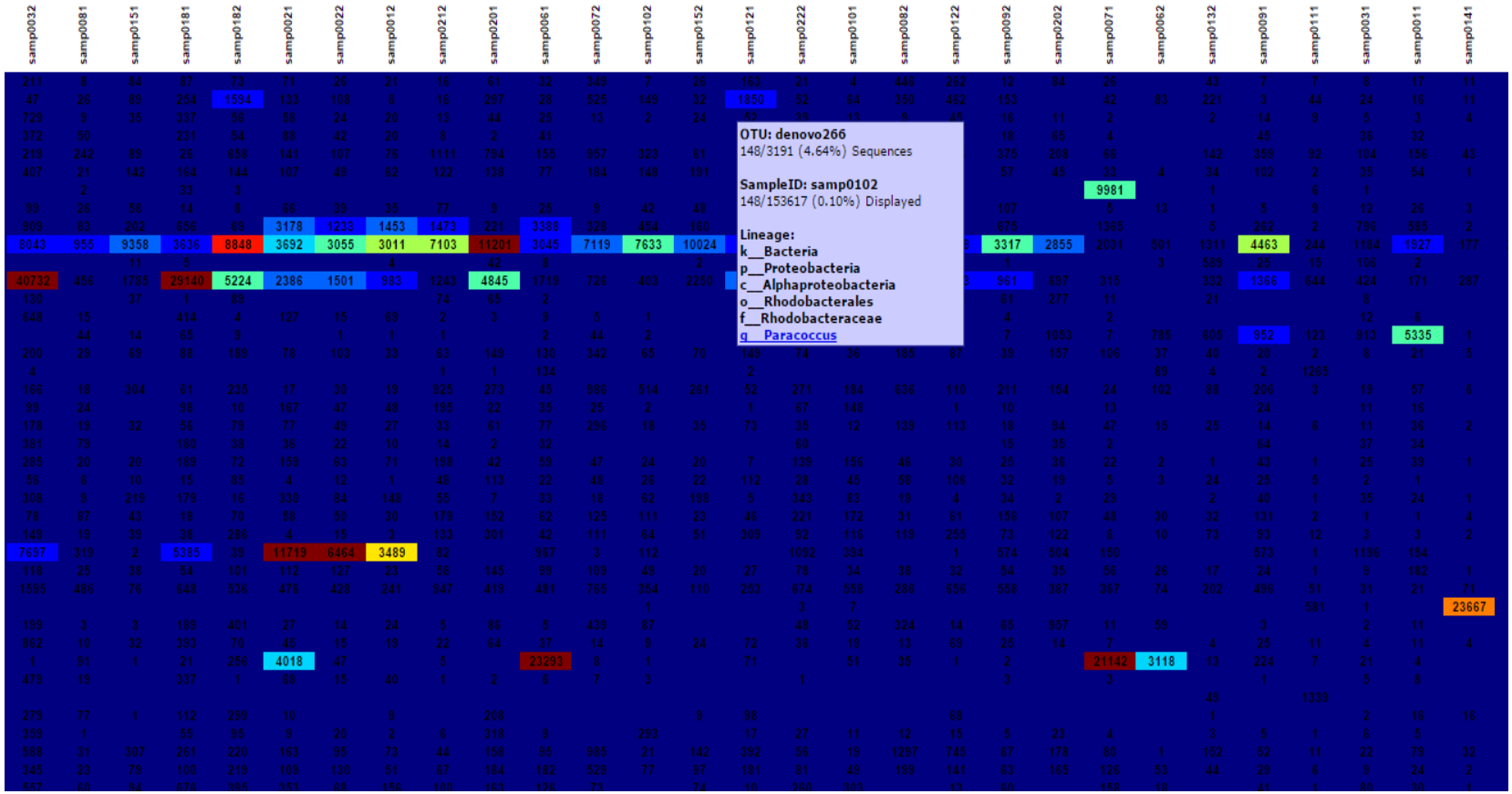

Figure 3.10: Screenshot of the heatmap.

3.4.3 Diversity analyses

3.4.3.1 Alpha diversity

In order to assess the diversity within the samples, alpha rarefaction diversity was calculated and displayed using the QIIME pipeline (Figure 3.11, Figure 3.12, Figure 3.13). A steep slope in the rarefaction curve indicates that further species richness can be revealed by additional sampling. If, however, the curve reaches an asymptote, this means that increased sampling efforts are likely to yield no new or only a few additional species (Siqueira *et al.*, 2011).

The diversity analyses of observed species, according to infection type (primary or secondary), sample IDs and sample type (S1 or S2), resulted in a similar pattern wherein the majority of curves are starting to plateau nearing the left side which suggests that further sampling may yield only limited additional species.

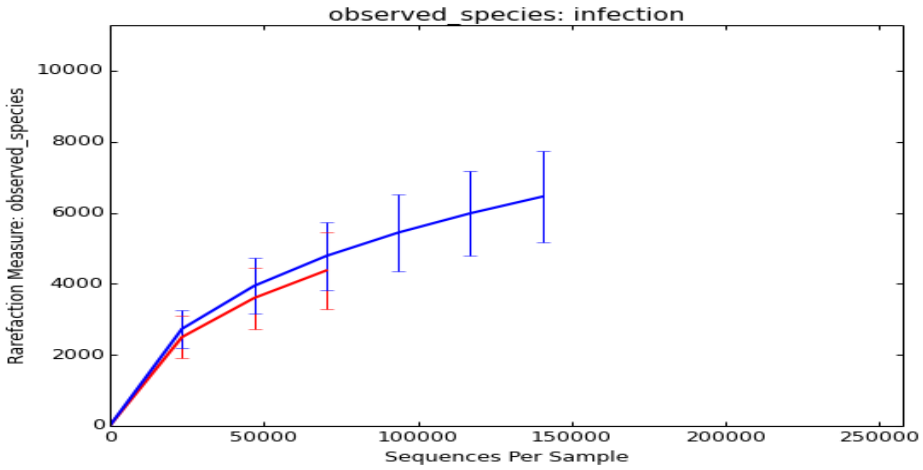

Figure 3.11: Alpha diversity rarefaction curve of observed species (at 0.3 identity cut off) in primary (red line) and secondary (blue line) infections. The curve suggests higher observed species diversity in secondary than primary infections. As the curves began to level off, this indicates that the diversity has almost been explored for both secondary and primary infections. Note that if the lines do not extend all the way to the right end of the x-axis, means that at least one of the samples in that category does not have sufficient sequences.

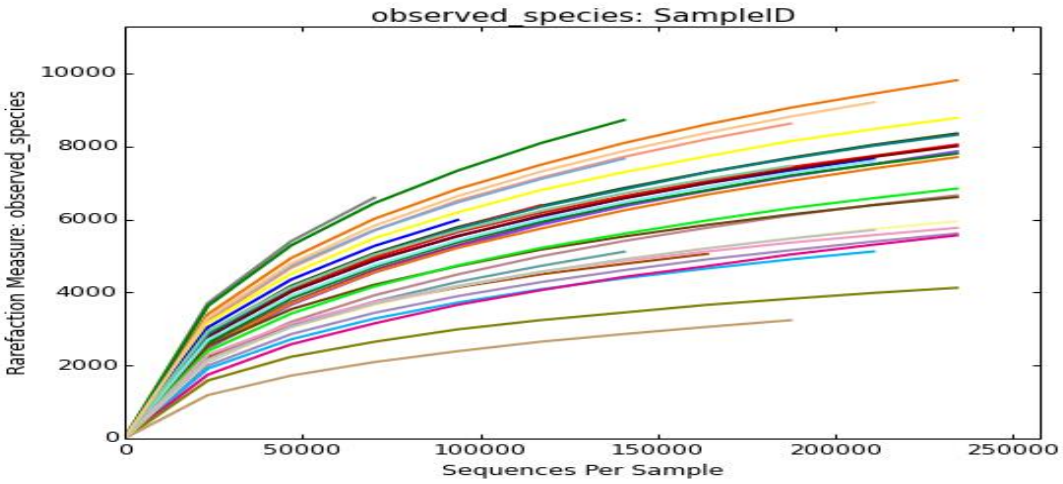

Figure 3.12: Alpha diversity rarefaction curve of observed species (at 0.3 identity cut off) in all samples. Most curves are starting to plateau but not completely which indicates that the diversity was nearly explored in most samples.

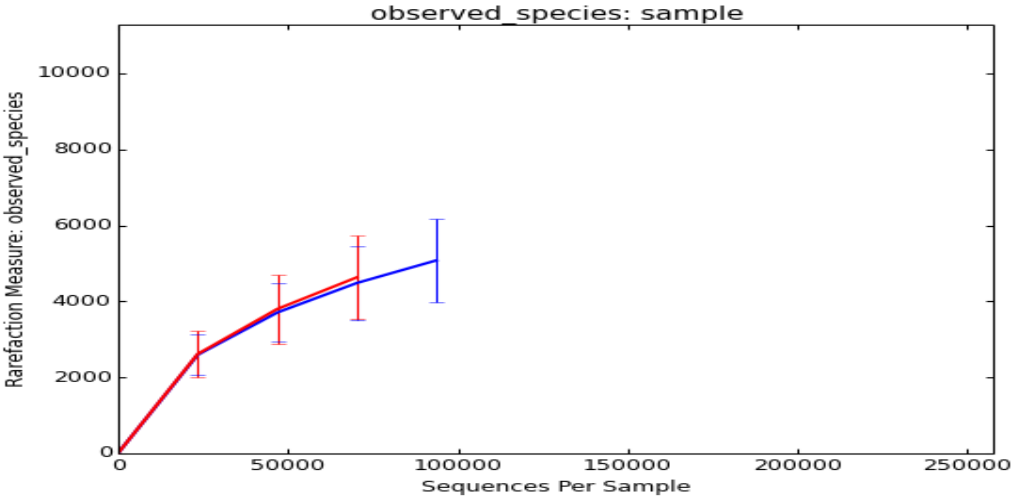

Figure 3.13: Alpha diversity rarefaction curve of observed species (at 0.3 identity cut off) in S1 (red line) and S2 (blue line).

3.4.3.2 **Beta diversity**

To assess the diversity between the samples, Beta diversity analysis was utilised using QIIME. A 3D PCoA based on weighted uniFrac plot was produced with Principal Coordinates PC1, PC2 and PC3 explained at 26%, 15% and 11%, respectively, of the overall variance among the samples.

In Figure 3.14, the plots were analysed according to:

- Sample number.
- Infection type and sample.
- Infection type, respectively, from top to bottom.

The general pattern shows short distances and some clustering of most samples, which may suggest that the samples had relatively similar microbial diversities.

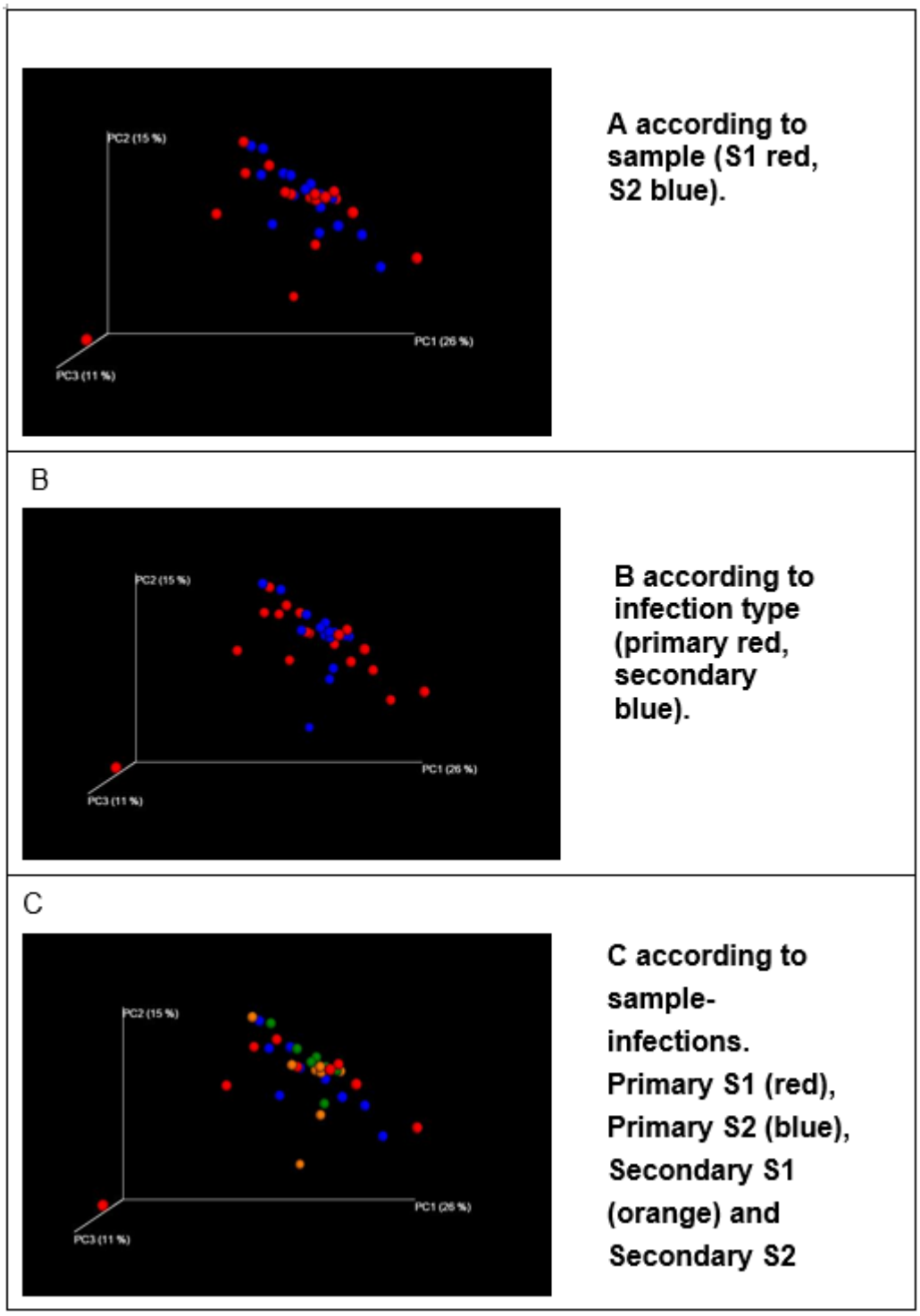

Figure 3.14: Beta diversity weighted uniFrac PCoA plots. Note the overall clustering and short distances between the dots apart from a few outliers.

3.4.4 Jackknifing analysis

Jackknifing analysis is a statistical test used to estimate the bias and standard error (variance) and so here it basically measures the robustness of the sequencing effort. In jackknifing analysis, a small number of sequencing data were chosen at random from each sample, and the resulting tree from this subset of data was compared with the tree representing the entire data set. This produced a 3D PCoA based on weighted uniFrac calculator with Principal Coordinates PC1, PC2 and PC3 explained at 26%, 15% and 11% of the overall variance among the samples, respectively (Figure 3.15). In addition, during this process, master and jackknifed trees were also produced (Figure 3.16 and Figure 3.17).

The general pattern of clustering of the vast majority of the examined samples in all three plots, analysed according to sample number, infection type and sample & infection type, respectively, from top to bottom as shown in Figure 3.15, indicates the sequencing effort used in this study was efficient and robust (QIIME).

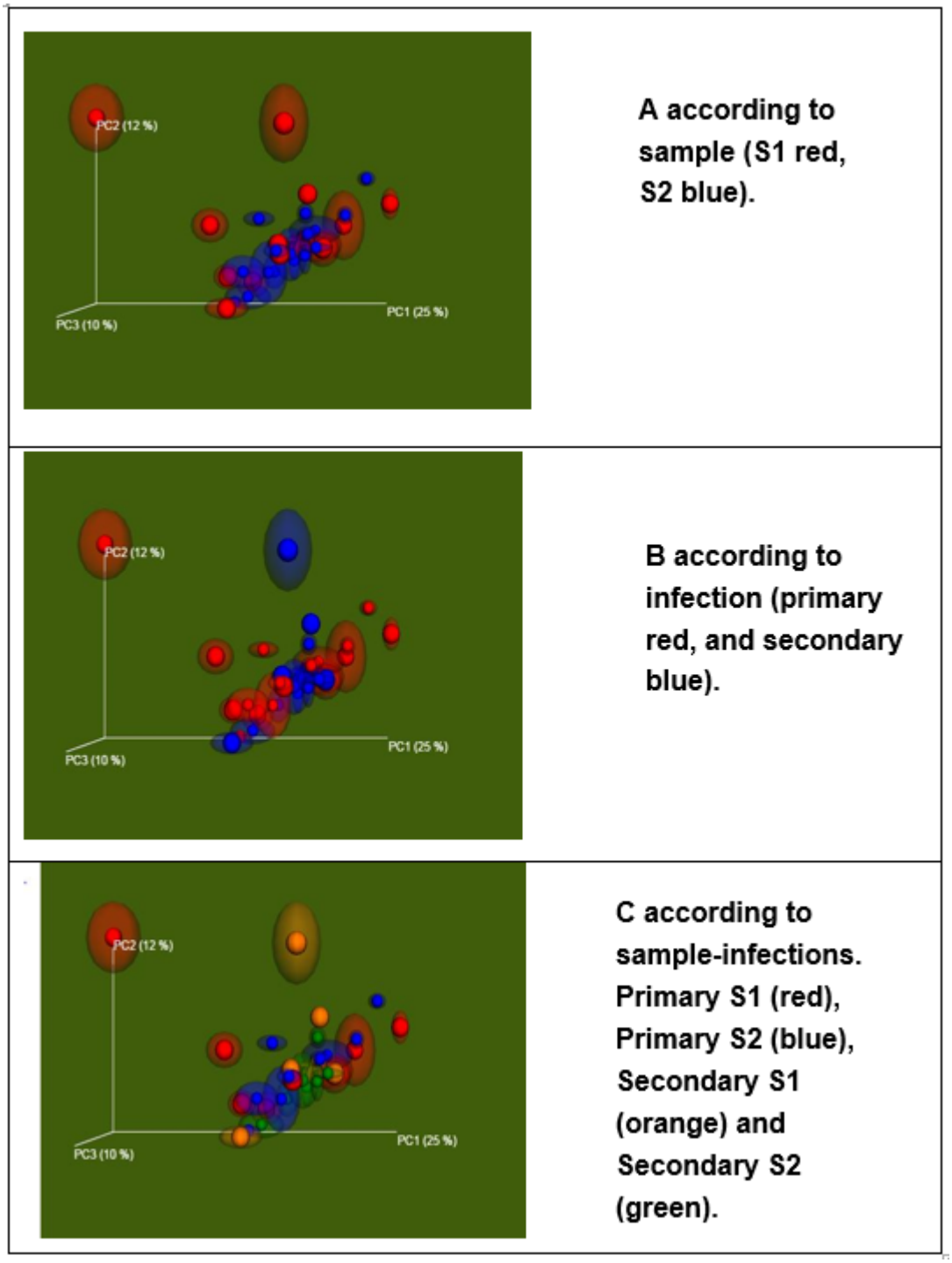

Figure 3.15: Jackknife weighted uniFrac PCoA plots. Note the overall clustering of dots apart from a few outliers.

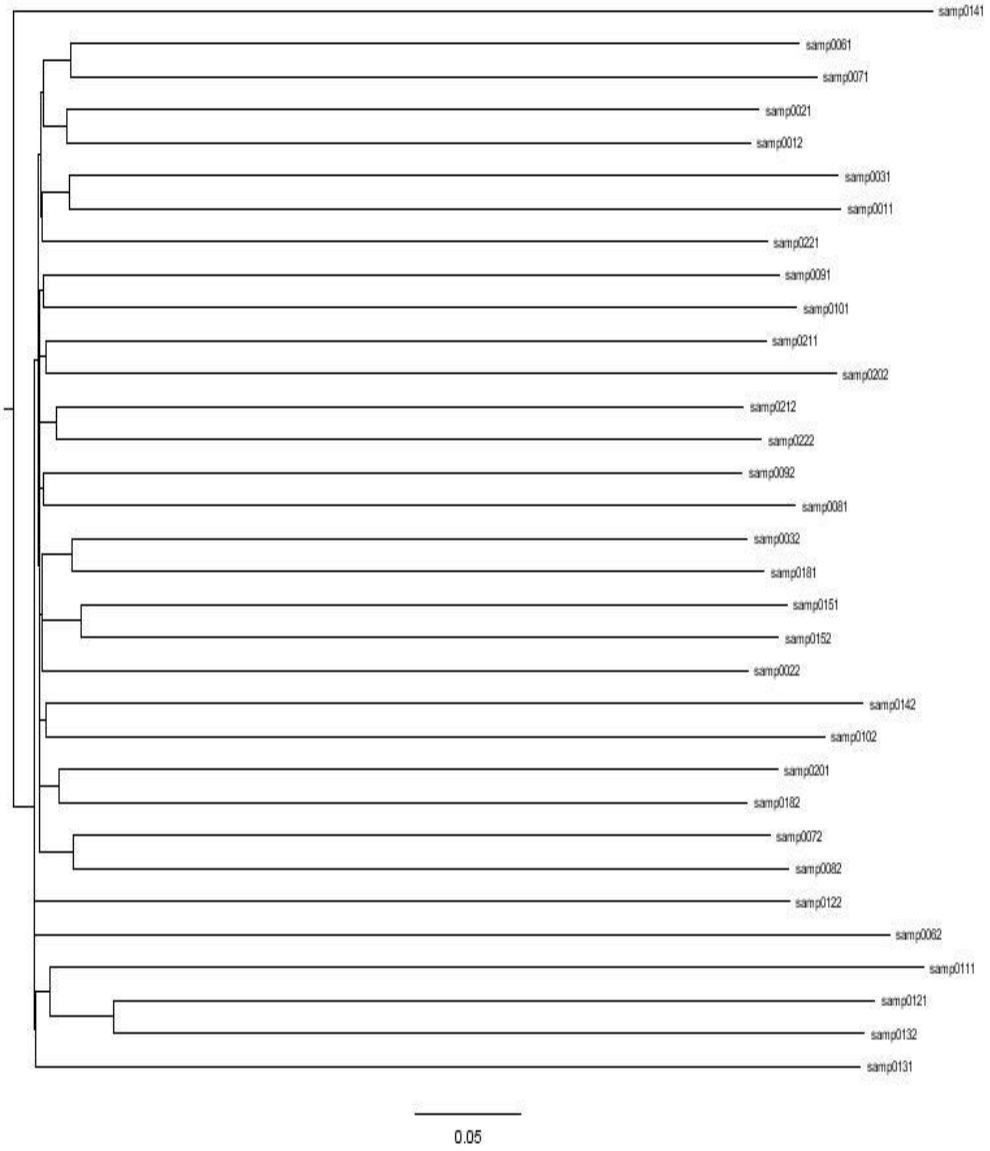

Figure 3.16: The master tree produced by jackknifing analysis.

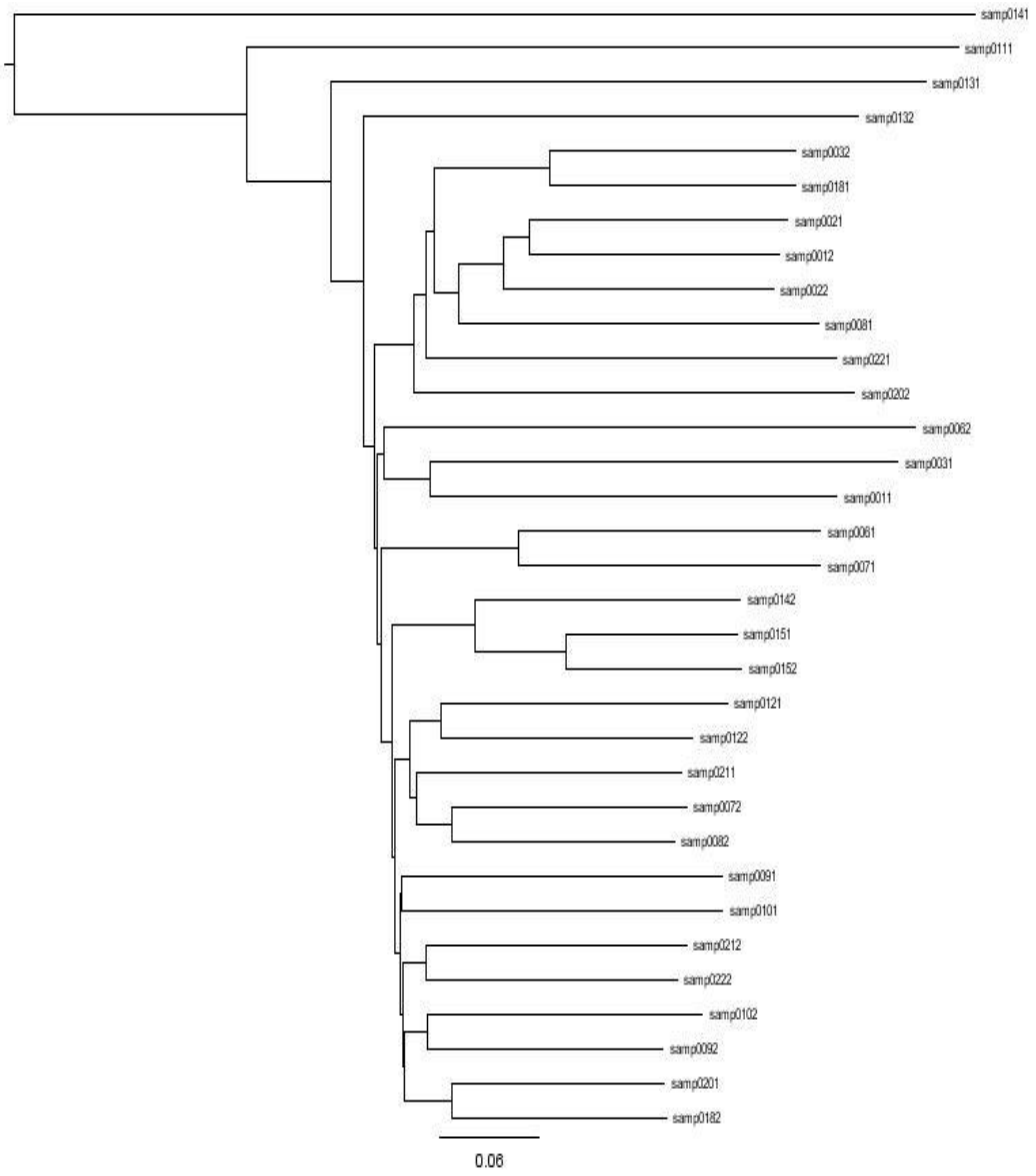

Figure 3.17: Jackknifed tree produced by the jackknifing analyses.

3.4.5 Microbial associations between samples

Using the OTUs table generated during the processing of data, scripts were constructed in QIIME to compute the shared OTUs at species level between the various samples. The output, generated as a text file, was then rearranged in Microsoft excel worksheet to compare primary and secondary S1 samples as described below.

3.4.5.1 Shared OTUs between Primary and secondary infection samples (S1)

Comparisons were drawn between primary and secondary S1 samples (Table 3.20). The mean percentages of shared OTUs between primary and secondary S1 samples were approximately 40% with a range from 18 % - 60%. Consequently, it is possible to state that less than half of the number of OTUS were shared between the samples.

Table 3.20: Shared OTUs (at species level) between S1 samples from primary (red) and S1 samples from secondary (black) infection. Figures above the blank diagonal line relate to the number of shared OTUs and the percentages are below the blank line.

	S0221	S0211	S0131	S0081	S0151	S0181	S0021	S0201	S0061	S0121	S0101	S0071	S0091	S0111	S0031	S0011	S0141
S0221		4408	2554	3585	3806	4574	4683	4139	3896	2941	4244	3099	3861	2068	3193	3100	2186
S0211	57%		2869	4096	4768	5228	5016	5251	4413	3662	5012	3399	4808	2166	3285	3509	1761
S0131	33%	33%		3051	2890	3057	3005	3031	2549	2748	2886	2105	3034	2196	2543	2558	1596
S0081	46%	47%	54%		3765	4238	4343	4031	3679	2996	4111	2821	4006	2039	3123	3113	1663
S0151	49%	55%	51%	51%		4860	4562	4980	3830	3616	4551	2868	4453	2318	3125	3204	1693
S0181	59%	60%	54%	58%	57%		5856	5140	4446	3467	5091	3346	4884	2283	3531	3656	1714
S0021	60%	58%	53%	59%	53%	62%		4949	4927	3321	4900	3627	4598	2256	3634	3697	1729
S0201	53%	60%	54%	55%	58%	55%	50%		4479	4249	4866	3311	4659	2397	3194	3421	1768
S0061	50%	51%	45%	50%	45%	47%	50%	49%		3199	4315	3543	4301	2244	3317	3288	1588
S0121	38%	42%	49%	41%	42%	37%	34%	46%	41%		3594	2473	3473	2445	2593	2515	1721
S0101	55%	58%	51%	56%	53%	54%	50%	53%	55%	59%		3265	5240	2489	3659	3376	1631
S0071	40%	39%	37%	38%	34%	36%	37%	36%	45%	41%	35%		3093	1675	2516	2628	1354
S0091	50%	55%	54%	54%	52%	52%	47%	51%	54%	57%	56%	59%		2331	3719	3514	1590
S0111	27%	25%	39%	28%	27%	24%	23%	26%	28%	40%	27%	32%	26%		2031	1773	1432
S0031	41%	38%	45%	42%	37%	38%	37%	35%	42%	43%	39%	48%	42%	35%		3359	1554
S0011	40%	40%	45%	42%	37%	39%	38%	37%	42%	41%	36%	50%	40%	31%	50%		1404
S0141	28%	20%	28%	23%	20%	18%	18%	19%	20%	28%	18%	26%	18%	25%	23%	21%	

3.4.6 Effect of root canal treatment on OTUs numbers

The number of OTUs detected in each sample, as shown in Table 3.14, was used to compare the effect of treatment on primary and secondary infections. Overall, the average number (± SD) of OTUs detected in S1 samples was 242,564 (± 64,250) OTUs/sample compared to 209,253 (± 68,323) in S2 samples. Table 3.21 provides summaries for each category. The effect is also illustrated using box and whisker plot (Figure 3.18).

Table 3.21: Number of OTUs detected from samples according to the category.

	Number of OTUs			
	Primary S1	**Primary S2**	**Secondary S1**	**Secondary S2**
Mean	248699	207465	235663	211297
Standard deviation	78502	84308	47851	50899
Minimum	118722	88757	149610	140622
Maximum	368848	334834	292914	273234

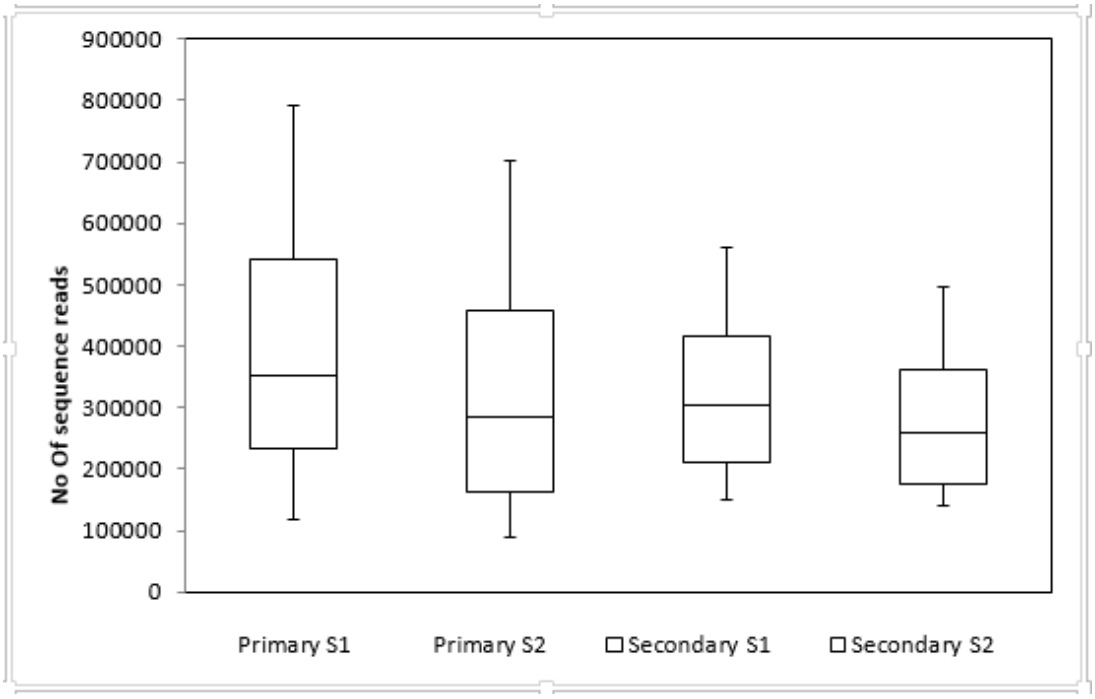

Figure 3.18: Box and whisker plot comparing the number of sequencing reads from all four categories. The top and bottom boundaries from the boxes show the 75th and 25th percentile and the end of the whiskers show the maximum and minimum values. The line within the box represents the median values (50th percentile). Note this plot was constructed after removing one outlier from primary S2 category.

Chapter 4 Conclusions

- High throughput sequencing on MiSeq managed to explore the diverse endodontic microbiology in particular low abundance species.

- Root canal infections are more diverse than previously demonstrated by culture and most molecular techniques.

- Contrary to previous indications, chronic secondary infections have similar diversity to chronic primary infections.

- Some species seem to be able to survive the standard root canal disinfection procedure.

- Strict aseptic procedures, more potent disinfection technique, irrigation and washing time may be recommended.

- Further studies are essential to further explore the diversity as well as the understanding the ecology within the infected root canal and apical regions.

Chapter 5 Discussion

5.1 The study design and methodology

5.1.1 Study population

The participants of this study consisted of patients with clinical and/or radiographic evidence of root canal infections. An important inclusion criterion was tooth restorability which is a requirement for root canal treatment (European Society of Endodontology, 2006). The opportunity to treat a restorable tooth and complete the treatment allowed us to obtain post chemo-mechanical preparation samples. Another important inclusion requirement was the absence of advanced or active periodontal disease. This is because of the known intimate relationship and communications between the pulp and periodontal space. The possible contamination of the root canal via periodontal microorganisms in advanced periodontal disease has been demonstrated previously (Kipioti *et al.*, 1984; Kobayashi *et al.*, 1990).

Routine root canal treatment is not contraindicated for most immunocompromised patients. However, because of possible health reasons, altered host response to treatment and the possible need for antibiotics, these patients were excluded from the study. Similarly, patients who were pregnant and those under 18 years of age, were excluded due to patient convenience and because it was ethically less acceptable to include such vulnerable individuals in a study of this type.

With regards to use of systemic antibiotics, over the years there has been a controversy surrounding this subject. Some researchers reported reduction of some species after the use of various forms of systemic antibiotic therapy (Haapasalo *et al.*, 1983; Yamamoto *et al.*, 1989). More recently, however, questions about the actual benefit of antibiotics for most endodontic cases, and concerns over bacterial resistance, have encouraged many authors to advocate strict and careful use of systemic antibiotics (Longman *et al.*, 2000). For this study, although the intra-radicular effect of antibiotics, should be extremely minimal, if any, especially in areas devoid of blood supply, such as the necrotic pulp, no risk, however minimal, was taken by including patients with recent antibiotic use.

Teeth with posts were also excluded from the study for several reasons: they would need to be removed to complete the root canal retreatment, and the removal procedures usually prolong the treatment. The removal procedures also bring higher risks of a number of complications, such as root or post fracture and perforation. In addition, in these cases it can be more difficult to ensure complete seal between appointments and in case of the provision of a temporary post, the additional surfaces of the post may even provide another challenge with sterilisation, canal contamination and act as a potential platform for microbial growth. Other exclusion cases included dental anomalies such as dilacerations, evidence of canal calcifications and severe root curvature because they present a huge challenge to microbiological sampling with paper points.

5.1.2 Sample size

The sample size of this pilot study was determined following statistical advice by a qualified biostatistician at the Centre of Epidemiology and Biostatistics, University of Leeds.

As mentioned previously, none of the Illumina technologies sequencing tools had been used to investigate endodontic microbiology at the start of this project (July 2012), although at that time some other human body meta-genomic studies were published. There are significant differences in the anatomy, ecosystem, infection nature and disease pathogenesis when comparing the root canal system and other body sites. Consequently, these studies were not appropriate to use for statistical calculation of a sample size and hence a decision was made to conduct a pilot study. There were, however, three studies that used a similar NGS technique in the form of pyrosequencing (Li *et al.*, 2010; Siqueira *et al.*, 2011; Santos *et al.*, 2011) and the sample sizes used were 7, 10 and 17 participants. The usual pilot study with a sample size of 30 does not apply here because our observed outcome was not expected to be normally distributed data. Therefore, based on this, previous literature and the time available for patient recruitment and sampling, we proposed to recruit 20 participants with an expected dropout rate of <15 %.

5.1.3 Microbiological sampling methodology

To investigate the microbiota of root canal infections in its natural habitat, the most common approaches are:

- Nonsurgical endodontic approach.
- Surgical endodontic approach, or
- The use of freshly extracted teeth.

The selection of a specific approach or methodology is determined by the primary and secondary questions being posed and this in turn generally dictates the microbiological sampling method.

A non-surgical approach during ortho-grade root canal treatment provides access to the root canal space through the clinical crown. One important advantage of this method is that the crown can be properly isolated from the oral environment using rubber dam, clamp and sealing agents which allow for superior operating site decontamination and reduce the likelihood of sample contamination. Also, using this method, more than one sample can be obtained on different occasions to, for example, assess disease changes and host response. Paper points are the most traditional and widely used method to collect samples from within the root canal space (Fouad, 2009). Sometimes an endodontic file is used to break up the biofilm and the file may be included in the sample, as was done in this study. Moller performed several comprehensive methodological experiments in this regard, the majority of which are still valid (Moller, 1966). A major drawback of using paper points, however, is that it does not permit the distinction of the root canal portion that is sampled (apical, middle, coronal), nor it is able to access hard to reach areas, such as isthmus and ramifications, which can also harbour bacteria (Siqueira *et al.*, 2010).

A surgical endodontic approach is used to gain direct access to the apical region through the soft and hard tissues. This constitutes an advantage over the use of conventional paper points as it gives direct access to the anatomically complex root apex as well as the enclosed apical lesion, allowing for a comprehensive analysis of this vitally important area, which is not possible via the nonsurgical approach. A historic accusation of this approach is concerned with the possible contamination of the periapical lesion during the reflection of the mucosal flap and microbial sampling (Fouad, 2009). However,

Sunde *et al.* (2000) surgically examined 30 root filled teeth with apical pathosis. They concluded that when care is taken during disinfection procedures, site contamination appeared to be very minor. Another limitation is that this approach can only be appropriate for selective analysis of apical infections. Also, because the revision of surgical approach is only indicated for limited cases, multiple samples cannot be normally obtained.

Unhindered by anatomical constraints, extraction of teeth provides direct access to the root canal and root surface. Sectioning of extracted teeth and analysing cryogenically-ground samples has a big advantage over the other two approaches as it allows the investigation of various, distinct segments of the root canal systems (Rocas *et al.*, 2010). However, contamination of the root from the periodontium and oral environment during tooth extraction is always a concern. This approach does not allow for the opportunity to longitudinally study disease progression.

Taken together, there is no one perfect approach for endodontic microbiological sampling and no method is without its inherent limitations, including the use of paper points. Nonetheless, paper points remain the most common method and the most appropriate option to answer the defined aims and objectives of this study. Further efforts are certainly required to develop more accurate endodontic sampling techniques or devices.

5.1.4 Root canal treatment procedures

As for the clinical treatment, it is always essential to provide patients with the best agreed practice and care. The root canal treatment procedures of this study were tailored to achieve this aim as well as to optimise the quality of the study in accordance with the ethics and regulations of the UK. Although the study population sample selected for this project was of a chronic nature, the definitive diagnoses varied and, hence, some details of the treatment needed to be tailored for each given case. In addition, other factors such as tooth morphology, the restorative status or those related to the patient were vital when judging the most appropriate treatment choice. Despite all of this, the clinical protocol was designed to be as similar as possible for all patients. This, in addition to collection of samples at exact time intervals, was aimed to obtain a more comparable, reflective picture of the microbiological status of the infected root canals.

One of the secondary objectives of this study was to investigate the microbial status of infected root canal after chemo-mechanical preparation. Opting for a multiple root canal treatment visit-approach allowed for this investigation as well as for comparison with pre-preparation samples. Although a Cochrane Review detected no significant differences in the effectiveness of root canal treatment between single and multiple visits, it concluded that the former is associated with higher frequency of symptoms (Figini *et al.*, 2008). In addition, for teeth with necrotic pulps and apical disease, as in this study, multiple visit root canal treatment is the traditional treatment option as it allows the use of inter-appointment medication which may be beneficial for the cases with more established infections (Sathorn *et al.*, 2009).

The adoption of aseptic methods is mandatory for the success of the treatment and an absolute necessity for the validity of the research (Fouad, 2009) . Aseptic techniques include wearing sterile surgical gloves for sampling, sterilisation of instruments and the use of rubber dam (European Society of Endodontology, 2006). In secondary cases, the removal of old root filling was performed mechanically except for two cases. Minimal solvent was applied to complete the removal of remaining root filling. This might have exerted some negative effect on the microbiota but it was vital to avoid jeopardizing the treatment outcome.

Although a mere manual instrumentation with sterile saline can reduce the number of bacteria in the canal by 100 to 1000 fold (Bystrom and Sundqvist, 1981), clinicians are expected to use an irrigant with antimicrobial properties (European Society of Endodontology, 2006). Sodium hypochlorite has been advocated as the irrigant of choice for most cases (Eliyas *et al.*, 2010). Its broad antimicrobial spectrum and other favourable properties such as tissue dissolving abilities, viscosity and low cost have been thoroughly reviewed (Mohammadi, 2008). The efficacy of NaOCl depends on many factors such as concentration, temperature, volume and contact time (Zehnder, 2006) . Various concentrations of NaOCl are in use ranging from 0.5% to full strength 5.25% (or 6% in US) but it remains a controversial issue. Generally, higher concentrations reduce microbes and dissolve necrotic tissues in less time, volume and temperature than lower concentrations. However, high NaOCl concentrations are extremely toxic and can cause severe complications (Eliyas *et al.*, 2010). A concentration of 2.5% was used in this research which properly provides a balance between effectiveness and safety.

Calcium hydroxide was used as an inter-appointment intracanal medication. Since its first dental application in the 1920s, it is still the most commonly used

endodontic medication throughout the world. This is because of its well documented and researched antibacterial, physical and biological properties (Athanassiadis *et al.*, 2007).

In the vast majority of cases, lateral compaction was used which is still the most widely used and taught technique of filling root canals, because it is simple, relatively cheap and can result in well-adapted root fillings (Patel and Barnes, 2011).

5.1.5 Tooth surface decontamination

Decontamination of the sampling field is a mandatory measure for correct sampling and to avoid false-positive results during microbiological analysis. Traditionally, the protocol recommended by Moller (1966) using 30% hydrogen peroxide followed by 5% or 10% iodine tincture has been considered as the gold standard. More recently Ng *et al.* (2003) compared the effect of 10% iodine tincture to 2.5% sodium hypochlorite (NaOCl) on the microbiota in vivo using culture and PCR. Analyses revealed no significant differences between them as decontaminating agents when microbial culture methods were used for detection. However, PCR showed that NaOCl was more effective as a tooth surface decontaminating agent than iodine. In our study, we used the later protocol with some modification by using a combination of 3% hydrogen peroxide and 2.5% NaOCl as described by other groups (Siqueira *et al.*, 2004; Sakamoto *et al.*, 2008).

5.1.6 The 16S rRNA gene and variable target regions as a bacterial identification tool

Metagenomic studies are commonly performed by analysing the prokaryotic 16S ribosomal RNA gene (16S rRNA) sequence. The 16S gene is ubiquitous in all bacteria and its main advantage is that it is long enough to be highly informative but also short enough to be easily sequenced (Fouad, 2009). The 16S rRNA gene consists of approximately 1,500 bp and contains nine variable regions (V) interspersed between conserved regions. Variable regions of 16S rRNA are frequently used in phylogenetic classifications. However, the choice of which variable region is open to debate because it depends on several factors such as experimental objectives, design, and sample type (Kuczynski *et*

al., 2012). Due to the high diversity of bacteria, it may be more desirable to target more than one variable region to increase sensitivity, specificity and reliability of the 16S gene study (Wahl *et al.*, 2013b). Consequently, this study opted to use the V3 and V4 regions. An experimental study showed that targeting this region yields high quality sequencing data (Fadrosh *et al.*, 2014). This is also recommended in the Illumina protocol manual (Illumina, 2013). Moreover, and in order to ensure high quality microbial classification, the study aimed to sequence two variable regions and to obtain paired-end reads of approximately 490 bp length with about 50 bp overlap. The primers used for amplification were 347F/803R which have been shown to be a suitable primer pair for classification of complex human microbiome (Nossa *et al.*, 2010).

5.2 Study results

5.2.1 Culture

Microbial growth was observed in 80% and 77.8% of samples from primary and secondary infections, respectively. In contrast, previous culture studies demonstrated that secondary infections are associated with fewer positive cultures than primary infections (Baumgartner, 2004). It was observed that in primary infections, anaerobes dominated whereas in secondary infections, total facultative and aerobic counts were more dominant. This may indicate that there are significant differences in the microbiomes derived from primary and secondary infections. This is agreement with previous studies (Bystrom and Sundqvist, 1981; Kvist *et al.*, 2004; Gomes *et al.*, 2004; Baumgartner, 2004).

Actinomyces spp. are usually detected in apical lesion infections (Ramachandran Nair and Schroeder, 1984). Molecular studies demonstrated that they are also found in dental caries lesions (Aas *et al.*, 2008). In root canal infections they have been isolated in both primary and secondary infections (Sjogren *et al.*, 1997; Tronstad and Sunde, 2003). Molecular studies showed that *Actinomyces spp*. were detected in 72 of 129 (55.8%) clinical samples (Xia and Baumgartner, 2003). In our study, *Actinomyces* spp. seem prevalent among the studied groups. They were detected in 50% and about 56% in primary and secondary S1 samples. However, there was a marked reduction in S2 samples as it was only detected in two out of the total 19 S2 samples. This

suggests that they may have survival properties. Findings from NGS also indicated that *Actinomyces* spp. is amongst the most prevalent genera.

Streptococcus spp. are also commonly found in the oral cavity (Aas *et al.*, 2005) and are traditionally mainly associated with dental caries (Aas *et al.*, 2008). It has been repeatedly isolated from both primary and secondary root canal infections (Abou-Rass and Bogen, 1998; Khemaleelakul *et al.*, 2002; Mindere *et al.*, 2010) and in this study it was observed in four out of the ten primary S1 samples and in only one of the nine secondary S1 samples. The ability of streptococci to survive root canal treatment has been previously reported (Chavez De Paz *et al.*, 2003). Findings from the culture experiments showed only two S2 samples containing *Streptococcus* spp.

5.2.2 Sequencing

Since NGS became available, there have been great advances in various aspects of the technology. Several metagenomics studies have been carried out in various fields such as environmental (Wahl *et al.*, 2013b), nutrition (Wahl *et al.*, 2013a), animals (Sturgeon *et al.*, 2014; Moreau *et al.*, 2014) and humans (Kong, 2011; Hattori and Taylor, 2009; Kistler *et al.*, 2013). Indeed, all of these studies, and many others, have revealed that microbial diversities in these environments were of magnitudes higher than previously described.

As mentioned previously, at the time this study was proposed (September 2012), to our knowledge, only six endodontic metagenomic studies had been published (Li *et al.*, 2010; Siqueira *et al.*, 2011; Santos *et al.*, 2011; Saber *et al.*, 2012; Ozok *et al.*, 2012; Lim *et al.*, 2011). Since then further four studies have been published (Hong *et al.*, 2013; Anderson *et al.*, 2013; Vengerfeldt *et al.*, 2014; Tzanetakis *et al.*, 2015). The sample sizes used in these studies were 6, 7, 10, 12, 13, 17, 18, 23, 40 and 48. Five studies examined in vivo primary and secondary infections (Hong *et al.*, 2013; Tzanetakis *et al.*, 2015; Lim *et al.*, 2011; Li *et al.*, 2010; Vengerfeldt *et al.*, 2014), three studies investigated apical regions in extracted teeth (Siqueira *et al.*, 2011; Saber *et al.*, 2012; Ozok *et al.*, 2012) , one compared chronic to acute primary infections (Santos *et al.*, 2011) and the final one studied secondary infections (Anderson *et al.*, 2013).

The primary goal of this study was to investigate the microbial diversity of primary (previously untreated) and secondary (previously filled) root canal infections. Although the clinical parameters of the endodontic infections included in this study were of a chronic nature, it was a relatively diverse

sample in terms of infection type, i.e. primary or secondary, stage of disease and clinical symptoms. However, the emphasis was on the “depth of coverage” that results from the use of NGS compared with a traditional method, in the form of culture, in analysing these infections.

From the initial 38 samples, 33 samples (including S1 and S2 samples) were used to carry out library preparation and sequencing procedures. Overall, the analysis detected a total of ten bacterial phyla while about 5% of the sequences could not be assigned to phyla level. At lower levels, 21 different bacterial classes, 35 orders, 87 families and 143 genera were represented by the root canal samples. A comprehensive review of findings from previous culture and molecular studies, prior to 2009, demonstrated that more than 460 bacterial species/ phylotypes belonging to 100 genera and 9 phyla have been detected in the different types of endodontic infections (Siqueira and Rocas, 2009a). The findings from our study alone suggest that these numbers may be underestimated. This is also supported by previous NGS studies. Using pyrosequencing, 13 bacterial phyla and 179 genera were detected in only seven teeth (Li *et al.*, 2010) while in the apical root canal infections, there were 84 genera and 10 phyla (Siqueira *et al.*, 2011). Ozok *et al.* (2012) studied 23 extracted teeth and compared apical and coronal segments and in total they detected 24 phyla. Each of the other seven NGS studies detected between 9 - 13 phyla (Hong *et al.*, 2013; Anderson *et al.*, 2013; Vengerfeldt *et al.*, 2014; Tzanetakis *et al.*, 2015; Lim *et al.*, 2011; Santos *et al.*, 2011; Saber *et al.*, 2012).

Regarding the unassigned phyla, about 5% were unclassified, which may be considered slightly high at the phylum level. This may be due to PCR artefacts, sequencing errors or possibly unknown bacterial phyla (Santos *et al.*, 2011).

A detailed examination of primary and secondary S1 samples revealed notable differences. The most dominant phyla in primary S1 samples were *Firmicutes* (50.5%) followed by *Actinobacteria* (14.4%) and *Bacteroidetes (*13.9%). While secondary infections were dominated by *Firmicutes* (46.2%), *Proteobacteria* (17.8%), *Actinobacteria* (11.9%), and *Bacteroidetes* (7.8%).

Firmicutes was also the dominant phylum in primary and secondary infection in a study that utilised the Illumina HiSeq2000 instrument (Vengerfeldt *et al.*, 2014). Other studies comparing primary and secondary infections revealed different findings. *Firmicutes* seem to dominate secondary infections (Anderson *et al.*, 2013), while *Bacteroidetes* were the most abundant phylum in both primary and persistent infections.

These differences in the dominant phyla and/or genera might be due to several aspects. These include the different clinical conditions and anatomical locations, different methods used for sampling, different NGS technologies and the use of different reading lengths. In addition, geography-related differences in the endodontic bacterial communities cannot be discounted as a possible cause for differences (Machado de Oliveira *et al.*, 2007; Siqueira *et al.*, 2008).

At the genus level, the most dominant in primary S1 sample were *Streptococcus*, *Bacillaceae* (5.7%) and *Eubacterium* (5.2%). *Prevotella* (4.3%), *Fusobacterium* (2.4%). *Enterococcus* (2.2%) and *Actinomyces* (1.5%) appeared in the top 20 most abundant genera. The *Streptococcus* genus was the most dominant in a number of culture, molecular and pyrosequencing studies (Rocas *et al.*, 2010; Siqueira *et al.*, 2002; Rocas *et al.*, 2008; Anderson *et al.*, 2013).

On the other hand, the most dominant genera in secondary S1 samples were *Alkalibacterium* (6.8%), *Bacillaceae* (6.6%) and *TG5* (6.0%). Interestingly, the former two genera were also the most dominant genera in Primary S2 and Secondary S2 samples. This may indicate survival properties of these genera. Other selected genera such as *Fusobacterium*, *Streptococcus*, *Actinomyces* and *Enterococcus* also appeared in the top 20 most dominant genera in secondary infections.

Alkalibacterium is an alkaliphilic bacterium isolated from marine organisms, salted foods, soft semi-hard cheese and edible-olive wash-waters (Ntougias and Russell, 2001; Ishikawa *et al.*, 2013). To our knowledge, this has not been previously isolated from endodontic infections. However, the detection of this species in this study should undergo further investigation. Although *Alkalibacterium* could have gained access to the root canal system via the oral cavity, through for example diet, it is important to exclude other possibilities such as sequencing errors. To exclude the latter, an experiment could be carried out using PCR with specifically designed primers to detect *Alkalibacterium*. Unfortunately, no further samples remained to conduct this verification experiment.

The *Synergistes* are a group of Gram-negative anaerobic organisms that have been frequently found in the oral cavity (Vartoukian *et al.*, 2007) as well as in endodontic infections (Siqueira and Rocas, 2005c). In this study, relatively abundant *Synergistes* spp. (6%) were identified in secondary infections, indicating that they may be an important member of the microbiota of teeth with failed root canal treatment.

Spirochete spp. are free-living, facultative, or obligate anaerobes, often found in ponds and marshes (Paster and Dewhirst, 2000). In this study they were detected in secondary infections but with low abundance. Pyrosequencing also detected *Spirochete* in Li et al (2010).

Prior to NGS studies, *Cyanobacteria, Acidobacteria,* and *Chloroflexi*, which are known bacterial phyla in water, soil, and wastewater plants, respectively, had not been detected in endodontic infections (Li *et al.*, 2010). Most of these were rather in low abundance and may have passed unnoticed in previous endodontic culture and molecular studies.

Enterococcus has been described as the most frequently detected bacterium in root-filled teeth with infections (Cheung and Ho, 2001). In the present study, *Enterococcus* genus appeared in the top 20 genera and constituted 2.2% and 1.1% from primary and secondary, respectively. The *Enterococcus* genus was detected as a low-abundance (0.7%) genus of secondary endodontic infections in the pyrosequencing study by Hong *et al.* (2013) whereas in Anderson *et al.* (2013) it was among the 15 most abundant genera (2.59%).

Contrary to early studies which advocated a vital role of *E. faecalis* in secondary infections (Portenier *et al.*, 2003), more recent studies have questioned that belief because it had not been detected or is rarely one of the most dominant species in root canal–treated teeth (Rocas *et al.*, 2004; Chugal *et al.*, 2011).

With regards to the number of species-level OTUs per canal, the mean number of species-level taxa in each canal was 63 (range from 34 – 80) in primary infections and 69.9 (range from 50 – 87) in secondary infections. Siqueira *et al.* (2011) reported a mean number of about 37 species-level taxa (range13-80) in the apical canal, whereas Ozok *et al.* (2012) reported a mean of 125 taxa (70-185) per canal. These figures are much higher than the average 3–5 species demonstrated by culture studies (Sundqvist, 1992; Siqueira *et al.*, 2007) and the 10–20 species per canal revealed by previous molecular analyses of samples taken from the main canal with no distinction of the sampling site (Munson *et al.*, 2002; Siqueira *et al.*, 2004; Siqueira *et al.*, 2008).

In terms of composition, examination of the heat map showed a marked inter-individual variability in the composition of bacterial communities. Each individual harboured a unique endodontic microbiota in terms of species richness and abundance. This was also evident between primary and secondary samples as demonstrated by shared OTUs percentages, which showed that in most samples the similarity level was less than 50%. The fact that the composition of microbiota differs consistently within and between

individuals with the same disease denotes a heterogeneous aetiology for apical periodontitis, where multiple communities can lead to similar disease outcomes. Despite this inter-individual variability, according to the weighted unifrac beta diversity analyses, many samples showed a tendency to cluster together. This suggests that some patterns of community structures may exist and these might be related to distinct clinical conditions.

Although alpha diversity analyses suggested that the diversity is nearly explored in the study, one must consider that the use of paper points in the present study may not be optimal to sample the whole microbiota in the complex root canal systems including dentinal tubules, isthmus, and lateral (accessory) canals, especially at apical third areas. This indicates that the overall bacterial diversity in both endodontic infections may be considerably higher than currently identified.

5.2.3 The effect of chemo-mechanical preparation and medication on the endodontic microbiota

The main goals of root canal treatment is to eradicate the microbial infection, or at least to reduce it to a level compatible with healing, and to prevent future re-infection of the root canal system (Sjogren *et al.*, 1997). It should be reiterated that evaluating the effect of the standard root canal chemo-mechanical preparation and medication procedures was only a secondary objective in this study.

Using microbial sampling to assess the microbial composition and load changes before and after treatment is referred to as a surrogate outcome. The method is certainly not an accurate method to predict treatment outcome but it may provide indications. Indeed, culture studies in the 1970s and 1980s advocated its use for clinical evaluation of treatment outcome (Fouad, 2009). Long term prospective cohort studies are more accurate in assessing treatment outcome. Yet, they still suffer from several patient and clinical confounding factors as well as clinician/ researcher bias.

In this study, culture S2 samples showed marked reductions in viable microbial load especially in secondary infection cases. Complete periapical healing occurred in 94% of cases that yielded a negative culture. Where the samples were positive prior to root filling, the success rate of treatment was just 68% (Sjogren *et al.*, 1997).

On the contrary, the NGS analysis outputs demonstrated that there was no significant difference in OTUs loads before and after root canal treatment. This may be explained by the fact that molecular techniques do not differentiate DNA from live or dead microorganisms. This finding emphasizes the current knowledge that existing root canal preparation procedures usually fail to disinfect and clean large parts of the root canal system (Siqueira, 2001) . The presence of high loads of OTUS in S2 samples may hold clinical implications. These OTUs may be the genetic material from dead species. Some components from these dead species remaining in the canal may serve as nutrition for surviving or, future, invading microorganisms leading to persisting or recurring infections. Moreover, other remnants of bacterial cells may be involved in inflammatory reactions.

Another interesting observation is that the sequencing analyses of S2 samples showed some genera that had not appeared in the primary samples. This could be simply because paper points failed to pick up these species in the S1 samples. The other probable explanation is that these microorganisms may have been hidden in the dentinal tubules, as some microbes are able to colonise dentinal tubules (Siqueira *et al.*, 1996), and only appear after being exposed by root canal wall instrumentation. Other reasons might be canal contamination during or between treatment visits, environmental contamination or sequencing errors.

As noted earlier, it was interesting to observe that the genera *Alkalibacterium* and *Bacillaceae* were the most dominant in Primary S2, Secondary S1 and S2 samples. This may indicate that some species of these genera are more difficult to remove from infected root canals. To our knowledge, this have not been previously isolated from endodontic infections.

5.2.4 Limitations of NGS

Another shortcoming of DNA based studies is the inability to discriminate dead from live microorganisms and hence all genetic material is assessed (Siqueira and Rocas, 2005a). This may overestimate bacterial load because it has been reported that DNA can persist for up to one year after cell death (Young *et al.*, 2007). It is argued, however, that an assessment of both live and dead microorganisms is important because these bacteria may have been predominant in the early phases of disease or played a part in biofilm formation (Saber *et al.*, 2012).

Furthermore, since the emergence of NGS, there has been doubt regarding the quality of taxonomic identification using short sequencing reads (Kuczynski *et al.*, 2012). This was particularly true with early NGS technologies. However, with recent advancement, several of the NGS technologies now offer various platforms/options which can sequence various read lengths from 50 bp and up to 700 bp, as for 2013 (Di Bella *et al.*, 2013).

Other common concerns include PCR and primer selection bias, sequencing errors and chimera formation and handling and interpretation of the massive data that can be produced by bioinformatics especially for human genomic studies (Quince *et al.*, 2009; Reeder and Knight, 2009).

5.3 General discussion

One of the major shortcomings of cross sectional microbiological studies is that they only provide a snapshot at a particular stage. Although this provides some information, it is difficult to understand the ecology within that given habitat. Nonetheless, a collection of data from various studies may provide a better picture.

Factors that dictate the microbial ecology in a given niche include the local pH, abundance and partial pressure of oxygen, redox potential, availability of selective nutrients, and the state of local host defences (Marsh and Devine, 2011).

Our findings from culture analyses indicated that primary infections were dominated by anaerobic species while secondary infections contained facultative species. However, from sequencing, it was observed that nine and ten of the top 15 genera isolated from primary infections and secondary infections, respectively, consisted mostly of anaerobic or facultative anaerobic species. It was thought that primary infections are dominated by anaerobic species while facultative anaerobes are more prevalent in secondary infections, (Baumgartner, 2004; Figdor and Sundqvist, 2007), whereas the more recent NGS endodontic studies showed mixed results (Di Bella *et al.*, 2013).

These conflicting findings may be because different bacterial dominances might contribute to different clinical expressions (Li *et al.*, 2010). For instance, previous studies comparing chronic and acute root canal infections have suggested the latter may contain more species and are associated with particular species (Siqueira and Rocas, 2009b; Siqueira *et al.*, 2004). Similar observations were also reported by pyrosequencing (Santos *et al.*, 2011). The findings in the present study both in terms of composition and diversity may suggest that both primary and secondary infections are associated with unique bacterial communities at different stages of the disease.

Therefore, a better understanding of the ecology and pathogenicity of a microbial community requires the thorough knowledge of every component involved, including identification of species present at low levels in the environment, especially considering that the dental pulp is a previously sterile environment (Santos *et al.*, 2011).

5.4 Summary and Clinical relevance

The study produced a number of findings that may have clinical implications.

- The sequencing analyses demonstrated that both primary and secondary infections have a diverse bacterial composition.
- The biofilm community consists of various species with a dominance of facultative anaerobic and obligate anaerobic in particular primary infections.
- A number of bacteria not normally detected in the oral cavity, were isolated.

Some of the clinical implications have already been highlighted earlier and, as mentioned, the findings show the importance of previous clinical recommendations that are not routinely followed by clinician in day to day practice.

The success of root canal treatment does not only depend on eliminating bacteria but also on preventing the introduction of new species. Therefore, the adoption of aseptic methods is mandatory in this regard. This may suggest the use of an antimicrobial oral rinse before the start of treatment. Tooth surface decontamination procedures are normally only used for research purposes to avoid false positive samples. It may be beneficial to use these procedures for routine cases. The use of an irrigant solution, or preferably a combination of irrigant, with full concentration may be recommended. Emphasis should also be placed to ensure adequate washing of canals to remove remaining debris. Finally, speedy placement of adequate permanent restoration is vital to reduce the chances of reinfection.

5.5 Future directions

First, the study would certainly benefit from a larger sample size. Improvement in root canal sampling techniques are necessary in order to provide a better representative picture of endodontic microbial community. This, with the ongoing advancement in NGS technologies, especially with regards to resolution at species level identification, can further refine our knowledge regarding the diversity of species as well as their association with different clinical conditions.

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Appendix

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Appendix 2: Participants information sheet.

Appendix 3: Sample letter to GDP.

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Appendix 1: Consent form.

Leeds Dental Institute
University of Leeds
Clarendon Way
Leeds LS29LU
T+44(0)1133436199
F+44(0)1133436165
E dentistry@leeds.ac.uk

UNIVERSITY OF LEEDS

Study Number: 2012003HA **(V.1-11/12/2012)**

Patient name:

CONSENT FORM

Title of project: Microbiological analysis of root canal infections

Name of Researcher: Mr Hussain Alenezi.

Please initial the box if you agree with the statement on the left

1. I confirm I have read and understand the information sheet dated 12/3/2013 (version .2) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that relevant sections of my medical notes and data collected during the study, may be looked at by individuals from Leeds Dental Institute, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐
4. I understand that the study involves taking samples from the root canal for microbial analysis. I give permission for the investigators to use them for the purpose of this research and future research. ☐
5. I understand if I withdraw consent, any samples or data which may have already been collected or used in the research before that date cannot be withdrawn. ☐
6. I agree to my GDP (dentist)/Referral being informed of my participation in the study. ☐
7. I agree to take part in the above study. ☐

Name of Patient	**Date**	**Signature**
Name of Person taking consent	**Date**	**Signature**

When completed: one copy for participant; one copy for researcher site file; one copy (original) to be kept in medical notes.

Appendix 2: Participants information sheet.

Leeds Dental Institute
University of Leeds
Clarendon Way
Leeds LS29LU

T+44(0)1133436199
F+44(0)1133436165
E dentistry@leeds.ac.uk

UNIVERSITY OF LEEDS

Study number: 2012003HA **(V.2-12/3/2013)**

Participant Information Sheet

Title of Study: Analysis of root canal infections

You are being invited to take part in a research project. Before you make a decision, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Please take time to decide whether you wish to take part. Thank you for reading this.

What is the purpose of this study?

When teeth are decayed or broken, bugs (bacteria) enter the inside of the tooth and cause infections. This is usually felt as pain and/or swelling and when this happens, root canal treatment is usually necessary to save the tooth. However, we still do not know exactly which bacteria cause these infections. This study will help us to know more about root canal infections and this may improve the standard of treatment in the future.

Who is doing the study?

I am a fully qualified dentist from the University of Leeds and have been practising dentistry for over 10 years. I am currently undertaking a full-time Doctorate Degree. This study will be carried out at Leeds Dental Institute and is part of my degree. I will be working with a fully trained research team and, in addition, Professors and members of staff will supervise all work.

Why have I been asked to participate?

The inside of your tooth is infected and so you have two options: either to remove the tooth; or to have root canal treatment. Your tooth can be saved and therefore root canal treatment is a better option for you. As long as you are generally healthy and can maintain good oral hygiene, you are eligible to participate in the study.

What will be involved if I take part in this study?

We will invite 20 patients, like yourself, with root canal infections who need root canal treatment. I will complete root canal treatment and this will take about 3 visits. The only difference from the normal treatment is taking samples of the bacteria from the inside of your tooth. Taking samples of the bacteria takes only 2 minutes and you will not even feel it. Basically, the water we usually use to clean the inside of your tooth is picked up on small pieces of paper. These papers are then taken to lab to study the bacteria. The study ends when the root canal treatment is finished but as a routine procedure, Leeds Dental Hospital will recall you for a check-up after 6-12 months.

Leeds Dental Institute
University of Leeds
Clarendon Way
Leeds LS29LU

T+44(0)1133436199
F+44(0)1133436165
E dentistry@leeds.ac.uk

UNIVERSITY OF LEEDS

What are the advantages and disadvantages of taking part?

Taking part in the study will not provide immediate benefits or money.
However, studying the bacteria that cause infections will provide valuable information and may help us to improve the standard of treatment in the future.

Can I withdraw from the study at any time?

You can withdraw at any time and the treatment will still be carried out. You do not have to give a reason. If you decide to withdraw, the samples already collected may be kept.

Will the information I give be kept confidential?

All personal data will be kept strictly confidential in accordance with the Data Protection Act 1998. Apart from the investigators and research team, no one will have access to these details.

What will happen to the results of the study?

The results of the research will be used for academic purposes and may be published in scientific journals. Some referring dentists may wish to know about the results, so we will provide them with a general summary, if they ask. However, these results will be anonymous and there is no way to identify participants. If you wish to know about the results, we will be delighted to provide you with a summary. At the end of the study, some of your bacterial samples may be kept for future research, but again, in no way could this be linked to you. Before we start the study, you will be asked to give your agreement.

Who has reviewed this study?

The study was carefully designed and has been reviewed by academic supervisors. Ethical approval was sought and obtained from NHS and National Research Ethics Committees (NRES).

If you would like more information or have any questions about the study, a meeting with the researcher can be arranged today. Alternatively, you can contact him to arrange an appointment on a later date. After meeting the student, you will have at least two more days to consider taking part or not.

Researcher:
Mr Hussain Alenezi.
Leeds Dental Institute
Email: efy6ha@leeds.ac.uk
T (research Unit): 0113- 3436127/3439400

Academic supervisor:
Professor Michael Manogue
Leeds Dental Institute
Email: m.manogue@leeds.ac.uk

Thank you for taking the time to read this information sheet.

Appendix 3: Sample letter to GDP.

The University Of Leeds
Leeds Dental Institute
Clarendon Way
Leeds, LS2 9LU
Web:
http://www.dentistry.leeds.ac.uk
Tel:

Patient Name:

D.O.B:

Date:

FOR INFORMATION ONLY

Letter to GP/Referral

Subject: research title: Microbial analysis of root canal infections.

Dear GDP:

Thank you for referring (Mr....) to Leeds Dental Institute for the consultation/treatment of (...). (Mr...) was seen on Prof .... consultant clinic on (date). The patient complained of pain on biting on LL4 that was root treated 3 years ago. Clinical examination revealed a draining sinus buccal to the LL4 and the tooth was TTP. Periapical radiographs showed (.....) associated with the LL4 and a short root filling. The most likely diagnosis is (.....). Mr has no medical issues and the tooth is functional and restorable and therefore non-surgical retreatment would be the most appropriate treatment option at this stage.

We are currently conducting clinical research to investigate these kinds of root canal infections. Mr... was eligible to be included and we have offered him that option. He kindly agreed to take part and we will commence treatment soon.

If you are interested in the results of the study, please contact us and we will provide you with a general summary as soon as it is available.

Kind regards

Hussain Alenezi

BChD, MFDSRCSI, DClinDent Student.
Leeds Dental Institute
Clarendon Way, LS2 9LU.
Email: efy6ha@leeds.ac.uk.

Appendix 4: REC approval

NHS
Health Research Authority
NRES Committee Yorkshire & The Humber - Leeds East
Yorkshire and Humber REC Office
First Floor, Millside
Mill Pond Lane
Meanwood
Leeds
LS6 4RA

Telephone: 0113 30 50126
Facsimile: 0113 855 6191

18 March 2013

Mr Hussain E Alenezi
Department of Restorative Dentistry,
Leeds Dental Institute.
Worsley Building, level 6,
Clarendon Way, Leeds.
LS2 9LU

Dear Mr Alenezi

Study title:	**Microbiological analysis of root canal infections using High Throughput Sequencing on the Illumina MiSeq Platform.**
REC reference:	**13/YH/0035**
Protocol number:	**2012003HA**
IRAS project ID:	**119427**

Thank you for your letter of 12 March 2013, responding to the Committee's request for further information on the above research and submitting revised documentation.

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

We plan to publish your research summary wording for the above study on the NRES website, together with your contact details, unless you expressly withhold permission to do so. Publication will be no earlier than three months from the date of this favourable opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to withhold permission to publish, please contact the Co-ordinator Mrs Anne Ward, nrescommittee.yorkandhumber-leedseast@nhs.net.

Confirmation of ethical opinion

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

Ethical review of research sites

NHS sites

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

Non-NHS sites

Conditions of the favourable opinion

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

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

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

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

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

Approved documents

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

Document	*Version*	*Date*
Covering Letter		14 January 2013
Covering Letter		12 March 2013
Evidence of insurance or indemnity		21 September 2012
Investigator CV	HAllenezi	11 December 2012
Letter from Statistician		11 January 2013
Other: Statistical Methodology	1	11 December 2012
Other: Academic Supervisor CV: M Manogue		

Other: Academic Supervisor CV: Thuy Do		
Other: Academic Supervisor CV: D Devine		
Other: Letter to GDP	2	12 March 2013
Participant Consent Form	1	11 December 2012
Participant Information Sheet	2	12 March 2013
Protocol	2	12 March 2013
REC application		11 January 2013
Response to Request for Further Information		12 March 2013

Statement of compliance

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

After ethical review

Reporting requirements

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

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

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

Feedback

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

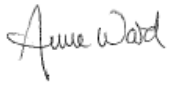
Further information is available at National Research Ethics Service website > After Review

13/YH/0035 **Please quote this number on all correspondence**

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

With the Committee's best wishes for the success of this project.

Yours sincerely



pp
Dr C E Chu
Chair

Email:nrescommittee.yorkandhumber-leedseast@nhs.net

Enclosures: "After ethical review – guidance for researchers" *[SL-AR2]*

Copy to: *Clare E Skinner, Faculty Head of Research Support, University of Leeds*
Mrs Anne Gowing, Leeds Teaching Hospitals NHS Trust

Appendix 5: R&D approval

The Leeds Teaching Hospitals NHS
NHS Trust

Michael Wood

16/05/2013

Mr Hussain E Alenezi

Leeds Dental Institute
Clarendon way
Leeds
LS2 9LU

Research & Development
Leeds Teaching Hospitals NHS Trust
34 Hyde Terrace
Leeds
LS2 9LN

Tel: 0113 392 2878
Fax: 0113 392 6397

r&d@leedsth.nhs.uk
www.leedsth.nhs.uk

Dear Mr Hussain E Alenezi

Re: NHS Permission at LTHT for: Microbiological analysis of root canal infections using High Throughput Sequencing on the Illumina MiSeq Platform.
LTHT R&D Number: DT13/10723
REC: 13/YH/0035

I confirm that *NHS Permission for research* has been granted for this project at The Leeds Teaching Hospitals NHS Trust (LTHT). NHS Permission is granted based on the information provided in the documents listed below. All amendments (including changes to the research team) must be submitted in accordance with guidance in IRAS. Any change to the status of the project must be notified to the R&D Department.

Permission is granted on the understanding that the study is conducted in accordance with the *Research Governance Framework for Health and Social Care*, ICH GCP (if applicable) and NHS Trust policies and procedures available at http://www.leedsth.nhs.uk/academic/research-development/

This permission is granted only on the understanding that you comply with the requirements of the *Framework* as listed in the attached sheet "Conditions of Approval".

If you have any queries about this approval please do not hesitate to contact the R&D Department on telephone 0113 392 2878.

Indemnity Arrangements

The Leeds Teaching Hospitals NHS Trust participates in the NHS risk pooling scheme administered by the NHS Litigation Authority 'Clinical Negligence Scheme for NHS Trusts' for: (i) medical professional and/or medical malpractice liability; and (ii) general liability. NHS Indemnity for negligent harm is extended to researchers with an employment contract (substantive or honorary) with the Trust. The Trust

only accepts liability for research activity that has been managerially approved by the R&D Department.

The Trust therefore accepts liability for the above research project and extends indemnity for negligent harm to cover you as investigator and the researchers listed on the Site Specific Information form. Should there be any changes to the research team please ensure that you inform the R&D Department and that s/he obtains an appropriate contract, or letter of access, with the Trust if required.

Yours sincerely

Dr D R Norfolk
Associate Director of R&D

Approved documents
The documents reviewed and approved are listed as follows

Document	*Version*	*Date of document*
NHS R&D Form	3.5	27/03/2013
SSI Form	3.5	15/05/2013
Directorate Approval		14/05/2013
REC Letter confirming favourable opinion		18/03/2013
Protocol	3.0	25/04/2013
Patient information sheet	2.0	12/03/2013
Consent form	1.0	11/12/2012
GP/Consultant information sheets	2.0	12/03/2013
Evidence of insurance		29/09/2012

Appendix 6: list of index primers.

Number	Primer Sequence	Tag
1	CAAGCAGAAGACGGCATACGAGATGATTGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	GATTGC
2	CAAGCAGAAGACGGCATACGAGATGGTAGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	GGTAGT
3	CAAGCAGAAGACGGCATACGAGATGCCAATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	GCCAAT
4	CAAGCAGAAGACGGCATACGAGATTGCACAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	TGCACA
5	CAAGCAGAAGACGGCATACGAGATACCACAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	ACCACA
6	CAAGCAGAAGACGGCATACGAGATCCAATCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	CCAATC
7	CAAGCAGAAGACGGCATACGAGATTGCAACGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	TGCAAC
8	CAAGCAGAAGACGGCATACGAGATGAAGGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	GAAGGA
9	CAAGCAGAAGACGGCATACGAGATCTGAAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	CTGAAG
10	CAAGCAGAAGACGGCATACGAGATTGGCTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	TGGCTA
11	CAAGCAGAAGACGGCATACGAGATAAGGCTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	AAGGCT
12	CAAGCAGAAGACGGCATACGAGATTAGGTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	TAGGTC
13	CAAGCAGAAGACGGCATACGAGATACATGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	ACATGC
14	CAAGCAGAAGACGGCATACGAGATACGAGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	ACGAGA
15	CAAGCAGAAGACGGCATACGAGATTGAAGGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	TGAAGG
16	CAAGCAGAAGACGGCATACGAGATTCTAGGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	TCTAGG
17	CAAGCAGAAGACGGCATACGAGATACCAGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	ACCAGT
18	CAAGCAGAAGACGGCATACGAGATCTTAGGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	CTTAGG
19	CAAGCAGAAGACGGCATACGAGATCTGAACGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	CTGAAC
20	CAAGCAGAAGACGGCATACGAGATGCTTACGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	GCTTAC
21	CAAGCAGAAGACGGCATACGAGATTCTGACGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	TCTGAC
22	CAAGCAGAAGACGGCATACGAGATTGAGTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	TGAGTC
23	CAAGCAGAAGACGGCATACGAGATCACAACGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	CACAAC
24	CAAGCAGAAGACGGCATACGAGATATCGGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	ATCGGT
25	CAAGCAGAAGACGGCATACGAGATAGAGCTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	AGAGCT
26	CAAGCAGAAGACGGCATACGAGATATTCGGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	ATTCGG
27	CAAGCAGAAGACGGCATACGAGATTAGGACGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	TAGGAC
28	CAAGCAGAAGACGGCATACGAGATACCAGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	ACCAGA
29	CAAGCAGAAGACGGCATACGAGATCATCACGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	CATCAC
30	CAAGCAGAAGACGGCATACGAGATGTTACCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	GTTACC
31	CAAGCAGAAGACGGCATACGAGATACCATGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	ACCATG
32	CAAGCAGAAGACGGCATACGAGATAACGCTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	AACGCT
33	CAAGCAGAAGACGGCATACGAGATGACTGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	GACTGA
34	CAAGCAGAAGACGGCATACGAGATCAAGACGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	CAAGAC
35	CAAGCAGAAGACGGCATACGAGATAGCAAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	AGCAAG
36	CAAGCAGAAGACGGCATACGAGATTGGACAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	TGGACA
37	CAAGCAGAAGACGGCATACGAGATCTTGTGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	CTTGTG
38	CAAGCAGAAGACGGCATACGAGATCTCAAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	CTCAAG

Appendix 7: List of all of genera detected

	Taxonomy
1	Unassigned
2	p__Actinobacteria;c__Actinobacteria;o__Actinomycetales
3	p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Actinomycetaceae;g__Actinomyces
4	p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Cellulomonadaceae
5	p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Cellulomonadaceae;g__Actinotalea
6	p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Cellulomonadaceae;g__Demequina
7	p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Corynebacteriaceae;g__Corynebacterium
8	p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Microbacteriaceae;Other
9	p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Microbacteriaceae;g__
10	p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Microbacteriaceae;g__Leucobacter
11	p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Micrococcaceae;g__Kocuria
12	p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Micrococcaceae;g__Micrococcus
13	p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Micrococcaceae;g__Rothia
14	p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Propionibacteriaceae;g__
15	p__Actinobacteria;c__Coriobacteriia;o__Coriobacteriales;f__Coriobacteriaceae;g__
16	p__Actinobacteria;c__Coriobacteriia;o__Coriobacteriales;f__Coriobacteriaceae;g__Atopobium
17	p__Actinobacteria;c__Coriobacteriia;o__Coriobacteriales;f__Coriobacteriaceae;g__Slackia
18	p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__;g__
19	p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Marinilabiaceae;g__
20	p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae;g__
21	p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae;g__Dysgonomonas

22	p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae;g__Paludibacter
23	p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae;g__Parabacteroides
24	p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae;g__Porphyromonas
25	p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;g__Prevotella
26	p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__RF16;g__
27	p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Rikenellaceae;g__Blvii28
28	p__Bacteroidetes;c__Cytophagia;o__Cytophagales;f__Cyclobacteriaceae;g__
29	p__Bacteroidetes;c__Cytophagia;o__Cytophagales;f__Cytophagaceae;g__
30	p__Bacteroidetes;c__Cytophagia;o__Cytophagales;f__Cytophagaceae;g__Dyadobacter
31	;p__Bacteroidetes;c__Cytophagia;o__Cytophagales;f__Flammeovirgaceae;g__
32	p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Cryomorphaceae;Other
33	p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Cryomorphaceae;g__
34	p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Cryomorphaceae;g__Fluviicola
35	p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Flavobacteriaceae;Other
36	p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Flavobacteriaceae;g__Capnocytophaga
37	p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Flavobacteriaceae;g__Flavobacterium
38	p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__[Weeksellaceae];g__
39	p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__[Weeksellaceae];g__Chryseobacterium
40	p__Bacteroidetes;c__Sphingobacteriia;o__Sphingobacteriales;f__;g__
41	p__Chloroflexi;c__Anaerolineae;o__S0208;f__;g__
42	p__Chloroflexi;c__Anaerolineae;o__SBR1031;f__A4b;g__
43	p__Chloroflexi;c__Thermomicrobia;o__JG30-KF-CM45;f__;g__
44	p__Cyanobacteria;c__ML635J-21;o__;f__;g__
45	p__Firmicutes;c__Bacilli;Other;Other;Other

46	p__Firmicutes;c__Bacilli;o__Bacillales;Other;Other
47	p__Firmicutes;c__Bacilli;o__Bacillales;f__;g__
48	p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae;Other
49	p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae;g__
50	p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae;g__Anaerobacillus
51	p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae;g__Bacillus
52	p__Firmicutes;c__Bacilli;o__Bacillales;f__Paenibacillaceae;g__Paenibacillus
53	p__Firmicutes;c__Bacilli;o__Bacillales;f__Staphylococcaceae;g__Staphylococcus
54	p__Firmicutes;c__Bacilli;o__Bacillales;f__[Exiguobacteraceae];g__
55	p__Firmicutes;c__Bacilli;o__Bacillales;f__[Exiguobacteraceae];g__Exiguobacterium
56	p__Firmicutes;c__Bacilli;o__Lactobacillales;Other;Other
57	p__Firmicutes;c__Bacilli;o__Lactobacillales;f__;g__
58	p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Aerococcaceae;Other
59	p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Aerococcaceae;g__Aerococcus
60	p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Aerococcaceae;g__Alkalibacterium
61	p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Aerococcaceae;g__Marinilactibacillus
62	p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Carnobacteriaceae;g__Granulicatella
63	p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Enterococcaceae;Other
64	p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Enterococcaceae;g__
65	p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Enterococcaceae;g__Enterococcus
66	p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae;g__
67	p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae;g__Lactobacillus
68	p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae;g__Pediococcus
69	p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae;g__Streptococcus
70	p__Firmicutes;c__Clostridia;o__Clostridiales;Other;Other
71	p__Firmicutes;c__Clostridia;o__Clostridiales;f__;g__
72	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;Other
73	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__
74	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__Alkaliphilus

75	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__Clostridium
76	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__Geosporobacter_Thermotalea
77	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Eubacteriaceae;g__Pseudoramibacter_Eubacterium
78	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;Other
79	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__
80	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Coprococcus
81	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Epulopiscium
82	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Moryella
83	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Oribacterium
84	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Peptococcaceae;g__
85	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Peptostreptococcaceae;g__
86	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Peptostreptococcaceae;g__Peptostreptococcus
87	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__
88	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__
89	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__Acidaminococcus
90	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__Megasphaera
91	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__Schwartzia
92	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__Selenomonas
93	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__Veillonella
94	p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Acidaminobacteraceae];g__Fusibacter
95	p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Mogibacteriaceae];g__
96	p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Mogibacteriaceae];g__Mogibacterium
97	p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Tissierellaceae];g__Parvimonas
98	p__Fusobacteria;c__Fusobacteriia;o__Fusobacteriales;f__Fusobacteriaceae;g__Fusobacterium
99	p__Fusobacteria;c__Fusobacteriia;o__Fusobacteriales;f__Leptotrichiaceae;g__Leptotrichia

100	p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Beijerinckiaceae;g__
101	p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Hyphomicrobiaceae;g__Devosia
102	p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae;Other
103	p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae;g__
104	p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae;g__Anaerospora
105	p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae;g__Loktanella
106	p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae;g__Paracoccus
107	p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae;g__Rhodobaca
108	p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae;g__Rhodobacter
109	p__Proteobacteria;c__Alphaproteobacteria;o__Rhodospirillales;f__Acetobacteraceae;g__
110	p__Proteobacteria;c__Alphaproteobacteria;o__Rhodospirillales;f__Acetobacteraceae;g__Roseococcus
111	p__Proteobacteria;c__Alphaproteobacteria;o__Rhodospirillales;f__Rhodospirillaceae;g__
112	p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Burkholderiaceae;g__Lautropia
113	p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae;Other
114	p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae;g__
115	p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae;g__Delftia
116	p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae;g__Hydrogenophaga
117	p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae;g__Variovorax
118	p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Oxalobacteraceae;g__

119	p__Proteobacteria;c__Betaproteobacteria;o__Neisseriales;f__Neisseriaceae;g__Kingella
120	p__Proteobacteria;c__Betaproteobacteria;o__Neisseriales;f__Neisseriaceae;g__Neisseria
121	p__Proteobacteria;c__Deltaproteobacteria;o__Desulfovibrionales;f__Desulfovibrionaceae;g__Desulfovibrio
122	p__Proteobacteria;c__Epsilonproteobacteria;o__Campylobacterales;f__Campylobacteraceae;g__Campylobacter
123	p__Proteobacteria;c__Gammaproteobacteria;o__Alteromonadales;f__[Chromatiaceae];Other
124	p__Proteobacteria;c__Gammaproteobacteria;o__Alteromonadales;f__[Chromatiaceae];g__Alishewanella
125	p__Proteobacteria;c__Gammaproteobacteria;o__Alteromonadales;f__[Chromatiaceae];g__Alkalimonas
126	p__Proteobacteria;c__Gammaproteobacteria;o__Alteromonadales;f__[Chromatiaceae];g__Rheinheimera
127	p__Proteobacteria;c__Gammaproteobacteria;o__Cardiobacteriales;f__Cardiobacteriaceae;g__Cardiobacterium
128	p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;g__
129	p__Proteobacteria;c__Gammaproteobacteria;o__Legionellales;f__Coxiellaceae;g__
130	p__Proteobacteria;c__Gammaproteobacteria;o__Oceanospirillales;f__Halomonadaceae;g__Candidatus Portiera
131	p__Proteobacteria;c__Gammaproteobacteria;o__Oceanospirillales;f__Halomonadaceae;g__Halomonas
132	p__Proteobacteria;c__Gammaproteobacteria;o__Oceanospirillales;f__Oceanospirillaceae;g__Nitrincola
133	p__Proteobacteria;c__Gammaproteobacteria;o__Pasteurellales;f__Pasteurellaceae;g__Haemophilus
134	p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Moraxellaceae;g__Acinetobacter
135	p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Pseudomonadaceae;Other
136	p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Pseudomonadaceae;g__

137	p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Pseudomonadaceae;g__Pseudomonas
138	p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Xanthomonadaceae;g__Aquimonas
139	p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Xanthomonadaceae;g__Stenotrophomonas
140	p__Spirochaetes;c__Spirochaetes;o__Spirochaetales;f__Spirochaetaceae;g__Treponema
141	p__Synergistetes;c__Synergistia;o__Synergistales;f__Dethiosulfovibrionaceae;g__TG5
142	p__TM7;c__TM7-1
143	p__TM7;c__TM7-3
144	p__TM7;c__TM7-3;o__EW055