The impact of implementing an enhanced infection control protocol on root canal treatment outcomes
in vivo and clinical studies

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The Impact of Implementing an Enhanced Infection Control Protocol on Root Canal Treatment Outcomes: *In vivo* and Clinical Studies

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Abstract

Aims:

This thesis was intended to determine the effectiveness of implementing an enhanced infection control protocol on primary root canal treatment. First, the effect was studied on the outcome of root canal treatment of molar teeth clinically and radiographically; teeth were assigned to one of the two protocols: a standard root canal treatment protocol (SP), and an enhanced infection control protocol (EP). Moreover, this project examined the impact of the EP on the microbial load and the composition of the microbial community after chemomechanical preparation.

Materials and methods:

The pilot study involved samples obtained from teeth diagnosed with irreversible pulpitis (IP). Samples were collected from different sites such as files, endodontic ruler surface, rubber dam surface, gloves and instruments (tip of the tweezer, DG-16 endodontic explorer, plugger and flat plastic instrument), as well as intracanal samples. Microbial load was investigated by quantitative polymerase chain reaction (qPCR). The microbial composition was evaluated by targeting the 16S rRNA V3-V5 hypervariable regions of the 16S rRNA and subjecting to next-generation sequencing (NGS). Moreover, the microbial load and composition of intracanal samples of vital teeth were investigated by similar molecular methods.

The randomised controlled clinical trial involved healthy patients at Guys' Hospital receiving primary root canal treatments. The patients were block randomised to a standard protocol (SP) and an enhanced infection control protocol (EP). Both treatment arms adhered to current best practice recommendations, while the EP comprised additional steps included replacing rubber dams, gloves, files, all instruments and surface barriers at the time of obturation to reduce the chances of iatrogenic contaminations. CBCT and PA radiographs were taken at baseline and

one-year follow-up to assess the outcome of treatment. The outcome was assessed clinically and radiographically using CBCT.

In addition, intracanal samples were taken at baseline (S1) and after completion of chemomechanical preparation (S2). Microbial 16S rDNA copy numbers were enumerated by qPCR. Bacterial composition and identification were performed targeting the 16S rRNA V3-V4 hypervariable regions and subjected to NGS.

Results:

Findings of the pilot study showed that around half of the rubber dam surfaces were contaminated with bacteria at time of obturation and 38% of initial files introduced into the canal showed significant levels of bacteria. Bacteria were also detected in 20-30% of gloves, instruments and rulers prior to obturation. *Streptococcus*, *Rothia*, *Propionibacterium*, and *Fusobacterium* were among the taxa found in such contaminated surfaces. The pilot study findings suggested the risk of introducing bacteria into the root canal space after chemomechanical preparations; higher bacterial loads were more frequently present in intracanal samples before root canal filling when instruments and surfaces were found to be contaminated.

Regarding the intracanal samples of IP teeth, half of the intracanal samples had a substantial bacterial load of bacteria within the vital pulp ($\geq 10^4$ 16S rRNA copies), as determined by qPCR. NGS microbial identification yielded 187 bacterial OTUs mainly belonging to the genera *Veillonella*, *Streptococcus*, *Corynebacterium*, *Cutibacterium*, and *Porphyromonas*.

At one-year follow-up, 115 teeth were analysed (54 in SP and 61 in EP), as a part of the clinical study. The probability of 12-month success was three times higher in the EP group compared to the SP group. The median bacterial reads were reduced to 3.5×10^3 in the SP group and to 1.3×10^3 in the EP group. The EP significantly reduced bacterial counts in pre-obturation samples when compared to the SP. Using a high-throughput sequencing approach, the findings

showed a trend of reduced diversity observed in pre-obturation samples of teeth treated within the EP compared to SP. Contaminants typically arising from saliva, skin or endodontic root canal spaces as *Actinomyces*, *Porphyromonas*, *Cutibacterium*, and *Haemophilus* were significantly reduced in their abundance in the EP pre-obturation samples.

Conclusion:

The evidence from the pilot study highlighted the risk of contamination during root canal treatment and generated a proof of concept to commence a clinical trial. Findings of the clinical randomised trial showed the effectiveness of implementing an enhanced infection control protocol on primary root canal treatment of molar teeth and suggested that the implementation of facile and simple procedural adaptations such as changing instruments, gloves and rubber dams during root canal treatment are important in improving asepsis and have a great impact on treatment outcomes.

Table of contents

List of figures

List of tables

List of abbreviations

AAE: American association of endodontists
ACE: Abundance-based coverage estimator
ADC: Acute dental care
AIP: Asymptomatic irreversible pulpitis
ALARA: As low as reasonably achievable
AP: Apical periodontitis
BIOM: Biological observation matrix
Ca(OH)2: Calcium hydroxide
CBCT: Cone-beam computed tomography
CC: Contamination control
Chao1: Number of species in a community
CHX: Chlorhexidine
CI: Confidence interval
Ct: Cycle threshold
DICOM: Digital imaging and communication in medicine
EDTA: Ethylenediaminetetraacetic acid
EPT: Electric pulp testing
ESE: European Society of Endodontology
EP: Enhanced infection control protocol
F: Endodontic file sample
FOV: Field of view
GCP: Good clinical practice
gDNA: Genomic DNA
GDP: General dental practitioner
GIC: Glass-ionomer cement
GP: Gutta-percha
GSTFT: Guy's St Thomas' foundation trust
H_2O_2: Hydrogen peroxide
HOMD: Human Oral Microbiome Database
HRA: Health Research Authority
In vivo: Within living organisms
IP: Irreversible pulpitis
kVp: Kilovoltage peak
LD: Lamina dura
mA: Milliampere-seconds
MDA: Multiple displacement amplification
MTA: Mineral trioxide aggregate
NaOCl: Sodium hypochlorite
NC: Negative control

NEC: Negative extraction control
NGS: Next-generation sequencing
NRES: National Research Ethics Service
NTC: Non-template control
OR: Odds ratio
OTUs: Operational taxonomic units
PA: Periapical radiograph
PAI: Periapical index
PARL: Periapical radiolucency
PCR: Polymerase chain reaction
PDL: Periodontal ligament
PI: Previously initiated
PN: Pulpal necrosis
PRIRATE: Preferred Reporting Items for randomised trials in endodontics
QC: Quality check
QIIME: Quantitative Insights into Microbial Ecology
qPCR: Quantitative polymerase chain reaction
R&D: Research and development
RCT: Root canal treatment
RD: Rubber dam
REC: Research ethics committee
rRNA: The bacterial 16S ribosomal RNA
S1: Initial intracanal sample
S2: Pre-obturation intracanal sample
SIP: Symptomatic irreversible pulpitis
SP: Standard protocol
T0: Baseline visit
T12: One-year follow-up visit
TAE: Tris-acetate-EDTA
TIFF: Tagged image file format
ZOE: Zinc oxide eugenol
δCt: delta Ct ($\delta Ct = Ct_{obturation(S2)} - Ct_{initial(S1)}$)
µSv: Micro Sievert

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I've come to believe that science is love and each research project is a love story. This research is no exception—it was a labour of love, done with the support of many individuals whom I had the privilege of having by my side throughout my PhD journey.

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Introduction

Overview

Asepsis in endodontics aims to control all potential sources of infection, including chemical disinfection of the operative field and the use of sterile instruments (Ørstavik, 2020). Inadvertent introduction of bacteria into the root canal system may occur when the aseptic chain is breached during treatment. Since the success of root canal treatment is critically dependent on the reduction of bacterial load present in the root canal system and the prevention of reinfection (Siqueira *et al.*, 1998), the risk of secondary infection might arise when root canal space is being exposed and manipulated by the clinician (Hargreaves *et al.*, 2016, Rotstein and Ingle, 2019). Therefore, measures and efforts should be taken towards the prevention of such microbial access and establishing an aseptic environment (Sathorn *et al.*, 2007, Bergenholtz *et al.*, 2013, Ørstavik, 2020). These measures include the use of rubber dam, scaling and polishing of tooth surfaces, caries removal of target tooth, chemical disinfection of the operative field, and the use of sterile instruments (Ørstavik, 2020).

Previous studies demonstrated significant clinical and preclinical contamination, from gloves, rubber dam, or dental materials (Williams *et al.*, 2003, Niazi *et al.*, 2016, Saeed *et al.*, 2017). However, clinical evidence supporting the effectiveness of these measures or other sterility protocols on root canal treatment outcomes is lacking.

Research aims and objectives

This clinical PhD project comprises two parts: a pilot clinical study and a randomised clinical trial.

The first part is a pilot study including a series of experiments to identify different sources and levels of contamination occurring during the process of root canal treatment. This was achieved by isolating microorganisms and investigating them quantitively and qualitatively from different sites throughout the endodontic treatment of 30 cases diagnosed as irreversible

pulpitis. The findings of these preliminary investigations provided information on the steps necessary to develop the enhanced infection control protocol and gain a mechanistic insight into the initial bacterial ingress into the root canal space.

The second part is a randomised clinical trial comparing the clinical and radiographical outcome of primary root canal treatment undertaken using the enhanced infection control protocol with that of root canal treatments undertaken using a standard protocol. Microbiological investigations were also undertaken as a part of the clinical trial.

The objectives of the pilot study were:

1. To generate proof-of-concept data to determine the feasibility of implementing an "enhanced infection control" protocol in root canal treatments.
2. To assess the presence, nature and level of bacterial contamination during root canal treatment from different sites throughout treatment of teeth presenting with irreversible pulpitis.
3. To identify unknown sources of iatrogenic contamination during RCT and forming the empirical basis of the complete study design.
4. To determine the microbiological status of vital cases, and early colonisation sites if present.

The objectives of the clinical trial were:

1. To compare the outcome of root canal treatments undertaken with the standard and with the enhanced infection contol protocol using CBCT.
2. To develop an evidence-based enhanced infection control protocol for endodontic treatment.
3. To minimise intra-operative cross infection and contamination of the root canal system.

4. To investigate the bacterial presence, load and types associated with diseased root canal systems.
5. To investigate the correlation between bacteriological status of root canal system and treatment outcomes after one-year recall.
6. Investigate the effect of the EP on microbial composition after chemomechanical preparation.

Structure of the thesis

This thesis is divided into seven chapters; a critical review of literature is provided in the first chapter, giving an overview of endodontic diseases and the microbiological approaches used to investigate endodontic microbiota with more focus on molecular methods such as qPCR and NGS. The review of literature also includes details on the microbiota associated with different stages of endodontic diseases. Next, the different radiological approaches to assess the outcomes, the outcomes of primary root canal treatments and factors affecting outcomes are also discussed. The last section of the literature review provides an overview of the studies examining the different possible sources of contamination during treatment, and the asepsis measures recommendation during treatment.

Chapter 2 is the pilot study commenced prior to the clinical study to provide a protocol for the EP used. The pilot study investigated the microbial load and composition of different possible sources of iatrogenic contamination.

Chapter 3 describes the microbiological load and composition of teeth diagnosed with IP using molecular methods.

Chapter 4 is dedicated to the randomised clinical trial, investigating the outcome of endodontic primary root canal treatments in molar teeth assigned to two treatment protocols: SP and EP. One-year follow-up was carried out clinically and radiographically using PA and CBCT. In addition, a molecular microbiological analysis was conducted (Chapters five and six).

Chapter 5 is an *in vivo* molecular study investigating the microbial load of the initial and pre-obturation intracanal samples taken as a part of the clinical study and explores the effect of EP on microbial load.

Chapter 6 investigates the change in the microbial composition of intracanal samples and the influence of the implemented protocols on specific microbiota remaining after chemomechanical preparation.

Finally, Chapter 7 gives an overview of the main findings of the pilot and clinical studies, together with recommendations for future work. Figure i-1 summarises the workflow of the research undertaken in this project.

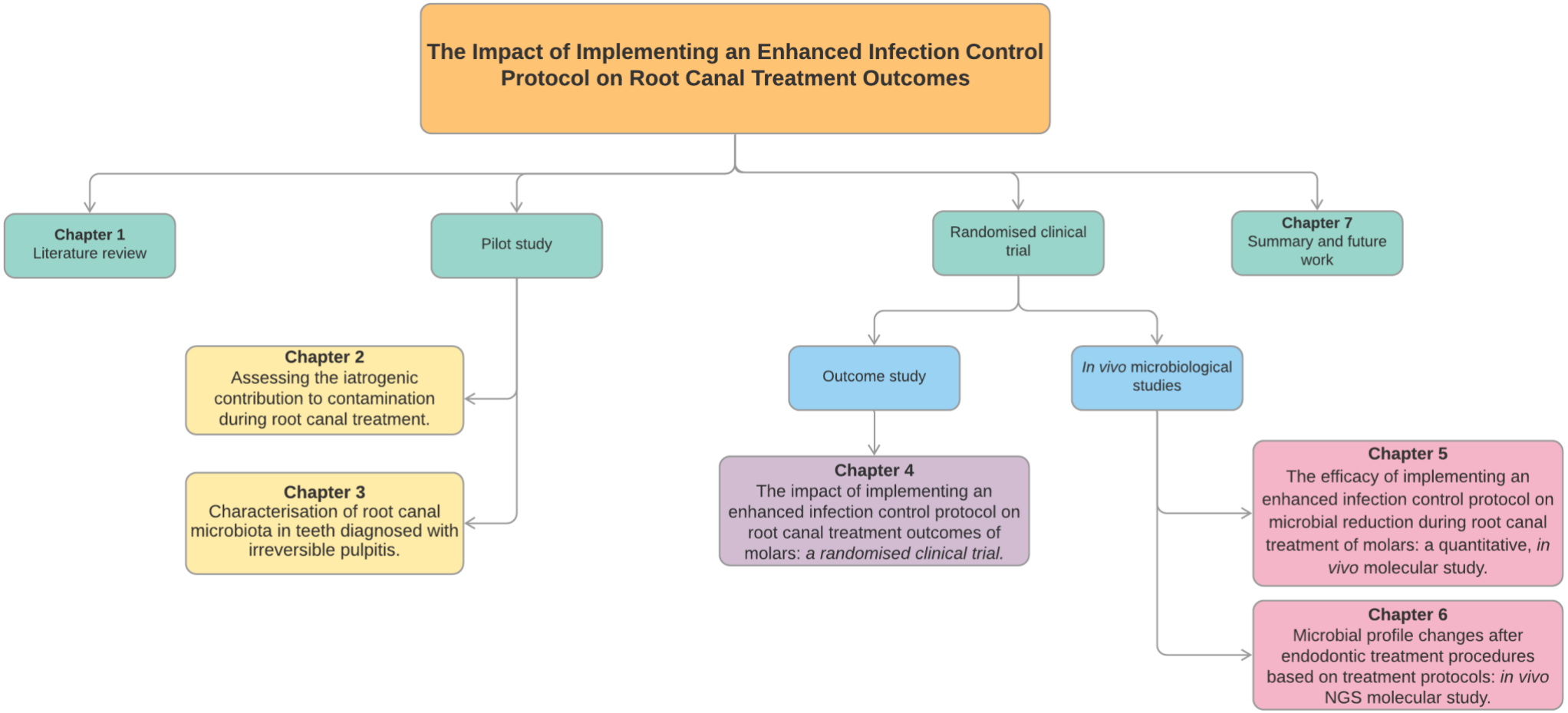


Figure i-1: The workflow of the research accomplished in this project.

Chapter One: Literature Review

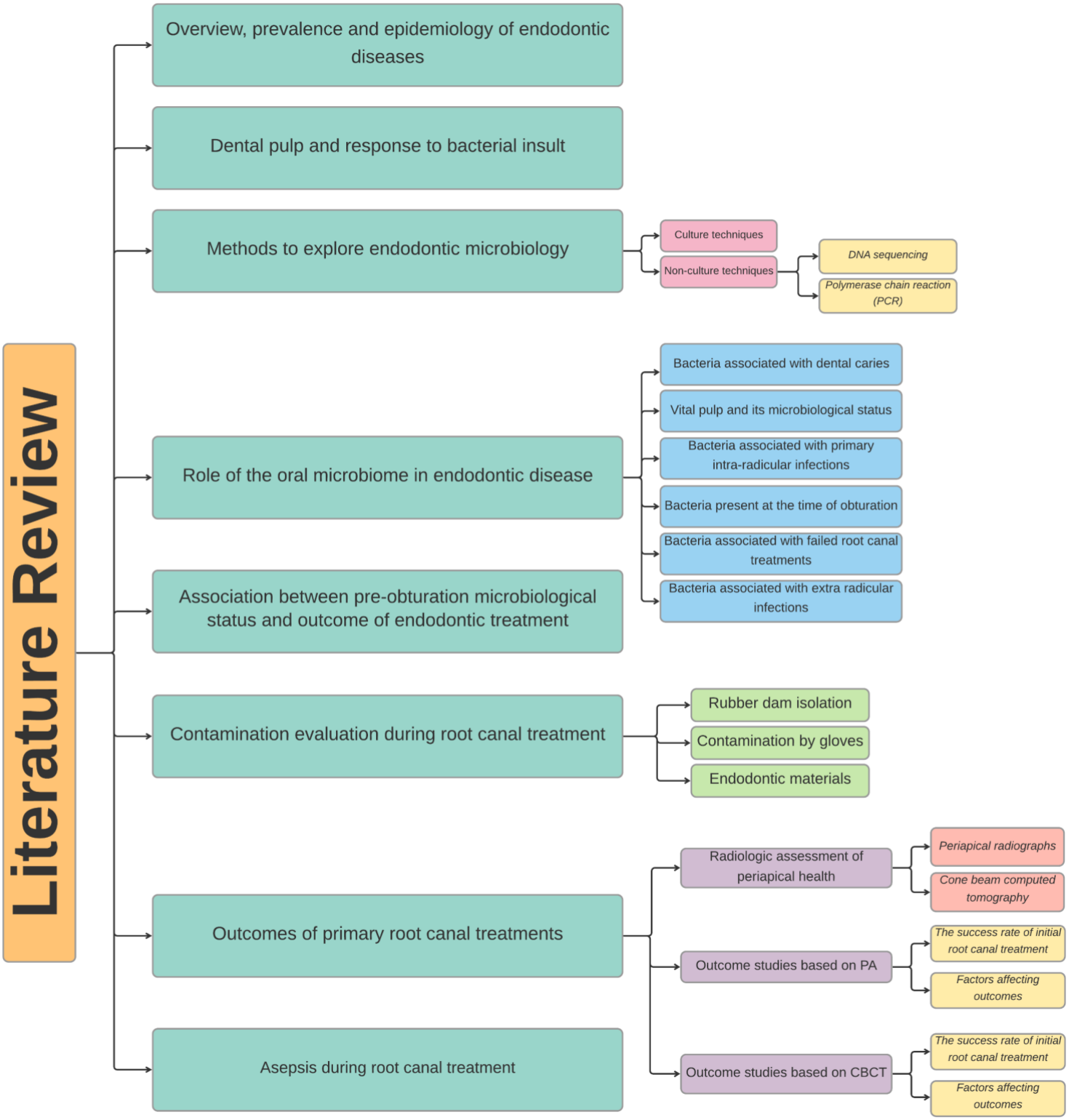


Figure 1.1: A summary of the literature review flow of Chapter 1.

1.1. Overview, prevalence and epidemiology of endodontic diseases

Endodontology is focused on the study of the pulp and periradicular tissue with respect to the anatomy and pathology, along with prevention and treatment of associated diseases (European Society of Endodontology, 2006). The goal of endodontic treatment is the prevention and/or elimination of apical periodontitis (AP), thus to restore or preserve the health of the periradicular tissues (European Society of Endodontology, 2006).

The oral microbiome is not only associated with endodontic diseases, but also with periodontal diseases and dental caries in its own, niche-specific communities. Host-immune response to microbiological colonisation in the root canal system will result in the pathologic effect of AP.

Apical periodontitis is the inflammatory process in the apical periodontium that develops and progresses as a response to the presence of microorganisms and their by-products within the root canal system of the tooth (Nair, 1997).

The diagnosis of AP is usually based on the clinical and radiographic manifestations. Clinical symptoms range from absence of clinical symptoms (no pain on percussion or palpation) to painful response to biting and/or percussion or palpation, while radiographic changes will occur depending on the stage of disease process presenting with periapical radiolucency. The radiographic diagnosis can be achieved with 2D periapical radiographs or 3D cone-beam computed tomography (Glickman, 2009).

In the US, it is estimated that 22.3 million endodontic procedures were performed annually, of which primary root canal treatment accounted for 15.1 million each year (American Dental

Association, 2007). Meanwhile in England, approximately more than 800,000 endodontic treatments were provided by the NHS yearly (NHS Business Services Authority Dental Practice, 2003).

The prevalence of AP has been reported in many studies, ranging from 2.9% to 7.3% of examined teeth in European countries (Ödesjö *et al.*, 1990, Lupi-Pegurier *et al.*, 2002). In the United States, Buckley and Spangberg (2005) found that 4.1% of all teeth and 31.3% of root-filled teeth were associated with periapical radiolucencies (Buckley and Spangberg, 1995). This percentage markedly increases with age: in the elderly, 7% of teeth had periapical radiolucency (PARL), 4% of those with PARL were associated with untreated teeth (Hamedy *et al.*, 2016). Findings of a systematic review covering studies spanning from 1968 to 2011 revealed that 5% of all examined teeth had PARLs, and a figure of 10% was attained when teeth were endodontically treated. The prevalence of PARL was very high, broadly equivalent to one radiolucency per patient (Pak *et al.*, 2012). More recently, a meta-analysis of cross-sectional studies published from 2012 to 2020 heightened an increase of the prevalence of apical periodontitis in the adult population. Overall, 6.3% of teeth had AP and 7.4% of all examined teeth had non-surgical root canal treatment. About forty percent of teeth which had root canal treatment were associated with apical periodontitis. It is predicted that the estimate of apical periodontitis will increase worldwide (Jakovljevic *et al.*, 2020).

Whilst PARL is conventionally examined by plain film radiography, when CBCT is used as the diagnostic method, 5.81% of all teeth examined had radiographic evidence of PARL (Dutta *et al.*, 2014). This figure was increased to 10.4% in another cross-sectional study (Meirinhos *et al.*, 2020). Additionally, almost half of the root-filled teeth were associated with PARL detected with CBCT (Gomes *et al.*, 2015a, Karabucak *et al.*, 2016, Meirinhos *et al.*, 2020).

1.2. Dental pulp and response to bacterial insult

It is generally agreed that the local immunological reaction of the pulp will eliminate early bacterial invasion. The pulp is well equipped to initiate innate immune reaction including antimicrobial peptides by pulp cells, and proteins that block bacterial invasion, followed by adaptive immune reaction which provides a more specific antigen response via T and B cells giving rise to cell-mediated and humoral immunity (Warfvinge and Bergenholtz, 1986, Hahn and Liewehr, 2007a). Even in early caries stages, such as white spot lesions, pulpal inflammatory reaction is evident way before bacteria reach the pulp tissue, as bacterial components can diffuse through dentinal tubules and induce localised pulpal inflammatory responses (Brannstrom and Lind, 1965, Warfvinge and Bergenholtz, 1986).

As caries progresses and demineralises enamel and reaches dentine, changes within the microflora are evident as bacteria become more anaerobic in deeper carious lesions as a response to the inflammatory reactions taking place in the pulp tissue (Chhour *et al.*, 2005).

Progression to a severe inflammatory reaction will result when the pulp is exposed to caries leading to micro-abscesses within the pulp chamber. This eventually will advance to irreversible pulpitis if the insult is not removed (Reeves and Stanley, 1966, Trowbridge, 1981, Levin *et al.*, 2009). Cells within the pulp such as odontoblasts, endothelial, and stem cells have receptors as the toll-like receptors that can recognise the microbial components such as lipopolysaccharides, lipoteichoic acids, and bacterial DNA. This cellular / bacterial interaction can initiate transcriptional regulatory pathways as those nuclear factor kappa B result in releasing antimicrobial molecules and cytokines to eventually control immune cell recruitment, activation, and differentiation. Examples of pulp inflammatory cytokines include interleukin (IL)-1a, IL1-b IL-4, IL-6, IL-8, IL-10, transforming growth factor beta 1 (TGF-b1), and tumour

necrosis factor alpha (TNF-a) all of which will activate more immune cells (Cooper *et al.*, 2014, Farges *et al.*, 2015). This induced inflammation can be further aggravated as the release of immune complexes and by-products from immune response, such as extracellular proteolytic enzymes released by phagocytosis aiming at bacterial elimination. Ultimately, the release of proteolytic enzymes by neutrophil granulocytes combined with oedema compromising vascular supply will lead to tissue necrosis if the infection was not controlled or arrested.

When the bacteria, their metabolites and virulence factors, such as lipopolysaccharides, together with the inflammatory response elicited exceed the pulpal regenerative potential, the pulp becomes necrotic and an intervention is required as primary root canal treatment (Langeland, 1987, Hahn and Liewehr, 2007a).

1.3. Methods to explore endodontic microbiology

1.3.1. Culture techniques

Until recently, the standard methods to explore disease-related pathogens as well as to identify endodontic microorganisms were restricted to culture-based approaches. With improving insights into growth media and the growth conditions, scientists were able to culture some microorganisms under specific laboratory conditions. This approach has its advantages and drawbacks. The main advantage is that this method allows studying microorganism susceptibilities, their physiology and pathogenicity (Siqueira and Rôças, 2005b). Drawbacks are summarised in Table 1.1.

Studies have reported that more than a third of oral microorganisms are uncultivable (Relman, 1999, Hugenholtz, 2002, Wade *et al.*, 2016). Regarding endodontic microorganisms, it has been reported that 66% of those in infected root canals are yet to be successfully cultivated (Ribeiro *et al.*, 2011). Moreover, the bias rises when these culture-based methods only show easier-to-culture genera / phyla. This consequently resulted in over-representation of some phyla such as *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Bacteroidetes* and genera such as *Streptococcus* (Hugenholtz, 2002).

1.3.2. Non-culture techniques

Over the past two decades, non-culture approaches have overcome the limitations of previous culture methods. The ability to isolate, amplify and interpret bacterial genotypic (DNA or RNA) rather than phenotypic features eliminated the shortcomings of culture techniques. Several advantages and drawbacks of this approach are explained in Table 1.1.

Molecular approaches target the genetic component, which allows microbial identification. This targeted gene is usually the gene encoding rRNA molecules which are present in all types of bacteria and have been not only used to identify bacteria, but all living organisms (Woese, 1987). The most useful and frequent target for a molecular approach is the 16S rDNA gene, which is the gene that encodes the 16S ribosomal RNA in prokaryotic DNA. The 16S rRNA molecule is part of the 30S ribosomal subunit of bacterial rRNA molecule. This molecule is approximately 1550 bp long. It has been widely used for microbial identification, characterisation and classification. The 16S rRNA gene includes both conserved and at least nine hypervariable regions (V1-V9). The conservative regions are identical in all members of a domain while the variable regions carry specific information about genus and species and substantially differ among bacterial taxa, hence those variable regions are used for organism identification (Woese, 1987, Madigan MT, 2000).

With this approach, the number of phyla identified rose from 11 in 1987 to almost 52 in 2003 (Rappe´ and Giovannoni, 2003). It also revealed that the oral microbiome is the second most complex microbiome after the gut, having more than 1000 taxa (The Human Microbiome Project Consortium, 2012). Within the field of endodontics, molecular methods widened the field of endodontic microbiology and broadened our knowledge to a greater extent (Zehnder and Belibasakis, 2015). Besides confirming the findings of the culture-based method, molecular techniques discovered new pathogens associated with periapical diseases, including *Tannerella forsythia, Dialister* species and *Spirochetes* (Conrads *et al.*, 1997, Siqueira *et al.*, 2000b, Rolph *et al.*, 2001). Collectively, up to date, more than 460 bacterial taxa from 100 genera and nine phyla were identified in the infected root canal space. Those uncultivated taxa were overlooked with culture-based approaches and might play an essential role in the pathogenesis, severity or symptoms associated with endodontic diseases (Siqueira and Rôças, 2009c).

Non-culture-based technologies include both closed-ended and open-ended molecular methods which have been developed to identify structure and diversity of the microbial community. Closed-ended approaches are more classical and designed to target 20–30 bacterial species (Siqueira and Rôças, 2005b). Examples of different microbiological approaches are shown in Figure 1.2.

Table 1.1: Advantages and drawbacks of culture-dependent and molecular methods.

Drawbacks of culture-dependent methods:

1. Inaccurate in detecting microorganisms with demanding environmental and nutritional needs as some bacteria are fastidious growing, and others are impossible to cultivate.
2. Difficulty in finding a suitable universal media as well as maintaining a fastidious nutritional requirement for bacterial growing as there is missing or very little knowledge about the growth conditions and nutritional needs of some pathogens.
3. Underestimation of the diversity and richness of the actual microbial community.
4. Time-consuming – might take up to weeks and be costly.
5. Rely on subjective judgements when determining bacteria phenotypic characteristics.
6. Misidentification of bacteria; some bacteria are genetically similar and might evolve differently phenotypically and vice versa (convergent and divergent strains).
7. Impossible to cultivate and identify a vast number of species as in endodontic disease.

Advantages of molecular methods:

1. The technique is more sensitive and accurate since it can detect both living and dead organisms.
2. Ability to detect bacteria that not yet have been cultured, as well as known species.
3. Considered faster, less time-consuming and can detect a large number of organisms.
4. More precise in organism identification and higher specificity (including divergent or convergent strains).
5. Sampling and transportation are less fastidious since it does not require controlled anaerobic conditions.
6. Does not require immediate processing of bacteria; large numbers of samples can be stored, processed and analysed at the same time.

Drawbacks of molecular methods:

1. The inability to differentiate cell viability is considered as the main drawback.
2. The possibility of contamination during sampling or DNA extraction procedures can dramatically alter results and overestimates the actual microbiological profiles.
3. There is no efficient way for decontamination in these molecular methods and positive DNA is still detected in some control samples.

(Relman and Falkow, 1992, Tanner *et al.*, 1992, Josephson *et al.*, 1993, Kell and Young, 2000, Rolph *et al.*, 2001, Wade, 2002, Sundqvist and Figdor, 2003, Wade, 2004, Siqueira and Rôças, 2005b, Figdor and Brundin, 2016).

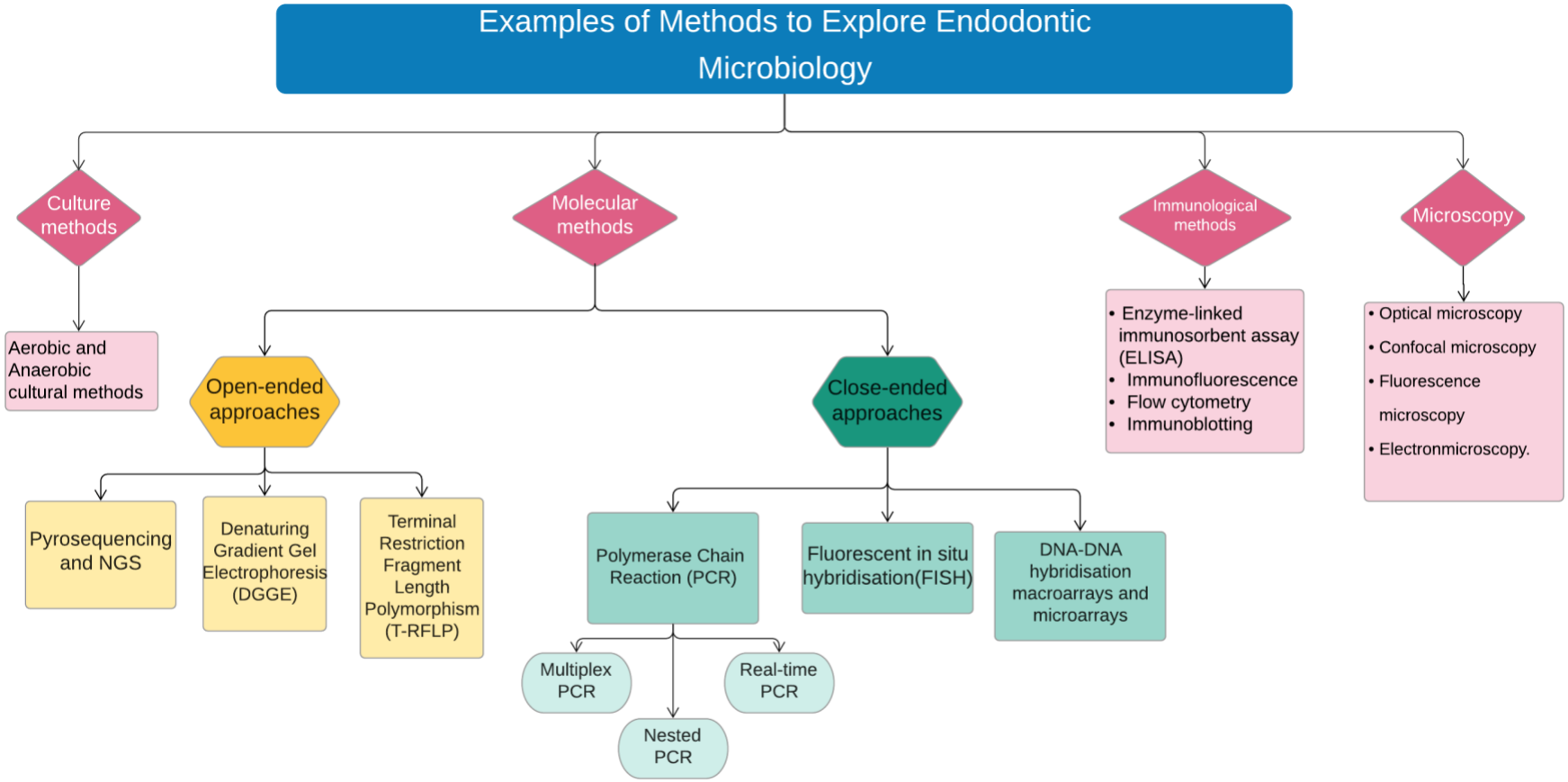


Figure 1.2: Examples of methods to explore endodontic microbiology.

Polymerase chain reaction (PCR)

In 1983, Kary Mullis invented the process of PCR. This invention enhanced current biological and medical research radically, and genomic studies rapidly developed afterwards. The basic concept of the PCR method is the *in vitro* replication of DNA through repetitive cycles of denaturation, primer annealing and extension (Mullis, 1994). In endodontics, the adoption of PCR-based approaches resulted in a revolution in the explored endodontic microbiota. Compared to other identification methods as cultures, DNA hybridisation or immunological methods, the PCR-based approach is at least 10–100 times more sensitive and more user-friendly. It can identify as few as 1–10 bacterial cells in a sample. This high sensitivity is particularly imperative in the field of endodontics due to difficulties encountered when sampling microbiota from the root canal. As many bacteria present in difficult-to-approach lateral canals, ramifications or deep within the dentinal tubules, sampling of the main canal results in very low numbers of bacteria. Factors affecting sampling effectiveness include, but are not limited to, the number and size of paper point, their depth of penetration, absorption power and the size of the root canal space (Siqueira and Rôças, 2003a).

The first report used a PCR non-culture method in endodontic infection targeting universal 16S rDNA found *T. forsythia* to be associated with many endodontic diseases, which had not been reported in previous culture-based investigations (Conrads *et al.*, 1997).

Following that, many PCR studies investigated unknown organisms in infected root canals. Nested PCR studies reported the presence of *Treponema socranskii*, *Treponema vincentii* and *Prevotella tannerae* in root canal infections (Xia *et al.*, 2000, Rôças *et al.*, 2003). Well-established periodontal pathogens such as *Treponema denticola* and *Tannerella forsythensis* were never detected by culture but identified by PCR among endodontic pathogens (Conrads *et al.*, 1997, Rôças *et al.*, 2001). Moreover, black-pigmented Gram-negative anaerobic bacteria,

such as *Porphyromonas endodontalis* and *Porphyromonas gingivalis* were detected by cultures, but noticeably higher prevalence was reported when assessed by PCR (Machado de Oliveira *et al.*, 2000).

Unlike conventional PCR methods which are qualitative, real-time polymerase chain reaction (qPCR) is a quantitative method used to estimate the number of bacterial rRNA gene copies present in samples by continuous measurement of amplification products. Three different approaches are available for the qPCR: SYBR-Green, TaqMan, and molecular beacon. SYBR-Green is currently the most direct, facile and simple method in which a fluorescent dye binds to double-stranded DNA. With continuous forming of double-stranded DNA during the reaction, the amount of fluorescence increases and is measured at the end of the extension step of every PCR cycle. This method, although highly sensitive, leads to false-positive reading results from dye binds to all double-stranded DNA present resulting in primer dimers and thus low specificity (Bustin, 2000, Siqueira and Rôças, 2005b). Limitations of this approach are shown in Table 1.2.

Table 1.2: Limitations of PCR and derivatives approaches

1. Most PCR assays only detect one or a few different species as well as target species, with the exception of broad-range PCR analysis.
2. The technique is laborious and costly, as well as sensitive to DNA extraction.
3. Contaminant DNA will be amplified, resulting in false-positive results.
4. Bias can arise from primer design and improper selection of primers and probes sets.

DNA sequencing

After PCR amplification of bacteria, the type of bacteria found in a selected community can be identified using many techniques. Examples are cloning and Sanger sequencing, molecular fingerprinting methods such as denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism analysis (T-RFLP). Although these techniques are very sensitive, their main drawback is the inability to detect low abundance microorganisms. They can only detect the most abundant bacteria in the community which results in overlooking less prevalent bacteria (Siqueira and Rôças, 2009b, Siqueira and Rôças, 2009c).

In the field of sequencing, the Sanger method is considered the first-generation technology developed in 1977 (Gomes and Korf, 2018). For more than ten years now, high-throughput DNA sequencing technologies have been developed and used to investigate the human mycobiome, including the oral cavity, thoroughly. This method is so-called next-generation sequencing (NGS). The technology refers to the deep, high-throughput, in-parallel DNA sequencing (Mardis, 2008), and is based on the 16S rRNA analysis. This massive parallel pyrosequencing technique became a powerful tool because it allows the detection of both dominant and low abundance microbial organisms by providing a large number of sequences reads in a single run (Hong *et al.*, 2013, Tzanetakis *et al.*, 2015). Low abundant microorganisms might have a crucial role in endodontic pathogenicity and might be easily overlooked with other less sensitive methods (Siqueira *et al.*, 2011). The principle of this method relies on 16S ribosomal RNA gene PCR amplification followed by sequencing (Mardis, 2008). Since this gene had different hypervariable regions, the analysis pipeline clusters these variable regions based on specific sequence into operational taxonomic unit (OTU) (Schmidt *et al.*, 2014) and these OTUs will be assigned to specific taxa when compared to 16S rRNA gene databases.

Compared to traditional Sanger sequencing, NGS provided a more comprehensive and broad-range open-ended analysis (Sogin *et al.*, 2006, Higuchi *et al.*, 2011, Siqueira *et al.*, 2012).

Further advancements in this technology resulted in whole-genome sequencing. Rather than targeted 16S rRNA amplification, the entire 16S gene is amplified and sequenced – "metagenomics" (Goodwin *et al.*, 2016). Furthermore, studies also incorporated metagenomes with meta-proteomes and metabolomes (multi-omics), which can reveal the interrelation between microbiome communities and their virulence factors as well as the host-immune response (Manoil *et al.*, 2020).

NGS has been employed in the field of endodontics within the last decade to explore the microflora of primary and infected root canal, as well as acute abscesses. It allowed not only the identification of uncultured taxa but also the taxa that do not belong to phylogenetically validly described taxa (Manoil *et al.*, 2020).

A number of studies adopted this technique in endodontics (Santos *et al.*, 2011, Siqueira *et al.*, 2011, Özok *et al.*, 2012, Anderson *et al.*, 2013, Hong *et al.*, 2013, Vengerfeldt *et al.*, 2014, Gomes *et al.*, 2015b, Tzanetakis *et al.*, 2015, Keskin *et al.*, 2017, Persoon *et al.*, 2017, Bouillaguet *et al.*, 2018, İriboz *et al.*, 2018). As a result, studies showed that there are no species exclusively presented in one endodontic infection, but all present with different relative abundances. It also allowed confirmation of the presence of previously reported taxa as well as countless low abundant unidentified taxa, enabling a better understanding of the microbial community. Such low-abundance taxa play an important role as they might favour environmental changes in the future, allowing them to dominate and become more pathogenic (Hong *et al.*, 2013, Vengerfeldt *et al.*, 2014, Tzanetakis *et al.*, 2015, Keskin *et al.*, 2017, Manoil *et al.*, 2020). Advantages and limitations of NGS methods are explicated in Table 1.3.

Table 1.3: Advantages and limitations of NGS methods

Advantages
1. Higher chance in detecting rare species through the ability to identify a low number of sequences per sample.
2. A large number of sequence reads, and information data can be acquired in a single run.
3. It is accurate, easy to automate, flexible, applicable, and uses parallel processing.
4. The sequencing accuracy of Illumina technology has been documented as more than 98.5%.
5. The application of NGS, particularly Illumina, in determining endodontic microbial community can be considered as relatively low cost if compared with the other techniques.
Limitations
1. Short-length reads can be considered as a limitation when microbial identification to species level and below is required, as too short sequencing can provide less phylogenetic information.
2. Primer selection and design.
3. One major shortcoming of DNA-based studies is the inability to discriminate dead from live microorganisms. Instead, all genetic material, including damaged and non-viable cells, are assessed.

(Ronaghi, 2001, Siqueira *et al.*, 2012, Ari and Arikan, 2016)

1.4. Role of the oral microbiome in endodontic disease

It is well-established that endodontic infection is polymicrobial in origin (Sundqvist *et al.*, 1989, Chávez de Paz, 2005). Nine out of thirteen phyla typically found as commensals in the oral flora have been identified within endodontic infections; these phyla were Firmicutes, Bacteroidetes, Actinobacteria, Fusobacteria, Proteobacteria, Spirochaetes, Synergistes, TM7, and Sulphur River1 (SR1) (Siqueira and Rôças, 2009c). Bacteria play important roles in endodontic infections, and the richness of species is much broader than viruses, fungi and archaea (Slots *et al.*, 2003, Waltimo *et al.*, 2003, Siqueira and Sen, 2004, Vianna *et al.*, 2006a). Investigating the whole endodontic microbiome is fundamental to understanding the development of different diseases and to raise the level of practice to a higher quality by providing approaches to control, prevent and manage those microorganisms (Siqueira and Rôças, 2009c).

1.4.1. Bacteria associated with dental caries

Dental caries is one of the most widespread chronic human diseases and the most common sequelae of an infectious aetiology worldwide (Selwitz *et al.*, 2007), affecting more than 2.4 billion people worldwide and considered the leading cause of oral pain and tooth loss (Langeland, 1987, O Fejerskov, 2003). Established carious lesions involve polymicrobial biofilms within the dentine substrate, leading to chronic, low-grade inflammatory responses within the confined pulp space that arise from diffusing microbial products (Bergenholtz, 1981). As dental caries is the main factor causing pulpal inflammation, investigations on caries microbiome are fundamental.

Both culture and molecular methods have demonstrated a predominant, facultatively anaerobic community in caries, with members of the Gram-positive genus *Lactobacillus* commonly enriched. Other often widely found carious taxa include *Prevotella, Selenomonas, Dialister* and *Fusobacterium* (Edwardsson, 1974, Hahn *et al.*, 1991, Martin *et al.*, 2002, Munson *et al.*, 2004, Chhour *et al.*, 2005, Lima *et al.*, 2011). Within the deeper layers of carious dentine, this microbiota shifts from facultative Gram-positive bacteria to anaerobic Gram-positive and negative cocci and rods (Hoshino, 1985).

Culture-based approaches have been used intensively to investigate carious microbiota conventionally. Those commonly associated taxa reported were from the genera *Streptococcus, Actinomyces, Lactobacillus, Bifidobacterium, Rothia, Arachnia, Eubacterium, Propionibacterium, Veillonella*, and *Prevotella* (Hahn *et al.*, 1991).

Molecular methods and sequencing of bacterial 16S ribosomal DNAs revealed higher complexity of microbiota in dental caries than conventional methods. Novel species have been reported, and around 50% were not cultivable (Becker *et al.*, 2002, Aas *et al.*, 2008). Cloning and 16S rRNA gene sequence analysis revealed around 100 different taxa, with each carious

lesion harbouring at least 30 different taxa. Gram-positive bacteria, particularly from the genera *Actinomyces, Lactobacillus, Propionibacterium*, and *Streptococcus* were identified, predominated by *Streptococcus* and *Lactobacillus* species (Becker *et al.*, 2002, Munson *et al.*, 2004).

As endodontic infection most commonly develops from caries, those bacteria in the deeper layer of caries lesion are of interest. Those bacteria might be initial colonisers, triggering the pulpal response and inflammatory reaction leading to irreversible pulpitis (Hahn *et al.*, 1991, Martin *et al.*, 2002, Rôças *et al.*, 2015).

Chhour and co-workers explored bacteria in ten advanced caries lesions using PCR followed by phylogenetic analysis. The most common species found were *Lactobacilli,* comprising 50% of the species, followed by *Prevotella*. Also, *Selenomonas* sp., *Dialister* sp., *Fusobacterium nucleatum*, *Eubacterium* sp., *Olsenella* sp., *Bifidobacterium* sp., *Propionibacterium* sp., and *Pseudoramibacter alactolyticus* were found to be of high abundance (Chhour *et al.*, 2005).

Rôças *et al*. investigated deep dental caries microbiome in 30 cases with pulpal exposure and diagnosed as symptomatic irreversible pulpitis. Using reverse-capture checkerboard analysis, the most frequent species were *Atopobium genomospecies C1* (53%), *Pseudoramibacter alactolyticus* (37%), *Streptococcus* species (33%), *Parvimonas micra* (13%), *Fusobacterium nucleatum* (13%) and *Veillonella* species (13%) (Rôças *et al.*, 2015).

When next-generation sequencing was used in similarly diagnosed cases, a more extensive bacterial diversity was reported. Bacterial taxa were assigned to 14 phyla and 101 genera. At the phyla level, 98% of the sequences belonged to *Firmicutes*, *Actinobacteria* and *Proteobacteria*. While at the genera level, *Lactobacillus, Olsenella, Pseudoramibacter* and *Streptococcus* were most commonly isolated. Those genera were widely reported in the infected root canal, with the exception of *Lactobacillus* species (Rôças *et al.*, 2016).

During the pathological process of caries, some microbes persisted, while others either appeared or were enriched in the later stage towards pulpal inflammation. The environmental changes, including the source of nutrients as glycoproteins from inflammatory exudate unlike to host diet in superficial caries, are among the ecological changes affecting microbial selectivity. Without longitudinal animal studies, these hypotheses regarding environmental changes during the carious pathological process are yet to be confirmed (Grenier and Mayrand, 1986). Previous studies also noted that some endodontic pathogens were simultaneously expressed only in advanced caries lesions and assumed to be the possible candidates triggering pulp inflammation and carrying the contributing bacteria (Rôças *et al.*, 2015, Rôças *et al.*, 2016).

Lactobacilli were the most dominating taxa in advanced caries, but their presence in infected canals is clearly diminished. It has been proposed that *Lactobacilli* are associated with caries progression rather than initiation (Minah and Loesche, 1977, van Houte, 1994). Another study found that half of the carious samples from cases diagnosed as irreversible pulpitis (IP) were dominated with *Lactobacillus* suggested as the pathogenic role of this microorganism causing IP. The same research indicated regression of *Actinobacteria* along with *Firmicutes* enrichment as caries progresses (Zheng *et al.*, 2019).

1.4.2. Vital pulp and its microbiological status

The rate of microbial penetration in vital and necrotic pulp is believed to differ significantly. This progression in a healthy tooth may be slow or impossible while rapid in necrotic pulp (Chirnside, 1961, Michelich *et al.*, 1980, Pashley *et al.*, 1984b, Nagaoka *et al.*, 1995).

Pashley *et al*. conducted an *in vivo* dog study to investigate dentine permeability. Cavities were prepared in molar teeth, and fluid-filtration was used to quantify dentine permeability. Their findings showed that permeability of dentine diminished by time only in vital intact teeth. On the other hand, there was no change or even slight increase in permeability when the pulp is removed (Pashley *et al.*, 1984b).

Following that, Nagaoka group carried out an ex-vivo study in which third molars planned for future extractions were either subjected to pulpectomy or kept with intact pulp. Class V cavities were prepared in both groups and left exposed to oral flora for 30 or 150 days. The depth and rate of bacterial penetration were measured after extraction and teeth were sectioned under microscopy and SEM. Following an exposure period of 150 days, the extent of microbial invasion was significantly lower in vital teeth than in non-vital (Nagaoka *et al.*, 1995).

Many reasons might contribute to this permeability difference. The dentinal tubules in healthy, vital teeth are filled with odontoblastic processes, collagen fibres, and dentinal fluid, thus resulting in reducing the dentinal tubular diameter and playing an essential role in resisting the microbial invasion by acting as a physical barrier. On the other hand, the dentinal tubules content in necrotic pulp changes, pulp tissue is typically removed, and the natural physical barrier exerted by the presence of odontoblastic process is diminished, facilitating bacterial advancement (Michelich *et al.*, 1980, Pashley, 1983, Trowbridge, 1984). Additionally, the intra-pulpal pressure in vital teeth causes an outward movement of the dentinal fluid opposing the inward movement of oral microorganisms (Olgart *et al.*, 1974, Michelich *et al.*, 1980).

Also, the plasma protein fibrinogen deposition on the tubular wall continues in the vital pulp and the diameter of the tubules decreases, while diameters remain unchanged in non-vital teeth (Pashley *et al.*, 1984a).

A recent study investigated the microbial profile in the root canal space of ten teeth diagnosed with irreversible pulpitis. Gram-positive and Gram-negative, cocci and bacilli, and facultative and strict anaerobes were evident. *P. micra, F. periodonticum, F. nucleatum, T. forsythia,* and *L. buccalis* were present in all samples (Arruda-Vasconcelos *et al.*, 2021). Another recently published paper raised the concern that the vital pulp tissue of healthy, virgin teeth might not be sterile, thus, challenging the belief that bacterial presence is incompatible with normal pulp vitality. In their investigation, 10 cases of radiographically healthy, caries-free, unrestored single-rooted teeth with no evidence of periodontal disease and completely formed apices were included. A highly-effective field decontamination protocol was followed. Bacterial DNA was found in all tested healthy pulp tissues sampled and sequenced. An average of 343 taxa per sample and 12 unique taxa were identified as being common to all pulp tissues sampled. The most represented taxa were the genera *Ralstonia*, *Acinetobacter*, and *Staphylococcus* (Widmer *et al.*, 2018). This is in contrast with the current understanding of pulp canal sterility before the ingress of bacteria from caries, cracks, periodontal space or exposed dentinal tubules (Siqueira JF Jr, 2011). They attributed that to the possibility of bacteria entering the canal space from the blood as bacteraemia resulting from dental procedures reflected in pulp tissue when using broach rather than a paper point for sample collection. In their study, possible environmental, reagent or sampling contamination cannot be excluded (Widmer *et al.*, 2018). Another study using NGS sampling root canal space of healthy control teeth similarly reported bacterial taxa (Qian *et al.*, 2019).

Histopathological examination of teeth clinically diagnosed as IP revealed localised areas of coagulation or liquefaction necrosis accompanied by inflammation and PMNs infiltration. Bacterial colonies were evident in approximately 85% of the coronal pulps of such cases (Ricucci *et al.*, 2014).

1.4.3. Bacteria associated with primary intra-radicular infections

Very much in keeping within the caries microbiome, primary endodontic infections are polymicrobial in origin. Microorganisms which invade the root canal predominantly arise from dental caries and colonise the necrotic pulp tissue. This microbiome is comprised of mixed communities dominated by approximately 10 to 30 anaerobic predominant species per canal, giving it the nature of heterogeneous aetiology (Munson *et al.*, 2002, Siqueira and Rôças, 2005a, Sakamoto *et al.*, 2007). It is also characterised by the variation between individuals and geographic locations (Siqueira and Rôças, 2009c). The total number of bacteria ranged from 10^3 to 10^8 cells per canal (Sakamoto *et al.*, 2007, Siqueira *et al.*, 2007d).

In a review by Siqueira in 2009, all datasets from culture and molecular studies were integrated. He reported that in infected root canal spaces, bacteria belonging to nine phyla, 82 genera and over 391 taxa were identified. From those, 261 taxa were unique for primary infection and were not reported in other types of endodontic infections (Siqueira and Rôças, 2009c). Remarkably, 40-55% of those microorganisms found in primary infected root canals were considered as-yet-uncultivated bacteria. Those uncultivable organisms were as abundant as 38% and 30% of the total sequenced clones in cases with chronic or acute apical abscesses (Munson *et al.*, 2002, Sakamoto *et al.*, 2006, Sakamoto *et al.*, 2007, Siqueira and Rôças, 2009c).

The collective term of *black-pigmented bacteria* was frequently used to describe predominant phenotypes of poorly-described bacteria associated with primary infections. This is a group of Gram-negative anaerobic rods which can form black colonies in blood agar, hence the name. This group was later classified into two genera: *Prevotella* and *Porphyromonas*. The most frequently reported species of the genera *Prevotella* were *P. intermedia*, *P. nigrescens*, *P. tannerae*, *P. baroniae*, and *P. denticola*, while the genera *Porphyromonas* was represented with *P. endodontalis* and *P. gingivalis* in primary infected root canals.

Although it is known that the most common microorganisms in primary infected root canals are Gram-negative bacteria, unlike caries in which Gram-positive *Lactobacilli* predominate, still, members of Gram-positive rods and Gram-positive cocci, specifically *Peptostreptococci* sp. and *Streptococci* sp. are frequently observed in infected root canals (Munson *et al.*, 2002, Sakamoto *et al.*, 2006, Sakamoto *et al.*, 2007, Siqueira and Rôças, 2009c).

Overall, Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria were the phyla with the most species richness. The most represented genera were *Prevotell*a, *Eubacterium*, *Streptococcus* and *Lactobacillus* (Siqueira and Rôças, 2009c). Figure 1.3 shows the most commonly found bacteria in primary endodontic infections.

Endodontic pathogens associated with primary endodontic infections were expanded after the introduction of molecular methods. The first PCR study identified *Tannerella forsythia* as a primary endodontic pathogen which has never been cultivated before (Conrads *et al.*, 1997). Following that, many non-culture methods studies identified further bacteria associated with primary endodontic infections; some were never identified by cultural methods such as *Haemophilus aphrophilus, Porphyromonas gingivalis, Corynebacterium matruchotii, Treponema denticola, Capnocytophaga gingivalis, Streptococcus intermedius, Fusobacterium naviforme,* and *Actinobacillus actinomycetemcomitans*. Also, members of known periodontal pathogens were present, such as *Porphyromonas gingivalis, Prevotella intermedia, Peptostreptococcus micros, Treponema socranskii*, and *Treponema denticola*. Additionally, some bacteria were only introduced with non-cultural methods as members of the genus *Olsenella*, new species of *Dialister*, unculturable clones of the phyla *Spirochaetes* and *Synergistetes*. Other species were reported as *Porphyromonas endodontalis*, *Fusobacterium nucleatum*, *Prevotella buccae*, and *Peptostreptococcus anaerobius* (Siqueira *et al.*, 2000b,

Siqueira *et al.*, 2000c, Rolph *et al.*, 2001, Fouad *et al.*, 2002, Munson *et al.*, 2002, Siqueira *et al.*, 2005, Vianna *et al.*, 2006a, Siqueira and Rôças, 2007).

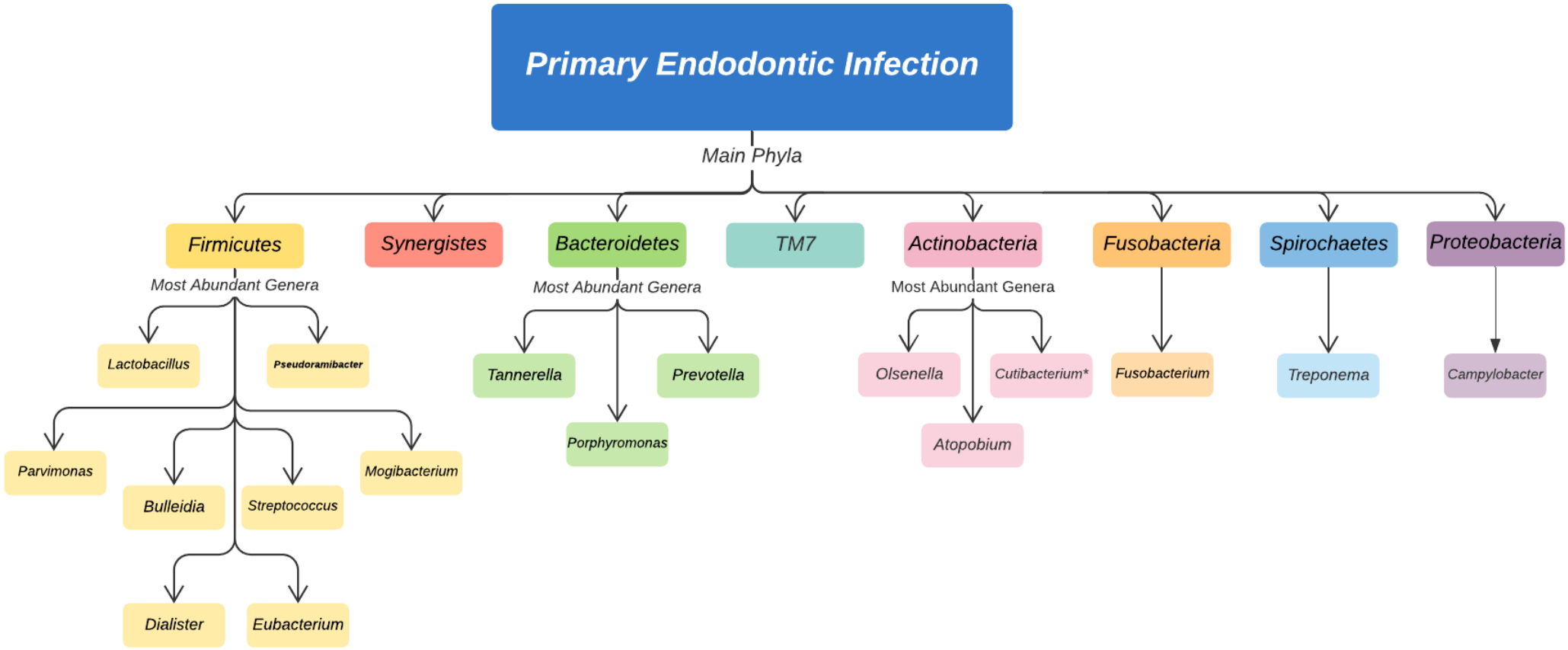
The apical third of the canal harbour microbiota is of particular interest in the development of AP. Siqueira *et al.* explored the apical microorganisms of 23 extracted primary infected teeth with periapical radiolucency. Using nested PCR targeting 11 bacterial species, commonly found microbes in infected root canal were *T. denticola, P. endodontalis, T. forsythia, P. alactolyticus, D. pneumosintes, F. alocis, P. gingivalis, P. propionicum* and *T. socranskii*. The most prevalent species was *P. alactolyticus* detected in 44% of the cases (Siqueira *et al.*, 2004).

Next-generation sequencing is a relatively recent technology to explore microbial presence. A number of studies analysed the microbiome present in primary infected root canals. Table 1.4 summarises the most predominant genera / species found in such cases (Santos *et al.*, 2011, Siqueira *et al.*, 2011, Özok *et al.*, 2012, Hong *et al.*, 2013, Gomes *et al.*, 2015b, Tzanetakis *et al.*, 2015, Keskin *et al.*, 2017, Persoon *et al.*, 2017, Bouillaguet *et al.*, 2018, İriboz *et al.*, 2018).

Results of NGS studies analysing endodontic microbiome are greatly diverse. Different reasons are responsible for such varied microbial findings. First, geographical or ecological factors are well recognised inherent factors to affect the radicular microbial community (Machado de Oliveira *et al.*, 2007, Siqueira *et al.*, 2008). Also, the stage of disease progression and clinical presentations might differ between studies and even within the samples of the same study.

Another source of differences is due to diverse DNA extraction methods, PCR amplification, and targeted regions. Although most studies targeted V1–V2 and V3–V4 regions, targeting V1-V2 regions reported to be less reproducible and can underestimate some taxa (Manoil *et al.*,

2020). Variations in results might finally originate from the bioinformatic analysis. Different pipelines were used, as well as different databases, clustering assignment and thresholds, and normalisation was not standard among studies.



* *Cutibacterium* formerly known as *Propionibacterium*.

Figure 1.3: Most common genera in primary endodontic infections.

Table 1.4: Summary of NGS studies' findings on primary endodontic infections.

Study	# of samples	Region	Most prevalent taxa (relevant abundance)
(Santos *et al.*, 2011)	8	V4	*Phocaeicola* (12.5%), *Eubacterium* (12%), and *Pseudoramibacter* (10%).
(Siqueira *et al.*, 2011)	10	V1-V2	*Fusobacterium* (15%), *Pseudoramibacter* (8%), *Novosphingobium* (8%), *Ralstonia* (6%), and *Bacterioides* (5%).
(Özok *et al.*, 2012)	23	V3-V4	*Lactobacillus* (14.3%), *Actinomyces* (12%), Unclassified *Actinobacteria* (7%), *Prevotella* (6.1%), *Parvimonas* (3.4%), *Pseudoramibacter* (3%), *Veillonellaceae* (2.5%), and *Fusobacterium* (2%).
(Hong *et al.*, 2013)	10	V1-V2	*Prevotella*, *Propionibacterium*, and *Pyramidobacter*.
(Tzanetakis *et al.*, 2015)	24	V1-V2	*Bacteroidacea*, Unclassified *Pyramidobacter*, and *Parvimonas.*
(Gomes *et al.*, 2015b)	15	V3-V4	*Enterococcus faecalis*, *Parvimonas micra*, *Bacteroidaceae* [G-1] sp. oral taxon 272, *Peptostreptococcaceae*, *Mogibacterium timidum*, and *Peptostreptococcus stomatis.*
(Persoon *et al.*, 2017)	23	V3-V4	*Prevotella* (12.7%), *Lactobacillus* (11.2%), *Actinomyces* (7.5%), *Fusobacterium* (7.2%), *Atopobium* (6.9%), *Streptococcus* (4.4%), and *Leptotrichia* (4.3%).
(Keskin *et al.*, 2017)	20	V3-V4	*Prevotella* (19.6%), *Porphyromonas* (16.5%), *Neisseria* (13.2%), *Lactobacillus* (11.7%), *Parvimonas* (11.1%), *Streptococcus* (10.7%), Enterococcus (3.5%).
(İriboz *et al.*, 2018)	20	V3-V4	*Dialister* (94%), *Paludibacter* (30%), *Agreggatibacter* (21.6%), *Porphyromonas endodontalis* (16.4%), *Tannerella* (13.8%), *Prevotella* (6.9%).
(Bouillaguet *et al.*, 2018)	21	V3-V4	*Fusobacterium nucleatum* (16%), *Parvimonas micra* (8%), *Porphyromonas endodontalis* (5.7%), *Dialister pneumosintes* (3.4%), *Prevotella oris* (5.7%).

1.4.4. Bacteria present at the time of obturation (microbiological status of pre-obturation intracanal samples)

Samples can be taken at the time of obturation to assess the effectiveness of chemomechanical preparation, intracanal medication or treatment-enhancing procedures. Also, microorganisms at root canal filling stage might be correlated to a future failure of the treatment or jeopardise treatment outcomes. Clinical studies demonstrated that complete sterility cannot be attained at the time of obturation and an average count of one to five bacterial species (10^2 to 10^5 cells per canal) were found (Byström and Sundqvist, 1985, Sjögren *et al.*, 1997, Williams *et al.*, 2006, Sakamoto *et al.*, 2007, Siqueira *et al.*, 2007b, Siqueira *et al.*, 2007c, Rôças and Siqueira, 2011b). Some studies showed that pre-obturation samples had approximately 20% to 80% positive cultures (Byström and Sundqvist, 1985, Ercan *et al.*, 2004) and 60-70% positive PCR signals to bacteria (Rôças and Siqueira, 2010, Rôças and Siqueira, 2011b), indicating that complete sterilisation of the root canal system is unapplicable, but the reduction of bacterial number and diversity is achievable during treatment (Siqueira and Rôças, 2009c).

Combined culture and molecular methods revealed more than 100 bacterial taxa at the time of obturation after chemomechanical preparation with or without the use of intracanal medication. Those detected taxa belonged to five phyla and 41 genera (Siqueira and Rôças, 2009c). Most detected species belonged to the phyla *Firmicutes*, *Proteobacteria,* and *Actinobacteria* followed by *Bacteroidetes* and *Fusobacteria* (Sjögren *et al.*, 1997, Chávez de Paz, 2004, Chu *et al.*, 2006).

Studies have shown that Gram-negative bacteria which are commonly overpopulated in the primary infection are usually eliminated after chemomechanical measures and are less resistant to treatment. Yet, members of Gram-negative anaerobic rods have been reported persisting after chemomechanical measures such as *F. nucleatum, Prevotella species*, and *C. rectus*.

Unlike Gram-negative bacteria, Gram-positive microorganisms usually resist the treatment measures and predominantly recovered after treatment (Chávez de Paz, 2005, Siqueira and Rôças, 2009c). Examples of the commonly reported taxa: *Streptococci* (*S. mitis, S. gordonii, S. anginosus, S. sanguinis, and S. oralis*), *Actinomyces* species (*A. israelii and A. odontolyticus*), *Propionibacterium* species (*P. acnes and P. propionicum*), *Lactobacilli* (*L. paracasei* and *L. acidophilus*). *E. faecalis, P. micra*, *Bifidobacterium* species, *Eubacterium* species, and *Staphylococci* were also reported (Byström and Sundqvist, 1985, Sjögren *et al.*, 1997, Chávez De Paz *et al.*, 2003, Chávez de Paz, 2004, Chu *et al.*, 2006, Sakamoto *et al.*, 2007, Rôças and Siqueira, 2011a, Rôças and Siqueira, 2011b).

Using NGS methodologies, a recent study examined pre-obturation samples of 20 necrotic teeth taken after root canal preparation, calcium hydroxide medication, 2.5% NaOCl and 17% EDTA. The reduction of the relative amount of *Agrobacterium* sp., *Aggregatibacter* sp. and *Porphyromonas endodontalis* was not significant, indicating their high persistence after treatment (İriboz *et al.*, 2018).

It is known that planktonic bacteria are easily susceptible to alkaline stress. On the other hand, bacteria arranging themselves into micro-communities and biofilms increases their resistance remarkably (Chávez de Paz, 2005). Besides biofilms arrangements, those persistent bacteria might be intrinsically able to resist antimicrobial agents, or their metabolic products can counteract the antimicrobial effects. Their presence in the inaccessible areas within the root canal system might be another contributing factor. Also, the inadequate exposure time to irrigant or inactivation of irrigant due to dentine present, inflammatory exudates or necrotic tissue might be another reason for bacterial persistence (Siqueira *et al.*, 2007d). Furthermore, bacteria presented with higher prevalence initially have more chance to survive after treatment (Rôças and Siqueira, 2011a, Rôças and Siqueira, 2011b).

Although these bacterial taxa have been found persisting endodontic treatment (Sundqvist *et al.*, 1998, Rôças *et al.*, 2008, Schirrmeister *et al.*, 2009), the importance of these bacteria on the treatment outcome is yet to be ascertained. Many of those bacteria might not survive after obturation and nutritional resources sealed off. Others might survive longer periods of starvation and harsh environmental conditions. Longitudinal clinical studies are needed to clarify an association between specific taxa and failure of root canal treatment using genomic methods (Rôças and Siqueira, 2010). Clinical studies are needed to investigate whether those remaining bacteria are insignificant in determining the treatment outcome, or our clinical procedures, irrigation techniques, instrumentation strategies, detection methods are yet to be improved (Rôças and Siqueira, 2010).

1.4.5. Bacteria associated with failed root canal treatments

Secondary endodontic infection is attributed to the introduction of microorganisms that were not present in the primary infection. These might arise from penetration into the root canal system during the treatment, between the appointments or after the treatment completion, followed by their ability to adapt and survive within this niche. On the other hand, persistent infection results from the presence of organisms that were members of the primary / secondary infection and were able to resist the antimicrobial intervention during the course of treatment (Siqueira, 2002).

Samples can be taken from failed previously treated cases due to the development / persisting symptoms and /or apical periodontitis. Findings of culture-based studies clearly revealed a different community in failed cases compared to primary infection, usually composed of a single species or significantly lower number of species in which Gram-positive bacteria predominate, particularly *Enterococcus faecalis*. Species ranges from one to five in failed, otherwise well obturated teeth, while up to 30 species were found in inadequately treated cases (Molander *et al.*, 1998, Sundqvist *et al.*, 1998, Pinheiro *et al.*, 2003, Rôças *et al.*, 2004a).

Within these samples, both molecular and culture methods revealed almost 160 bacterial taxa. Molecular methods detected 109 taxa whilst 72 were confirmed using conventional bacterial culture (Siqueira and Rôças, 2009c). Still, as-yet-uncultivated phylotypes in molecular methods corresponded to 55% of the taxa identified (Sakamoto *et al.*, 2008). Those bacteria belonged to seven phyla and 58 genera. Similar to primary infection, Firmicutes, followed by Actinobacteria, Proteobacteria and Bacteroidetes were the phyla with greater richness. Members of Spirochaetes and Synergistes were also reported (Siqueira and Rôças, 2009c).

Using 16S rRNA gene clone library analysis, one study reported very high inter-individual variability in detected taxa among failed cases. A total of 74 taxa were found, but a few were encountered in more than one sample, such as *Bacteroidetes* oral clone X083, *Prevotella oris*, *Pseudomonas aeruginosa*, *Streptococcus mutans*, *Synergistes* clone BA121, *Peptostreptococcus* clone FG014, *Dialister* clone 9N-1, and *E. faecalis* (Sakamoto *et al.*, 2008).

Adopting next-generation sequencing methods, studies have expanded knowledge regarding the microbiota associated with failed cases. One of the first reported studies was the work by Anderson and co-workers that reported 277 genera associated with failed cases as *Streptococcus, Prevotella* and *Lactobacillus* predominated (Anderson *et al.*, 2013). Subsequently, Siqueira revealed *Fusobacterium* and *Pseudomonas* followed by *Enterococcus* to be highly abundant in such cases (Siqueira *et al.*, 2016). Zandi and co-workers also reported *Fusobacterium* among the most abundant genera followed by *Streptococcus*, and *Actinomyces* (Zandi *et al.*, 2018).

Sequencing methods were used to compare primary and secondary infection cases. The microbiota of secondary infected cases was reported to be similar to primary infection in regard to composition and diversity in two studies (Hong *et al.*, 2013, Keskin *et al.*, 2017), and differ in three (Vengerfeldt *et al.*, 2014, Tzanetakis *et al.*, 2015, Bouillaguet *et al.*, 2018). Unlike the commonly believed concept, one study reported a higher bacterial diversity among persistent compared to primary endodontic infections (Tzanetakis *et al.*, 2015).

Comparing the primary and secondary infected cases at the phyla level, Proteobacteria and Tenericutes were enriched in secondary in one study, while Actinobacteria in another. At the genus level, *Lactobacillus, Streptococcus, Sphingomonas* and *Ralstonia* predominated failed

cases in one study, while *Enterococcus faecalis* was found significantly more abundant or only present in failed cases (Vengerfeldt *et al.*, 2014, Tzanetakis *et al.*, 2015, Bouillaguet *et al.*, 2018). Table 1.5 summarises the findings of NGS studies of post-treatment failed cases.

E. faecalis has been frequently reported in studies using culture and non-culture methods as the most commonly encountered species in failed cases. Culture methods revealed it as the only organism present in the canal of all failed cases, with a prevalence of up to 90% of the cases (Molander *et al.*, 1998, Sundqvist *et al.*, 1998, Pinheiro *et al.*, 2003, Rôças *et al.*, 2004b, Sedgley *et al.*, 2006). PCR studies confirmed that this species was the most prevalent organism, present in 64% to 77% of the cases (Siqueira and Rôças, 2004). Furthermore, nested PCR study found that *E. faecalis* was strongly associated with failed cases, unlike primary infections. It was nine times more likely to retrieve *E. faecalis* in failed cases compared to primary infected cases (Rôças *et al.*, 2004b, Williams *et al.*, 2006). On the other hand, other PCR studies revealed the *E. fecalis* in failed cases was never among the most dominant taxa, arguing its aetiology never as a single organism but within a mixed community (Rôças *et al.*, 2004a, Sakamoto *et al.*, 2008). NGS studies also reported the presence of *Enterococcus*. In one study, it was the most abundant genera (13.9%) though present in only two out on 10 cases (Zandi *et al.*, 2018). In accordance with Zandi, Bouillaguet *et al*. detected *E. fecalis* abundances ranging from 17% to 99.9% in 7/22 cases which was significantly higher than in primary infections (Bouillaguet *et al.*, 2018). Another study reported their presence only in failed cases and never in primary (Tzanetakis *et al.*, 2015), while others reported its presence only at very low abundance (Hong *et al.*, 2013, Siqueira *et al.*, 2016).

Table 1.5: Summary of post-treatment failed cases microbiome (NGS studies).

Study	#of samples	Region	Abundant Phyla	Abundant Genera
(Hong *et al.*, 2013)	8	V1-V2	10 phyla Bacteroidetes (29.6%), Firmicutes (23.2%), Actinobacteria (10.5%), and Fusobacteria (13%).	133 genera *Fusobacterium* (14.6%), *Porphyromonas* (10.6%), *Prevotella* (8.6%), *Peptostreptococcaceae* (5.4%), and *Dialister* (4.9%).
(Anderson *et al.*, 2013)	40	V1-V2	14 phyla Firmicutes (30%), Proteobacteria (26%), Actinobacteria (22.7%), Bacteroidetes (13.3%), and Fusobacteria (4.5%).	277 genera *Streptococcus* (10.9%), *Prevotella* (8.21%), *Lactobacillus* (8.06%), *Kocuria* (5.17%), Neisseria (3.38%), and *Enterococcus* (2.59%).
(Tzanetakis *et al.*, 2015)	24	V1-V2	11 phyla Bacteroidetes (37%), Firmicutes (30%), Fusobacteria (11%), Actinobacteria (7.6%), Synergistetes (7.4%), and Proteobacteria (6.4%).	109 genera *Fusobacterium* (11%), *Bacteroidaceae* (10%), and *Prevotella* (9.4%).
(Siqueira *et al.*, 2016)	10	V3-V4	11 phyla Proteobacteria (46%), Firmicutes (18%), Fusobacteria (15%), and Actinobacteria (8%).	103 genera *Fusobacterium* (15%), *Pseudomonas* (15%), *Klebsiella* (5%), and *Stenotrophomonas* (4%).
(Zandi *et al.*, 2018)	10	V3-V5	9 phyla Firmicutes (47%), Fusobacteria (14%), Bacteroidetes (12%), Proteobacteria (12%), Actinobacteria (9%), and Synergistetes (4%).	59 genera *Enterococcus* (13.9%), *Fusobacterium* (12.7%), *Streptococcus* (9.8%), *Actinomyces* (8.2%), *Desulfobulbus* (5.2%), and *Fretibacterium* (3.6%).
(Keskin *et al.*, 2017)	20	V4	15 phyla Proteobacteria (35.8%), Firmicutes (32.3%), Bacteroidetes (26.3%), and Fusobacteria (4.2%).	157 genera *Prevotella* (15.7%), *Porphyromonas* (16.5%), *Neisseria* (13.2%), *Lactobacillus* (11.7%), *Parvimonas* (11.1%), *Streptococcus* (12%), and *Enterococcus* (5%).
(Bouillaguet *et al.*, 2018)	22	V3-V4	16 phyla Firmicutes, Bacteroidetes, Actinobacteria, Fusobacteria, and Synergistetes.	177 genera *Fusobacterium* (5.3%), *Enterococcus* (18.9%), and *Streptococcus* (3.5%).
(Qian *et al.*, 2019)	8	V3-V4	11 phyla Firmicutes (26.2%), Bacteroidetes (17.3%), Actinobacteria (13.6%), Fusobacteria (8.6%), and Proteobacteria (28.7%).	Abundance of genera were not clearly reported.

1.4.6. Bacteria associated with extra-radicular infections

The invasion of intra-radicular microorganisms into the inflamed periradicular tissue is known as extra-radicular infection, and this condition is one of the recognised controversies in endodontics (Tronstad *et al.*, 1987, Bergenholtz and Spangberg, 2004). The extra-radicular infection might be dependent or independent of the intra-radicular infection (Siqueira and Rôças, 2009c). These types of infections are noticeable frequently as recalcitrant to orthograde endodontic treatment (Tronstad *et al.*, 1987). Some microorganisms have the ability to develop pathogenicity, allowing them to invade and survive the periradicular tissue such as *Actinomyces* species and *P. propionicum* (Siqueira Jr, 2003).

Culture studies revealed limited types of bacteria associated with treatment failures in such lesions as *Actinomyces*, *Staphylococcus epidermidis, Bacteroid* and *Streptococcus* species (Tronstad *et al.*, 1987, Sunde *et al.*, 2000a). Recent molecular studies showed way more organisms surviving this harsh environment. Using checkerboard DNA-DNA hybridisation taken from pathological samples, 11 to 34 species per sample were found. The most commonly found organisms were *Fusobacterium*, *Porphyromonas, Prevotella*, *Campylobacter*, and *Treponema* and half of the examined lesions were infected with *P. endodontalis.* Confirming findings of culture-based studies, Gram-positive anaerobes such as *Actinomyces*, *Propionibacteria, Peptostreptococcus* were commonly detected (Gatti *et al.*, 2000, Sunde *et al.*, 2000b). Using the FISH technique, rods, spirochaetes and cocci were observed in 20 of 39 periradicular lesions. Taxa such as *Streptococcus, T. forsythia*, *P. gingivalis*, and *P. intermedia* were found to be physiologically active in these lesions (Sunde *et al.*, 2003). Overall, Firmicutes, Bacteroidetes followed by Proteobacteria were the most represented phyla in such lesions out of six phyla reported. The most reported species belonged to 38 genera, including

Actinomyces species, *P. acnes, P. propionicum, P. gingivalis, P. intermedia, Prevotella oralis, P. micra,* and *F. nucleatum* (Siqueira and Rôças, 2009c).

Table 1.6: Summary of different common taxa reported in primary, secondary, and extra-radicular endodontic infections.

Primary endodontic infections	Secondary endodontic infections	Extra-radicular infections
Bacteroidetes	*Enterococci*	*Actinobacteria*
Treponema	*Actinobacteria*	*Cutibacterium**
Porphyromonas	*Streptococcus*	*Prevotella*
Prevotella	*Cutibacterium**	*Staphylococcus*
Fusobacterium	*Staphylococcus*	*Porphyromonas*
Parvimonas	*Pseudomonas*	
Streptococcus		
Eubacterium		
Actinobacteria		
Campylobacter		
Mogibacterium		

*Formerly known as *Propionibacterium*

1.5. Association between pre-obturation microbiological status and outcome of endodontic treatment

Sampling the root canal space just before obturation has been commonly used in clinical endodontic studies to predict the treatment outcomes. The question of whether this method is sensitive enough to predict the root canal treatment outcomes is still uncertain (Sathorn *et al.*, 2007). An example to clarify the point; two-visit endodontic treatment and intracanal medication has proven to reduce the bacterial load and yielded fewer bacteria in many studies (Law and Messer, 2004). Subsequently, randomised clinical trials and meta-analysis did not prove that intracanal medication could lead to better outcomes, hence the negative bacterial culture did not equate to better healing (Trope *et al.*, 1999, Weiger *et al.*, 2000, Peters and Wesselink, 2002, Sathorn *et al.*, 2005).

Previous studies have explored the relationships between the microbiological status of the canal before obturation and root canal treatment outcomes. A considerable number of cases healed clinically, even with a positive culture at the time of obturation and vice versa, where clinically failed cases did not show bacterial growth (Sathorn *et al.*, 2007).

Historically, many studies showed that the microbiological status of the root canal at the time of obturation does not affect the healing of apical periodontitis and cannot be used as a prognostic factor for treatment outcome (Zeldow, 1963, Bender *et al.*, 1964, Engstrom and Lundberg, 1965, Heling and Shapira, 1978). The main drawback of these old studies is the lack of proper microbiological techniques in anaerobic culturing, resulting in underrepresenting the actual microbiological status (Sathorn *et al.*, 2007). Thereafter, some studies demonstrated that the outcomes of root canal treatment were significantly determined by the presence / absence of bacteria at the time of obturation (Sjögren *et al.*, 1997, Waltimo *et al.*, 2005, Fabricius *et al.*, 2006).

Sjögren and co-workers were the first to report this correlation. In their five years follow-up longitudinal study, the healing rate of 53 cases was 94% and 68% in negative and positive pre-obturation samples, respectively. A significant difference was observed, and they emphasised the microbial status of the root canal at the time of obturation as a critical factor determining the treatment outcomes. In their study, teeth with negative cultures at the time of obturation had a 6.8-fold higher likelihood to completely heal after treatment when compared to teeth with positive cultures (Sjögren *et al.*, 1997, Sathorn *et al.*, 2007).

Similarly, another one-year follow-up trial reported that negative cultures after chemomechanical preparations were strongly associated with success rate. In their study, 30 teeth were followed up for one year; the mean reduction of PAI score was 1.53 in the bacteria negative group and 0.79 in the bacteria positive group. The difference was statistically significant, confirming the notable impact of bacterial absence at the time of obturation on healing (Waltimo *et al.*, 2005).

In a monkey study model, Fabricius *et al*. highlighted the importance of having a bacteria-free sample before obturation. In their study, 175 infected root canals were endodontically treated and followed up radiographically after 2-2.5 years. When bacteria were present in the root canal system at the time of obturation, 79% of AP did not heal. On the other hand, only 28% of failures were observed in bacteria-free canals. They also stated that the quality of root canal filling did not affect the outcome in bacteria-free canals (Fabricius *et al.*, 2006).

In contrast to previously mentioned studies, other clinical and animal investigations did not confirm this association. In a dog experiment of 44 roots with AP, healing occurred even if bacteria present at the time of obturation (Allard *et al.*, 1987).

Three clinical trials also concluded the same, in which healing was not significantly impacted by bacterial conditions. One study examined 85 teeth for two to three years' follow-up. The

success rate was 76% and 73% in cases with negative and positive cultures, respectively. The difference was not significant (Matsumoto *et al.*, 1987). The second report had only 38 teeth, in which surprisingly greater success (87%) was noted in the positive culture group compared to negative cultures, which were associated with a 74% success, though a significant difference was not reached. Since the sample size of the two groups in the comparison was considerably different (30 vs. eight), the higher success in positive culture cases should be critically examined (Peters and Wesselink, 2002). Finally, Molander and co-workers showed that although positive bacteriological samples at time of obturation reduced the healing rate, the difference was not significant. They followed up 88 root canal-treated teeth for two years. Eighty percent of teeth with negative cultures at the time of obturation completely healed compared to 44% in cases with positive cultures. Also, the outcome "uncertain" was higher in positive samples (52% of the cases) in comparison to negative samples (7%) (Molander *et al.*, 2007). Figure 1.4 summarises the findings of studies associating microbiological findings at obturation stage to outcomes.

There are two main limitations of previous, earlier-mentioned studies. Firstly, all used culture-based methods in bacterial detection, in which limitations are recognised. Secondly, only a limited number of studies considered CBCT as a follow-up diagnostic method to assess periapical tissue healing. None of these studies correlated CBCT-based outcomes to the microbiological status before obturation. It is obvious that the low number of teeth involved in the above-mentioned trials, combined with the low level of sensitivity of the periapical radiographs used to assess the outcome, resulted in an insufficient power for the detection of differences between positive and negative pre-obturation cultures studies.

The current evidence we have indicates a very limited value of microbiological status in predicting the outcomes. The need for more clinical studies exploiting modern microbiological

and radiographic methods is evident. Additionally, it has always been a concern whether the current sampling procedures (paper point soaking in the root canal space) can accurately reflect the microbial status of the root canal (Sathorn *et al.*, 2007). This might contribute to the absence of bacteria in failed cases. One study compared the culture status of previously treated teeth. Four years after treatment completion, samples were collected upon access of failed cases and 32% of failed cases had negative cultures (Molander *et al.*, 1998), raising a concern regarding the reliability of paper points sampling and / or microbiological methods.

Factors affecting sampling effectiveness include the number and size of paper point, their depth of penetration, absorption power, and the size and shape of the root canal space (Siqueira and Rôças, 2003a). Finally, the possibility of contamination during sampling procedures may alter results, leading to overestimation of the actual microbiological profile.

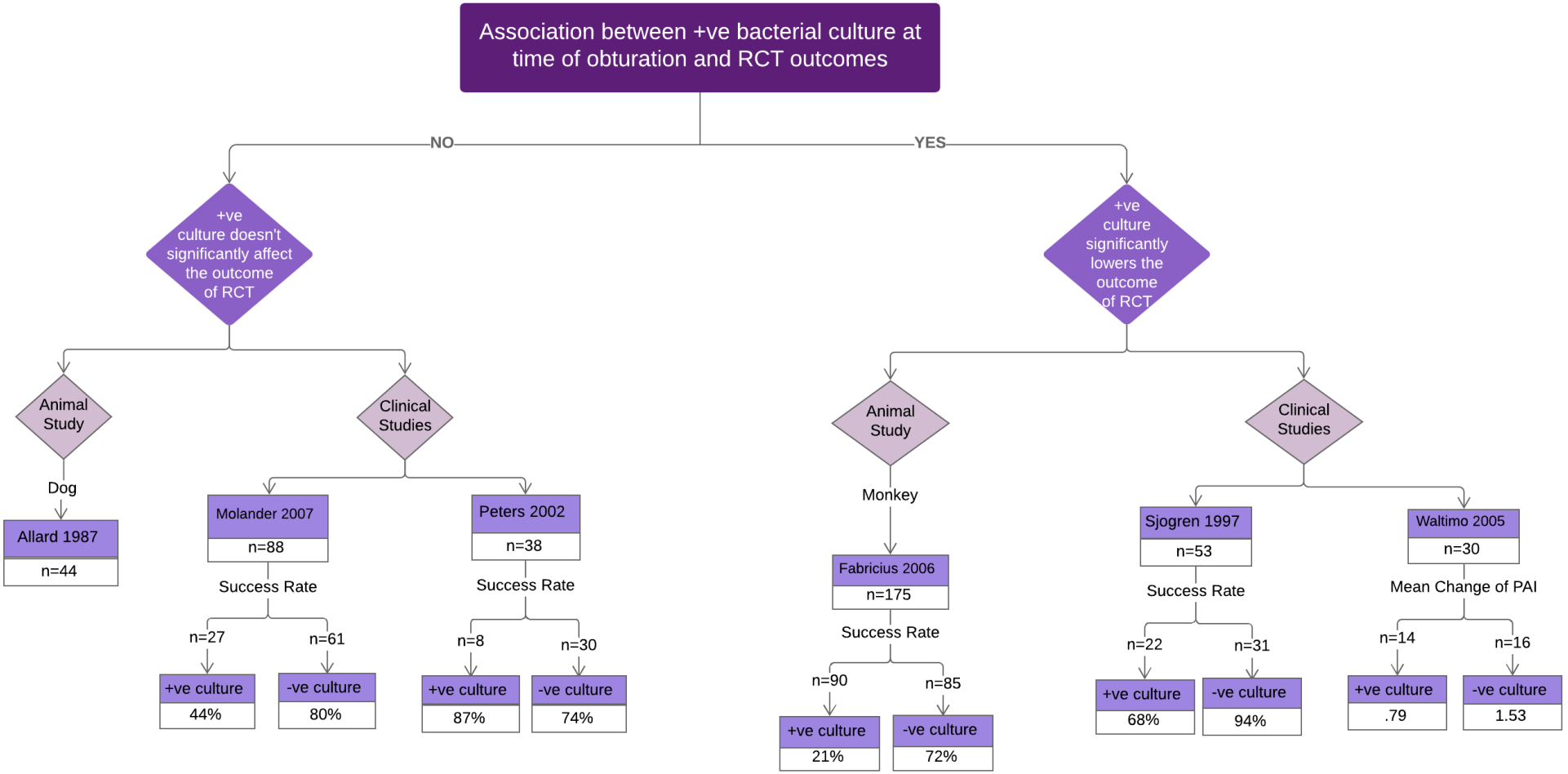


Figure 1.4: Summary of different studies associating outcomes to microbiological status of cultured pre-obturation samples.

1.6. Contamination evaluation during root canal treatment

Microorganisms and their by-products are involved from the initial carious process to the development of apical periodontitis (Kakehashi *et al.*, 1965, Möller, 1966, Möller *et al.*, 1981, Fabricius *et al.*, 1982). Therefore, treatment is principally targeting the removal of the aetiological agent(s). As a result, current treatment protocols (isolation, canal preparation, antibacterial irrigants, and intracanal medicaments) are directed toward bacterial elimination (Sathorn *et al.*, 2007).

Additionally, it is of paramount importance to prevent the introduction of microorganisms into the root canal system (Siqueira *et al.*, 1998, Schirrmeister *et al.*, 2007, Peciuliene *et al.*, 2008). This is achieved when aseptic measures are applied during treatment, and a sterile protocol is maintained during the treatment to prevent the adverse effect of bacteria and reinfections (Möller *et al.*, 1981, Ramachandran Nair, 1987).

It has been reported that iatrogenic contamination of the root canal space may occur during root canal treatment. The source of contaminating microorganisms may include the patient's saliva, gloves worn by the dentist, operating surfaces, the use of non-sterile materials and instruments, bacteria in the surrounding environment or bacterial leakage in between visits (Williams *et al.*, 2003, Niazi *et al.*, 2010, Niazi *et al.*, 2016, Saeed *et al.*, 2017). Thus, a substantial effort should be devoted to maintaining aseptic conditions during treatment.

1.6.1. Rubber dam isolation

As many endodontic pathogens are oral commensals, it is crucial to prevent the ingress of such organisms to the canal space. Thus, the use of a rubber dam is a mandatory quality guideline and a gold standard of care in endodontic practice to protect the patient as well as prevent bacterial contamination from saliva (European Society of Endodontology, 2006).

Isolation with the rubber dam was introduced in 1864 by Dr Sanford C. Barnum (Elderton, 1971). The survival of root canal treatment, as well as the outcome of retreatment, was reported to be significantly improved with the use of a rubber dam (Nieuwenhuysen *et al.*, 1994, Lin *et al.*, 2014, Lee *et al.*, 2017, Kwak *et al.*, 2019). In a population-based study, including 3,040,178 teeth receiving primary and secondary root canal treatments, the use of rubber dam resulted in significantly fewer extractions five years after treatment. The five-year survival rate of root canal treatment (retention in the oral cavity) was 92.9% and 90.6% with and without the use of rubber dam, respectively. While for retreatment, the survival rate dropped from 89.98% to 88.14% when rubber dam was not used (Kwak *et al.*, 2019).

While the use of rubber dam has been identified as a substantial factor in endodontic outcomes (Nieuwenhuysen *et al.*, 1994, Lin *et al.*, 2014, Lee *et al.*, 2017, Kwak *et al.*, 2019), still, the compliance to its use during root canal treatment is reported to be low, ranging from 11% to 90% worldwide (Ahmad, 2009). In the UK, self-reported surveys revealed that less than one-fifth of general dentists use the rubber dam regularly and 60% never used it during root canal treatments (Whitworth *et al.*, 2000, Jenkins *et al.*, 2001).

Despite the high rate of rubber dam use by endodontists (92-100%) when compared to general dentists (44%), the concern is whether a leakage-free field can be assured and maintained during treatment when the rubber dam is used (Whitten *et al.*, 1996, Ahmad, 2009, Anabtawi *et al.*, 2013). Studies acknowledged the difficulty in maintaining a sterile field during the course of treatment. The literature revealed that maintaining asepsis within the operative field was not predictably achieved after initial disinfection in any study (Baumgartner *et al.*, 1975, Fors *et al.*, 1986, Hermsen and Ludlow, 1987, Ng *et al.*, 2003, Malmberg *et al.*, 2016, Rorslett Hardersen *et al.*, 2019).

One study reported that positive culture was evident in 31.75% of 63 rubber dam surface sampled immediately after placement (Ng *et al.*, 2003). Other studies reported an increase in bacteria detected on the rubber dam surface after storage (Saeed *et al.*, 2017). For those reasons, studies suggested the use of disinfecting materials such as hydrogen peroxide, iodine, chlorhexidine, alcohol, and NaOCl for operative field disinfection (Malmberg *et al.*, 2016).

More than five decades ago, Möller found that disinfecting the operative field using 30% hydrogen peroxide (H_2O_2) resulted in significantly higher rates of culture-negative samples from tooth surfaces and tooth/rubber dam junction when compared to 5% iodine tincture (Möller, 1966). Alternatively, Ng *et al*. compared the operative field disinfection performance of 2.5% NaOCl and 10% iodine. None of the two solutions revealed a sterile, bacteria-free operative field when PCR was used as a means to detect potential microbial detection. No difference between the two methods in decontaminating the rubber dam surface was noted (16% positive bacteria in iodine and 13% in NaOCl group at the final stage). NaOCl was significantly more effective only on reducing bacteria on the tooth surface rather than the rubber dam when compared to iodine. The study recommended a second decontamination stage using NaOCl, which significantly reduced the number of bacteria compared to after first decontamination and access preparation (Ng *et al.*, 2003). Another more recent study using PCR showed that the rubber dam surface remained aseptic at the time of obturation in 81% of the cases after disinfection with 0.5% chlorhexidine in alcohol and 1% NaOCl. The study using gene sequencing also reported that common microorganisms on the rubber dam surface were *Bacteroidales* sp., *Propionibacterium* sp., Bacteroidetes sp., *Prevotella nigrescens*, *Haemophilus parainfluenzae*, *Fusobacterium nucleatum* and *Streptococcus mitis* (Rorslett Hardersen *et al.*, 2019).

1.6.2. Contamination by gloves

As suggested by the European Society of Endodontology (2006), the use of gloves is mandatory and forms part of the basic infection control regimen to prevent the two-way contamination between patient and practitioner. Operatory surfaces, such as the light handles and chair control switches, and even the air, carry the risk of harbouring bacteria. The source of bacteria was shown to be environmental or salivary (Vidana *et al.*, 2015) and this contamination was proven to increase during the day (Monarca *et al.*, 2000, Williams *et al.*, 2003). Touching these common surfaces with gloves might result in picking up those organisms and thus increase the possible risk of their transmission. Furthermore, manipulating gutta-percha (GP) points with the gloves resulted in 100% microbial growth on the GP point surfaces, specifically with *Staphylococcus* (Gomes *et al.*, 2005). Thus, evaluating gloves worn by the dentist from the microbial aspect is required. It has been demonstrated that freshly unused gloves might harbour bacteria, and the treatment procedure will increase the bacterial count (Fiehn and Westergaard, 1993, Berthelot *et al.*, 2006). One study indicated that bacterial counts increased 10-fold in the glove surface after rubber dam application. The mean colony count increased from 1.58 in fresh gloves to 158 after the rubber dam application (Luckey *et al.*, 2006). Another study showed a progressive increase in microbial load on gloves surface during the course of root canal treatment. The viable count of bacteria was significantly increased at the end of the treatment session compared to at the beginning. This significant increase was also noticed before and after taking periapical radiographs. The common taxa on glove surfaces were *P. acnes* and *Staphylococcus epidermidis* (Niazi *et al.*, 2016). These studies recommended the frequent change of gloves, especially after rubber dam placement, radiograph taking, and at time of obturation (Luckey *et al.*, 2006, Niazi *et al.*, 2016).

1.6.3. Endodontic materials

As a part of operative fields infection control measures during root canal treatment, all instruments and materials used should be bacteria-free (Malmberg *et al.*, 2016). Saeed and co-workers in 2017 investigated the microbiological status of endodontic materials during clinical use. They examined GP points, rubber dams, paper mixing pads, caulking agents, and sponges. Anaerobic and aerobic culture and qPCR was carried out to determine bacterial load followed by identification of bacteria types using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS). Results showed that all materials tested immediately from freshly sealed packages were contaminated when qPCR was carried out. After seven and 14 days of clinical storage, significantly more bacterial DNA was observed, indicating bacterial contamination upon storage. Over 100 taxa were identified from those samples, and the most common isolated genera were *Propionibacterium* and *Staphylococcus* in 42% and 32% of the samples, respectively (Saeed *et al.*, 2017). Those same microorganisms were also reported in GP points (Gomes *et al.*, 2005).

To gain further insight into the possibility of iatrogenic contamination, several studies focused on the microorganisms on gutta-percha obturation points and their disinfection effectiveness. While storage of GP cones did not change its sterility conditions in some studies (da Motta *et al.*, 2001, Gomes *et al.*, 2005, Seabra Pereira and Siqueira, 2010), others showed the opposite (Montgomery, 1971, Namazikhah *et al.*, 2000, Kayaoglu *et al.*, 2009, Saeed *et al.*, 2017). Gomes *et al*. reported that 10% of new GP points were contaminated once the box is opened. After 2.5 years of storage, 94.5% of the remaining GP points maintained sterility. However, all sterile cones became contaminated with gloves handling and manipulation (Gomes *et al.*, 2005). Other studies pointed out that GP points taken directly from manufacturer's sealed packages were contaminated (Kayaoglu *et al.*, 2009, Saeed *et al.*, 2017). Low numbers of

bacteria were found when detected by cultural methods (Kayaoglu *et al.*, 2009), but none of the stored cones were free from bacterial DNA when tested with qPCR. The bacterial levels were increased with storage time, suggesting non-culturable bacteria present or DNA from dead bacteria (Saeed *et al.*, 2017).

It was suggested that complete disinfection of GP cones can be achieved with 5.25% NaOCl for one minute (Senia *et al.*, 1975, Frank and Pelleu, 1983, Gomes *et al.*, 2005, Gomes *et al.*, 2007) or 2.5% NaOCl for five minutes (da Motta *et al.*, 2001). None of the studies adopted the non-culture method to examine the effectiveness of NaOCl in disinfecting GP cones.

1.7. Outcomes of primary root canal treatments

1.7.1. Radiologic assessment of periapical health

Periapical radiographs

For over a century, conventionally, periapical (PA) radiographs have been used to assess the periapical health and thus determine the incidence/prevalence of AP as well as endodontic treatment outcomes. However, the two-dimensional images are unable to reflect the complex three-dimensional anatomy and actual pathological conditions. Moreover, radiographic interpretation is influenced by adjacent anatomical structures overlapping, bone thickness, and x-ray angulation, resulting in missing or underestimating the periapical pathosis (Bender and Seltzer, 1961, Brynolf, 1967, Halse *et al.*, 2002, Huumonen and Ørstavik, 2002). Various studies have suggested that the appearance of PARL on conventional radiographs obliges cortical plate involvement and at least 30-50% of mineral bone loss (Bender *et al.*, 1982, Bender and Seltzer, 2003).

To prevent the subjective nature and bias raised from periapical radiographs evaluation, scoring systems were used. There are three different indices used to evaluate periapical health based on the two-dimensional radiographs. Those are the periapical index (PAI), Strindberg index, and probability index (Strindberg, 1956, Reit and Grondahl, 1983, Ørstavik *et al.*, 1986). The earliest method is the Strindberg index in which the outcome is classified into "success", "failure", and "uncertain" (Strindberg, 1956). The most commonly used index is the PAI, which is a scoring system introduced by Østravik. The five-scoring system ranges from healthy periapical bone "score 1" to severe apical periodontitis with exacerbating features "score 5" (Ørstavik *et al.*, 1986). This system was modelled based on the histological and radiographical findings of Brynolf studying maxillary incisors only (Brynolf, 1967). The validity of using the same method comes into question in more complex areas of the jaw as maxillary molars which have different thickness of overlying cortical bone and different positions of root tips in association with the cortex (Wu *et al.*, 2009).

Cone-beam computed tomography

Around the late 1990s, cone-beam computed tomography (CBCT) was introduced to generate three-dimensional images of the maxillofacial skeleton at a significantly lower radiation dose than traditional CT (Mozzo *et al.*, 1998, Arai *et al.*, 1999, Patel *et al.*, 2009). In endodontics, there have been several studies in the literature reporting improved diagnostic accuracy of PARL with CBCT compared to PA radiographs (Patel *et al.*, 2009, Patel *et al.*, 2012b, Leonardi Dutra *et al.*, 2016). The pooled sensitivity and specificity of CBCT imaging were 0.95 and 0.88, respectively, while PA radiographs had 0.58 and 0.70 sensitivity and specificity, respectively, based on meta-analysis findings (Leonardi Dutra *et al.*, 2016).

CBCT detected 11-39% more lesions compared to PA radiographs in primary treatments and 20-34% additional lesions in secondary treatments (Patel *et al.*, 2019). In a systematic review of endodontic outcomes, the ability of CBCT to determine the failure of the root canal treatment was found to be two times higher than traditional periapical radiograph (Aminoshariae *et al.*, 2018). It is worth noting that only three of the six clinical studies included in the review had a CBCT image at baseline, therefore the results of this systematic review are substantially flawed. Studies have shown that approximately 20% of periapical radiolucencies were missed when examined with periapical radiographs compared to CBCT (Liang *et al.*, 2013, van der Borden *et al.*, 2013, Al-Nuaimi *et al.*, 2018, Torabinejad *et al.*, 2018).

The superior reliability of CBCT in detecting pathosis is also supported by the excellent inter-examiner and intra-examiner agreement usually obtained with CBCT compared to a generally very low agreement with PA radiographs (Goldman *et al.*, 1972, Lennon *et al.*, 2011, Patel *et al.*, 2012b, Pope *et al.*, 2014, Aminoshariae *et al.*, 2018).

Several cadaver and animal studies have correlated the histological findings of lesions with corresponding CBCT radiography. Kanagasingam and co-workers in 2017 compared CBCT

images to film and digital, straight and angled PA radiographs. The gold standard was the histopathological examinations of 86 roots of fresh cadavers. Their findings showed high agreement between CBCT and histological findings. The accuracy was measured through the area under the curve, which was ranging from 0.56 to 0.68 in PA radiographs, whereas the accuracy of CBCT was 0.94. This accuracy was significantly higher in CBCT than PA radiographs. One of the main downsides of PA radiographs is the lower negative predictive value (NPV) ranging from 0.36 to 0.44, which limit the ability to rule out periapical disease, resulting in missing around 56-61% of roots with AP. In contrast, CBCT had significantly higher NPV of 0.81, in which only 20% of the cases which appeared healthy in the scan were histologically inflamed using this modality (Kanagasingam *et al.*, 2017a, Kanagasingam *et al.*, 2017b). This was in accordance with a subsequent cadaver study (Kruse *et al.*, 2019) and an animal study (de Paula-Silva *et al.*, 2009).

For the above reasons, using CBCT in detecting pre-operative PARL as well as assessing the outcome of root canal treatment are essential in this era of technology advancement.

1.7.2. Outcome studies based on periapical radiographs

The success rate of initial root canal treatment

A considerable amount of literature has been published on the outcome of initial root canal treatment (Friedman, 2002). Observational cohort studies, as well as clinical trials, are carried out to evaluate the success of different endodontic procedures. This success is evaluated through clinical examination and radiographic evaluation (Strindberg, 1956, Sjögren *et al.*, 1990, de Chevigny *et al.*, 2008, Ng *et al.*, 2008, Ng *et al.*, 2010).

A systematic literature review was conducted on outcome studies of primary root canal treatment after at least one-year follow-up. The success rates (of 63 studies) ranged from 31% to 96% when the complete absence of PARL was defined as clinical success, whereas the mean reduction of periapical radiolucency size ranged between 60% and 100%. The meta-analysis weighted pooled success rates (from 40 studies) ranged from 68% and 85% when the complete resolution of PARL was considered (Ng *et al.*, 2008). The success rate of primary root canal treatment outcome studies is summarised in Table 1.7.

Table 1.7: Outcome studies examining success rates of primary root canal treatment.

Study	**Sample size (teeth)**	**Follow-up period**	**Success rate (complete healing)**
(Strindberg, 1956)	529	4 years	80%
(Seltzer *et al.*, 1963)	2921	0.5 years	80%
(Sjögren *et al.*, 1990)	356	8-10 years	91%
(Molven *et al.*, 2002)	265	20-27 years	88%
(de Chevigny *et al.*, 2008)	510	4-6 years	86%
(Ng *et al.*, 2011)	702	≥ 2 years	83%
(Azim *et al.*, 2016)	291	2 years	77%

Factors affecting outcomes

The results of outcome studies are very diverse due to the methodological differences, such as: study designs, sample size, tooth type and anatomy, diagnosis, providers, treatment techniques, final restorations, recall rate and radiographic interpretation. Moreover, the criteria used to define success or failure are different among studies (Friedman, 2002).

Strindberg in 1956 was among the earliest reported cohort observational studies in which the success rate of initial root canal treatment was reported to be 80%. Factors having a positive impact on outcomes were absence of PARL, and root canal obturation 0-2mm from the radiographic apex. While the patient's age, PARL size, and filling material did not impact the outcomes (Strindberg, 1956). In Sjogren's study (1990), after an eight- to 10-year follow-up period, the success rate of initial root canal treatment was 96% and 86% in vital cases and necrotic with PARL respectively. The most important prognostic factor was the pre-operative periapical status. The length of instrumentation and obturation was the second most significant factor in cases with pre-operative lesions (Sjögren *et al.*, 1990).

Endodontic treatment involves many steps and procedures, starting from tooth isolation to chemomechanical preparation and ending with the obturation and coronal restorations. To explore those highly interrelated factors impacting outcomes, a thorough systematic review and meta-analysis was published in 2008, including 63 outcome studies published from 1922 to 2002. The factors with the strongest effect on outcome were the presence of periapical radiolucencies, the apical extent of fillings, quality of fillings and the quality of post-treatment coronal restorations (Ng *et al.*, 2008). A summary of the main prognostic factors is presented in Table 1.8. Following the systematic review by Ng *et al.*, other cohort studies investigated many prognostic factors influencing the outcomes (Table 1.9).

Some investigated patient-related factors did not significantly affect the outcomes in most studies, such as gender and age. Although a trend of lower success with advanced age was reported, the difference in success between the older and younger groups did not reach levels of significance (Ng *et al.*, 2008). The general medical health was poorly investigated and was not among the factors influencing the success in meta-analysis (Ng *et al.*, 2008). However, cohort studies showed that diabetes and impaired non-specific immune diseases negatively impacted the success rate and were considered as an important pre-operative factor impacting the outcomes (Fouad and Burleson, 2003, Marending *et al.*, 2005, Segura-Egea *et al.*, 2016, Nagendrababu *et al.*, 2020). Additionally, delayed healing was substantial in the medically compromised patients (Azim *et al.*, 2016). An enlarged apical size preparation was associated with a higher success rate only in necrotic cases (Saini *et al.*, 2012), and improved the overall healing time (Azim *et al.*, 2016), but other clinical studies did not agree with the former two studies (Kerekes and Tronstad, 1979, Hoskinson *et al.*, 2002, Souza *et al.*, 2012).

The type of irrigant used or the concentration of NaOCl did not significantly affect the outcomes in many studies (Cvek *et al.*, 1976, Byström and Sundqvist, 1983, Ng *et al.*, 2011). Whilst the use of EDTA had a marginal effect on primary treated cases (Ng *et al.*, 2011), it did however, significantly improve the success of retreatment cases (Ng and Gulabivala, 2008).

With regard to the filling material used and techniques of placement, the warm vertical obturation technique was reported to have a positive impact on success rate compared to cold lateral in cases with PARL (de Chevigny *et al.*, 2008); however, the results of this particular trial are largely invalidated by the extremely low recall rate. On the other hand, the type of filling material (Ng *et al.*, 2008) or sealer extrusion did not affect the outcome (Schaeffer *et al.*, 2005, Sari and Duruturk, 2007, Ricucci *et al.*, 2016).

The number of visits to complete treatment has been subject to considerable debate in the literature (Trope *et al.*, 1999, Weiger *et al.*, 2000, Peters and Wesselink, 2002, Sathorn *et al.*, 2005, Molander *et al.*, 2007). The latest Cochrane review on the subject concluded that the difference between the two treatment regimens was not significant in terms of healing rate (Manfredi *et al.*, 2016). Intra-appointment pain and flare-ups were significant factors influencing the outcomes in one study (Ng *et al.*, 2011); however, 60% of teeth in that prospective clinical study were treated in three or more visits, which might have introduced a bias when compared to other studies where treatment was carried out in either one or two visits. Flare-ups were not proven to be a significant outcome predictor in other studies (Kerekes and Tronstad, 1979, Byström *et al.*, 1987, Sjögren *et al.*, 1990).

Table 1.8: Summary of the main factors affecting primary root canal treatment outcomes from meta-analysis (Ng *et al.*, 2008).

Prognostic factor	Studies showing a significant effect on the outcome	Meta-analysis result
Pre-operative factors		
Tooth type Mandibular molars showed the lowest success rate.	(Swartz *et al.*, 1983, Benenati and Khajotia, 2002)	No significant difference in odds of success.
Pre-operative pulpal status (vital vs non-vital) Higher success rate in vital teeth.	(Smith *et al.*, 1993, Hoskinson *et al.*, 2002)	Odds of success of vital teeth were 1.77 times higher than those of non-vital.
Pre-operative periapical status Presence of PARL reduced the success rate.	(Swartz *et al.*, 1983, Sjögren *et al.*, 1990, Chugal *et al.*, 2001, Hoskinson *et al.*, 2002)	Odds of success of non-vital teeth without a lesion were two times higher than those of non-vital with a lesion.
Size of lesion Better success with smaller lesion sizes.	(Friedman *et al.*, 1995, Chugal *et al.*, 2001, Hoskinson *et al.*, 2002)	Odds of success of teeth with smaller lesions were higher but not statistically significant when compared to teeth with larger lesions.
Intra-operative factors		
The taper of canal preparation Wider taper associated with higher success.	(Smith *et al.*, 1993)	Insufficient data to analyse the effect.
Canal obstructions and technical errors Technical errors including iatrogenic perforations reduced success.	(Cvek *et al.*, 1982, Sjögren *et al.*, 1990)	Insufficient data to analyse the effect.
Intracanal medication with CH Better outcome when intracanal medication used between visits.	(Trope *et al.*, 1999)	Insufficient data to analyse the effect.

Bacterial status at the time of obturation	(Engstrom and Lundberg, 1965, Oliet and Sorin, 1969, Sjögren *et al.*, 1997)	Odds of success of teeth with negative cultures were not significantly different from those with positive cultures at time of obturation.
Use of sealer: ZOE-based sealer associated with higher success compared to other sealer types.	(Nelson, 1982)	Insufficient data to analyse the effect.
The apical extent of root filling	(Bender *et al.*, 1964, Jokinen *et al.*, 1978, Sjögren *et al.*, 1990, Smith *et al.*, 1993)	Teeth with AP: The highest success was in teeth with flush fillings while the lowest was in teeth with short fillings, and the odds of success of teeth with flush fillings were 2.3 times higher than long and 1.6 times higher than short fillings. Teeth without AP: Odds of success of teeth with flush fillings were 3.7 times higher than long fillings and there was no difference in odds of success between flush and short fillings.
Post-operative factors		
Quality of root filling (presence of voids)	(Heling and Kischinovsky, 1979, Nelson, 1982)	Odds of success of teeth with satisfactory root fillings were four times higher than teeth with unsatisfactory root fillings.

Quality of coronal restoration	(Heling and Kischinovsky, 1979, Swartz *et al.*, 1983, Sjögren *et al.*, 1990, Friedman *et al.*, 1995)	Odds of success of teeth with satisfactory coronal restorations were 1.8 times higher than teeth with unsatisfactory coronal restorations.
Use of abutment: Lowered success when root canal treated teeth used as a bridge or denture abutments.	(Matsumoto *et al.*, 1987, Sjögren *et al.*, 1990)	Insufficient data to analyse the effect.

Table 1.9: Summary of the main factors affecting primary root canal treatment outcomes after 2002 (PA radiographs).

Prognostic factor	Effect on outcome	Study
Pre- and intra-operative factors		
Tooth type	Higher success in single-rooted teeth.	(de Chevigny *et al.*, 2008, Azim *et al.*, 2016)
Root type	More favourable outcome observed in roots with one canal compared to those having two.	(de Chevigny *et al.*, 2008, Ng *et al.*, 2011, Azim *et al.*, 2016)
Periapical status	The presence of PARL negatively impacted the outcomes.	(de Chevigny *et al.*, 2008, Ng *et al.*, 2011, Azim *et al.*, 2016)
Size of lesion	The smaller the lesion size, the better the treatment prognosis.	(Ng *et al.*, 2011)
The apical extent of filling	0-2mm from radiographic apex had the highest success rate, followed by short then long fillings.	(de Chevigny *et al.*, 2008, Azim *et al.*, 2016, Garcia-Guerrero *et al.*, 2020)
Apical size of canal preparation	A positive association between the master apical preparation size and an improvement in PAI scores as well as an increase in the average healing time.	(Saini *et al.*, 2012, Azim *et al.*, 2016)
Density of obturation	Significantly better outcomes in teeth with proper obturation densities.	(Azim *et al.*, 2016, Garcia-Guerrero *et al.*, 2020)
Procedural errors	In cases with lesions, the presence of root perforation at the coronal or mid-root level was found to reduce the odds of success significantly.	(de Chevigny *et al.*, 2008, Ng *et al.*, 2011, Azim *et al.*, 2016)
Post-operative factors		
Quality of coronal restoration	The presence of a satisfactory coronal restoration was associated with greater success.	(Ng *et al.*, 2011)

1.7.3. Outcome studies based on CBCT

The success rate of initial root canal treatment

Clinical studies using CBCT to determine root canal treatment outcome are becoming increasingly popular. More clinical studies are directed towards this more accurate method in reporting their treatment outcomes.

Patel *et al.* found a significant difference between the two radiographic modalities when assessing root canal treatment outcomes. Whether a pre-operative PARL is present or absent, CBCT images tend to increase the failure compared to conventional radiographs. After one-year follow-up, 17.6% of examined teeth with healthy periapical area pre-operatively developed PARL in CBCT scans. On the other hand, the development of new radiolucencies was noticed in only 1.3% of PA radiographs. Similarly, cases with pre-operative PARL had 13.9% and 10.4% failures in CBCT and PA, respectively. Overall complete resolution of primary root canal treatment was reported to be 87% in PA radiographs and dropped to 62.5% in CBCT. The same scenario was noted when success was defined as "healing", where the success rate was 95% and 84.7% in PA and CBCT, respectively (Patel *et al.*, 2012a). In accordance with Patel *et al.*, another group found a success rate of 88.7% in PA radiographs compared to 77.5% in CBCT when following up cases with pre-operative PARL for 10-37 months (van der Borden *et al.*, 2013). Liang and co-workers evaluated the initial root canal treatment outcome using CBCT of anterior teeth. Favourable outcomes were observed in 91% of teeth, with 19% of PARL completely resolved while 72% reduced in size (Liang *et al.*, 2013). After two years of initial treatment, the same group re-evaluated teeth with lesions and found that reduction of PARL was observed in 63% of the cases, while unfavourable outcomes were defined radiographically as unchanged periapical areas and increase lesion sizes were observed in 33% and 3%, respectively (Zhang *et al.*, 2015).

It is worth mentioning that studies pointed out that the difference in detecting pathosis between PA and CBCT is mainly noticed in molar teeth (Patel *et al.*, 2012a, Al-Nuaimi *et al.*, 2018). This difference is attributed to the anatomical structure in posterior regions challenging the detection with 2D modality (Patel *et al.*, 2020). Also, CBCT scans did not show a significant difference in the radiographic outcomes between initial treatment and retreatment, as the overall success rate was 76% for primary treatments and 75.3% for retreatments (Al-Nuaimi *et al.*, 2018). Studies on CBCT outcomes are shown in Table 1.10.

Table 1.10: Clinical studies adopting CBCT as a radiographic measure of the outcome of primary root canal treatment.

Study	Number of teeth	Type of teeth	Diagnosis	Follow-up period	Healed (%)		Healed +healing (%)	
					PA	CBCT	PA	CBCT
(Liang et al., 2011)	74	All except maxillary molars	Vital	2 years	87.4	74	--	--
(Patel et al., 2012a)	123	All teeth	Different	12 months	87	62	95	84.7
(van der Borden et al., 2013)	50	All teeth	All with PARL	10-37 months	45	15.5	88.7	77
(Liang et al., 2013)	84	All single-rooted	All with PARL	10-19 months	32	19	94	91.7
(Zhang et al., 2015)	61	All single-rooted	All with PARL	2 years	63 reduction in size of PARL			
(Kamburoglu et al., 2017)	21	Only maxillary first molars	All with PARL	12 months	Significant reduction of lesion volume and mucosal thickening after treatment			

Factors affecting outcomes

In line with outcome studies based on PA radiographs, studies adopting CBCT showed great variability in the outcomes of initial treatment. The success rate ranged from 19% to 92% (Liang *et al.*, 2011, Patel *et al.*, 2012a, Liang *et al.*, 2013, van der Borden *et al.*, 2013, Zhang *et al.*, 2015). A handful of cohort studies evaluated the prognostic factors influencing the CBCT-based outcomes. CBCT studies further confirmed the agreed fact of pre-operative periapical status as the most prognostic factor influencing the outcomes (Liang *et al.*, 2012, Patel *et al.*, 2012a). Since the CBCT would allow more accurate lesion measures, the size of the pre-operative lesion was found to influence the outcomes in some studies. In one study, none of the teeth with lesions larger than 65mm^3 (lesion diameter of 5mm) completely healed after 10-19 months follow-up (Liang *et al.*, 2013). In contrast, another study did not find such an association between pre-treatment lesion volumes and post-treatment outcomes (Kamburoglu *et al.*, 2017).

Regarding the patient's related predictors and agreeing with previous reports, the patient's systemic health did not significantly change the success rate of initial treatment. Other patient-related factors such as age and gender were also not significant (Friedman *et al.*, 2003, Liang *et al.*, 2011). Tooth or root type were not among the success-predicting factors in some studies (Liang *et al.*, 2011, Kamburoglu *et al.*, 2017), whereas in others, molars were found to be less successful than premolars and anterior teeth (Al-Nuaimi *et al.*, 2018).

Some treatment-related factors were found to be strongly associated with success, such as proper length and density of obturation, proper and immediate placement of coronal restoration (Liang *et al.*, 2011, Liang *et al.*, 2012). Other treatment-related factors such as the adjunct ultrasonic activation did not affect the outcome (Liang *et al.*, 2013); however, this particular study was limited to anterior teeth which are likely to be successfully treated irrespectively of

the technique used. A summary of the treatment outcome predictors based on CBCT studies is shown in Table 1.11.

It is important to note that in some CBCT-based studies, the pre-operative lesions were assessed on PA radiographs and CBCT scans were not taken at baseline, and thus the success was evaluated based on the changes on CBCT scans and compared to less sensitive PA radiographs at baseline. This could result in possibly underestimating the actual number of cases with pre-operative lesions and could clearly lead to an overestimation of the number of failures (Fernandez *et al.*, 2013, Fernandez *et al.*, 2017, Restrepo-Restrepo *et al.*, 2019).

Table 1.11: Summary of the main factors affecting primary root canal treatment outcomes (CBCT).

Prognostic factor	**Effect on outcome**	**Studies**
Pre-operative		
Presence of PA lesion	Presence of pre-op PARL has a negative impact on outcome.	(Liang *et al.*, 2012, Patel *et al.*, 2012a)
PARL size/ volume of the lesion	Better prognosis of root canal treatment in teeth with small lesions compared to larger lesions.	(Liang *et al.*, 2013)
Intra-operative		
Length of filling	Fillings 0–2 mm short of the apex had a significantly higher success rate.	(Liang *et al.*, 2012, de Sousa Gomide Guimaraes *et al.*, 2019, Meirinhos *et al.*, 2020)
	Prevalence of AP was higher in cases filled more than 2mm shorter of the apex.	(Gomes *et al.*, 2015a)
Size of master cone	Master cone ≤#45 were associated with complete healing compared to larger sizes.	(Liang *et al.*, 2013)
Density of obturation	An adequate density of obturation associated with completely healed periapical areas.	(Liang *et al.*, 2011, Gomes *et al.*, 2015a)
Post-operative		
Quality of coronal restoration	Satisfactory quality of coronal restoration positively influenced treatment outcomes.	(Liang *et al.*, 2011, Liang *et al.*, 2012, Gomes *et al.*, 2015a)

*Based on cross-sectional studies: (Gomes *et al.*, 2015a, de Sousa Gomide Guimaraes *et al.*, 2019, Meirinhos *et al.*, 2020)

1.8. Asepsis during root canal treatment

Endodontic failures develop from either persistence of microorganisms or secondary infections (Nair *et al.*, 1990, Haapasalo *et al.*, 2003). Secondary endodontic infection develops from microorganisms that were not present in the primary infection if the aseptic chain is breached during treatment (Hargreaves *et al.*, 2016, Rotstein and Ingle, 2019), or as a result of coronal leakage (Nair *et al.*, 1990, Haapasalo *et al.*, 2003, Hargreaves *et al.*, 2016).

In endodontic therapy, potential sources of microbial introduction into the pulp chamber and root canal space include dental biofilms, calculus, saliva and gingival exudate, and caries on the crown. Other sources of contamination are infected debris, leakage from rubber dam, non-sterile instruments or contaminating endodontic instrument (Bergenholtz *et al.*, 2013, Hargreaves *et al.*, 2016).

Different measures were advocated and suggested to maintain the operative field bacteria-free. These methods include (1) the use of rubber dam, (2) mechanical preparation of the involved tooth, (3) chemical disinfection of the operative field, and (4) the use of sterile instruments (Ørstavik, 2020).

The use of rubber dam is considered the main step to achieve an aseptic field (European Society of Endodontology, 2006). The mechanical preparation of the tooth includes removal of calculus and plaque from tooth surface with scaling and polishing (Engstrom and Lundberg, 1965, Ørstavik, 2020). Mechanical preparation also comprises caries excavation before entering the pulp chamber to prevent the penetration of bacteria from infected dentine into the pulp space causing inflammatory reaction (Bergenholtz *et al.*, 2013, Ørstavik, 2020). Due to the higher chance of leakage underneath restorations, removal and replacement of defective fillings prior

to access is also considered an important measure in maintaining asepsis (Bergenholtz *et al.*, 2013, Ørstavik, 2020).

Operative field disinfection is the step that follows tooth cleaning and rubber dam isolation. Different disinfections protocols were advocated to disinfect the rubber dam and tooth surface. Möller used 30% H_2O_2 followed by 5% or 10% iodine (Möller, 1966). Alternative protocols include the use of 5-10% iodine or CHX in alcohol without H_2O_2 (Bergenholtz *et al.*, 2013), or disinfection with CHX or iodine combined with H_2O_2 or ethanol (Tronstad, 2003). Moreover, NaOCl was also suggested to swab the operatory surfaces after disinfection with H_2O_2 (Hermsen and Ludlow, 1987, Walton and Torabinejad, 2008). Some even recommended an extra second disinfection step when treating carious lesion exposing the pulp of vital teeth (Ørstavik, 2020). In this case, the cavity and pulp wound are swabbed with iodine alone or 0.5% CHX in alcohol. Such an extra step is recommended to prevent the transmission of microorganisms from the caries and coronal pulp to the radicular space in cases of vital pulp where bacteria are not expected to penetrate deeply (Ørstavik, 2020). It is worth mentioning that the ESE quality guidelines for root canal treatment indicated operative field disinfection but did not specify any protocol to be followed (European Society of Endodontology, 2006). The Italian Endodontic Society on the other hand had advised the operative field disinfection for two minutes with 5% NaOCl or 80% ethanol (Italian Endodontic Society, 2020).

Another essential part of asepsis in endodontics is the use of sterile instruments as well as avoiding contaminating instruments by hand manipulation or contacting non-sterile items (Bergenholtz *et al.*, 2013). Foreign body reaction or delay healing might emerge from dislodged debris from instrument surfaces to the canal space (Ørstavik, 2020). For these reasons, suggestions are, after access cavity, to remove all instruments used for rubber dam

application and access preparation, and a new sterile tray to be used for canal instrumentation was advised (Tronstad, 2003). Others proposed discarding only burs used for caries excavation and avoid their usage for access cavity preparation (Ørstavik, 2020).

These aseptic measures are likely to be more critical in vital cases, in which bacteria are expected to be present only in the coronal portion of the pulp, to prevent bacterial introduction to the root canal (Ørstavik, 2020), while in necrotic cases, asepsis is aimed to prevent the introduction of bacteria that are not members of root canal microbiota and believed to be more resistant to treatment (Bergenholtz *et al.*, 2013).

Although such suggested methods are simple but are believed to be effective in reducing the chance of contaminating the root canal space (Ørstavik, 2020), scientific evidence is lacking about the effectiveness of including these measures in root canal treatment outcomes as well as the actual application of these measures among practitioners.

Clinical studies involving sampling of the root canal space for microbiological procedures typically report their disinfection protocol in order to ensure reliability and accuracy of microbial findings (Figdor and Brundin, 2016). On the other hand, operative surface disinfection or asepsis protocols followed were not clearly described in most clinical outcome studies (Friedman *et al.*, 1995, Chugal *et al.*, 2001, Hoskinson *et al.*, 2002, Liang *et al.*, 2011, Patel *et al.*, 2012a, Saini *et al.*, 2012, van der Borden *et al.*, 2013, Zhang *et al.*, 2015, Azim *et al.*, 2016, Fernandez *et al.*, 2017, Restrepo-Restrepo *et al.*, 2019, Garcia-Guerrero *et al.*, 2020). Rubber dam disinfection was reported in some studies with H_2O_2 and iodine following Möller's protocol (Möller, 1966, Byström and Sundqvist, 1983, Sjögren *et al.*, 1990, Chugal *et al.*, 2003, Kvist *et al.*, 2004, Molander *et al.*, 2007), or operative field disinfection with H_2O_2 only

(Kamburoglu *et al.*, 2017). Prospective studies reported following the ESE guidelines (Ng *et al.*, 2011), or a generic disinfection of operative field (de Chevigny *et al.*, 2008). Other studies reported the establishment of asepsis during treatment without specifying which protocol was used (Sjögren *et al.*, 1997, Trope *et al.*, 1999, Friedman, 2002, Waltimo *et al.*, 2005).

Despite the above recommendations and the importance of maintaining asepsis during root canal treatment, the actual compliance of dentists is unknown. Many surveys addressed the substandard use of rubber dam especially among general dentists (Ahmad, 2009), while operative field disinfection and instruments changing were not the centre of interest of researches carried out on the subject (Malmberg *et al.*, 2016).

A survey was designed to assess infection control routine and asepsis maintenance measures taken by general dentists and endodontists during root canal treatment (Shuen *et al*., in press). The survey was sent to over a thousand participants. Out of the 928 practitioners who completed the questionnaire, 28.8% were endodontists.

Regarding changing gloves during treatment, more than half of practitioners reported changing their gloves only when torn or visibly soiled (61% of general dental practitioners (GDPs), and 55% of endodontists), while 30% changed their gloves after taking radiographs. More endodontists tend to change their gloves before obturation (25%) compared to GDPs (9%). In general, changing gloves before canal obturation or after caries removal was not commonly done (17% and 10% of respondents, respectively).

Although rubber dam isolation is a standard of care, only 65% of GDPs reported rubber dam isolation all the time during endodontic treatment, compared to 93% of endodontists. Sealing the gap around the isolated tooth was not as common, with 37% of GDPs and 6% of

endodontists not attempting to use any means of additional isolation after rubber dam placement. Others do so only when there is a visible gap between the tooth and the rubber dam (40% of respondents).

Disinfecting the operative field was not frequent despite the international recommendation (European Society of Endodontology, 2006). Almost half of respondents did not disinfect the endodontic operative field at all (44% of total, 55% of GDPs, and 31% of endodontists). It was more common among endodontists to disinfect the field after tooth isolation (55%) compared to GDPs (21%). To a lesser extent, disinfection was carried out either after caries removal (18%) or before obturation (13%).

Using a new sterile set of endodontic instruments during treatment was not routinely carried out among practitioners. Just over half (54%) reported not changing instruments during treatment while 35% disinfect instruments with alcohol. Replacing instruments after caries removal or before obturation was seldom (7% and 5%, respectively) although 22% reported finishing treatment in one visit.

Data from the above conducted survey showed that the use of infection control measures although acceptable in some aspects such as rubber dam use and changing gloves, yet other measures are still below standard, especially when it comes to operative field disinfection and the use of new instruments.

Chapter Two: Assessing the Iatrogenic Contribution to Contamination During Root Canal Treatment: A Pilot Study

2.1. Introduction

As set out in Chapter 1, the main objective of endodontic treatment is to prevent the development of, and treat, apical periodontitis. Since microorganisms and their by-products are involved from the initial carious process and successive tissue destruction to the development of periapical inflammation, the treatment should be directed towards the removal of the aetiology (Kakehashi *et al.*, 1965, Möller, 1966, Möller *et al.*, 1981, Fabricius *et al.*, 1982). As a result, current treatment protocols (isolation, canal preparation, antibacterial irrigants, and intracanal medicaments) are directed toward bacterial elimination (Sathorn *et al.*, 2007). Additionally, it is of paramount importance to prevent the external (re)introduction of microorganisms into the root canal system (Siqueira *et al.*, 1998, Schirrmeister *et al.*, 2007, Peciuliene *et al.*, 2008), achieved when aseptic measures are applied during treatment, and protocols as close to sterile as possible are maintained during the treatment (Möller *et al.*, 1981, Ramachandran Nair, 1987), prior to placement of definite obturation and restorative materials to prevent reinfections at a later stage. Furthermore, some organisms described in persistent endodontic infection and extra-radicular lesions such as *Staphylococcus*, *Rothia* and *Cutibacterium* (formerly *Propionibacterium)* are not typical endodontic bacteria, and this might raise a concern about iatrogenic contamination (Sunde *et al.*, 2000a, Siqueira and Rôças, 2004, Dioguardi *et al.*, 2020).

It has been reported that iatrogenic contamination of the root canal space may occur during root canal treatment, from materials used, airborne microorganisms, leakage in between visits or even contamination from operating surfaces, caries or saliva during the treatment (Williams *et al.*, 2003, Niazi *et al.*, 2010, Niazi *et al.*, 2016, Saeed *et al.*, 2017). Thus, a substantial effort should be devoted to maintaining aseptic conditions during root canal treatment, as well as identifying potential routes of iatrogenic contamination.

Since many endodontic pathogens are oral commensals, it is crucial to prevent the (re-)ingress of such organisms into the root canal space, which can be achieved primarily with the use of rubber dam (European Society of Endodontology, 2006). The use of rubber dam has been identified as a substantial factor in endodontic outcomes (Nieuwenhuysen *et al.*, 1994, Lin *et al.*, 2014, Lee *et al.*, 2017, Kwak *et al.*, 2019). Despite this, recent studies have demonstrated that its use is not even close to best practice as recommended by international committees of experts, with self-reported figures of 3%-90% of use in practice (Ahmad, 2009). Discrepancies were noted in rubber dam usage prevalence between students, general practitioners and endodontists (Ahmad, 2009). Less than half of the general dentists use rubber dam during root canal treatment, compared to 92-100% among endodontists (Whitten *et al.*, 1996, Ahmad, 2009, Anabtawi *et al.*, 2013). However, a major concern is whether a leakage-free field can be assured and maintained within the rubber dam during treatment, where studies reported the presence of bacteria on the rubber dam surface to be increased during endodontic treatment, especially after access cavity preparation (Ng *et al.*, 2003), and thus recommended the disinfection of rubber dam surfaces.

In recognising the potential reinfection from the rubber dam, studies have investigated operative field disinfection techniques, including hydrogen peroxide, iodine, chlorhexidine, alcohol, and sodium hypochlorite (Möller, 1966, Ng *et al.*, 2003, Malmberg *et al.*, 2016). Möller recommended the use of 30% H_2O_2 as an effective method in reducing culturable bacteria from rubber dam surface when compared to 5% iodine (Möller, 1966). Others showed that decontaminating with NaOCl after access preparation significantly reduced the number of contaminated rubber dam surfaces. Yet, culturable bacteria were still present, and a greater bacterial contamination was detected when PCR was adopted to quantify bacteria (Ng *et al.*, 2003). Another study showed that after the disinfection with 0.5% chlorhexidine in alcohol

followed by 1% NaOCl, 19% of the rubber dam surfaces had positive amplification with PCR universal primers (Rorslett Hardersen *et al.*, 2019). Taxa detected after decontamination included *Propionibacterium* sp., and *Haemophilus parainfluenzae* (Rorslett Hardersen *et al.*, 2019). Finally, a review of the literature revealed that asepsis was not achieved entirely in the operative field after initial disinfection in any study (Malmberg *et al.*, 2016).

The materials used during root canal treatment are another possible source of contamination. Although these materials are not expected to be in direct contact with saliva, a concern was raised when materials such as gutta-percha (GP) points, rubber dams, paper mixing pads, caulking agents, and sponges were contaminated with bacteria when tested immediately from freshly sealed packages (Saeed *et al.*, 2017).

A number of researches have reported that gloves worn by the dentist are another potential source of contamination, especially since new gloves might harbour bacteria (Fiehn and Westergaard, 1993, Berthelot *et al.*, 2006). Bacterial growth on gloves was shown to be significantly increased after rubber dam application, access cavity preparation, and taking radiographs (Luckey *et al.*, 2006, Niazi *et al.*, 2016), suggesting contamination by the patient's saliva. Another study highlighted the risk of transmitting bacteria from operatory surfaces to the gutta-percha points if manipulated by gloves (Gomes *et al.*, 2005).

One of the limitations of previous studies on iatrogenic bacterial contamination of the root canal system is the inclusion of teeth with different clinical diagnoses (Ng *et al.*, 2003, Niazi *et al.*, 2016, Rorslett Hardersen *et al.*, 2019). Including teeth with endodontic infection could result in the inability to differentiate infected root canal space microbiota from the iatrogenically introduced ones, particularly because endodontic microflora is comprised of

select members of the salivary microbiome confirmed by high-throughput studies (Manoil *et al.*, 2020). Since microbial ingress is believed to be minimal or absent in vital cases (Nagaoka *et al.*, 1995), it would be optimal to include vital cases only in studies assessing the iatrogenic contamination of root canals.

There is a significant lack of well controlled studies exploring the timing and clinical impact of iatrogenic contamination of the root canal space. While previous studies discovered endodontic pathogens on contaminated surfaces, yet the clinical relevance of contaminated operative fields is lacking. Moreover, instruments, endodontic rulers and files have not been investigated for their microbial status during treatment.

2.2. Study design and aim of the study

This pilot clinical study included a series of experiments to identify different sources and levels of contamination occurring during the process of root canal treatment. This was achieved by isolating and investigating microorganisms quantitively and qualitatively from seven sites throughout the treatment of 30 cases diagnosed with irreversible pulpitis, in which the presence of bacteria within the root canal space before the treatment is believed to be minimal.

Finding of these preliminary investigations provided the information on the steps necessary to develop an enhanced infection control protocol to be implemented in a clinical trial with the objective of improving the outcome of root canal treatments. Also, we aimed to optimise laboratory techniques for sampling and extraction of small quantities of microbial DNA and optimal amplification.

2.3. Materials and methods

2.3.1. Subjects and patients

Samples were obtained from 34 adult patients who visited King's College London Dental Institute, Guy's Hospital dental emergency department, between May and September 2018. The clinical study was conducted in accordance with the Helsinki Declaration. Before commencing the study, ethical approval was granted by the London - Surrey Research Ethics Committee (reference no. 18/LO/1661). Patients were recruited after informed, written consent was obtained. All included teeth were diagnosed with irreversible pulpitis (IP) based on clinical and radiographic findings and according to the reports of the American Association of Endodontists consensus conference on diagnostic terminology (Levin *et al.*, 2009). Patients presented to the dental emergency unit with sharp, spontaneous, lingering pain, and over-the-counter analgesics did not help to relieve the pain. When tested by thermal sensibility test (Endo-Frost, Coltène, Switzerland), teeth responded with lingering pain (lasting more than 30 seconds after stimulus removal) when compared to control teeth. Response to palpation/percussion tests was either normal or showing increased tenderness compared to unaffected teeth. Periapical radiographs showed a normal thickness of the periodontal ligament and no evidence of osseous changes. Inclusion and exclusion criteria are shown in Table 2.1.

Table 2.1: Inclusion and exclusion criteria of study participants.

Inclusion criteria	Exclusion criteria
1. Age range 18-60 years old. 2. Healthy patients with no significant medical history. 3. Diagnosed with symptomatic irreversible pulpitis. 4. Confirmed clinical diagnosis of vital pulp by bleeding upon access. 5. Radiographic analysis showing mature root apexes, and normal width of the apical periodontal ligament spaces. 6. Restorable teeth.	1. Patients younger than 18. 2. Patients of compromised medical health affecting the outcome of root canal therapy. 3. Patients with a clinical and radiographic diagnosis of necrotic pulp, previously treated or initiated root canal treatment. 4. Evidence of external or internal root resorption. 5. Pregnant women. 6. Patients unable to give consent. 7. Non-restorable teeth.

2.3.2. Sample collection

Sample collection was carried out as described elsewhere (Siqueira and Rôças, 2003b), with minor modifications. Identifiers of samples taken throughout root canal treatment are given in Table 2.2 and Figure 2.1.

Briefly, samples were collected under aseptic conditions by a single, trained operator. After administration of local anaesthesia, each target tooth was cleaned with pumice and isolated with a rubber dam (UnoDent, UK) with appropriate clamp and sealed with gingival barrier OpalDam Green™ (Ultradent, South Jordon, UT, USA). The operative field including the tooth, rubber dam surface and clamp were decontaminated with a small cotton pellet immersed in 2.5% NaOCl, swabbed for at least 30 seconds. Removal of caries and or pre-existing restoration was carried out using high-speed and low-speed burs. The pulp chamber was then accessed with a sterile bur under sterile saline irrigation and the access bur was immediately collected (B). The operative field, including the pulp chamber, was then decontaminated again with 2.5% NaOCl and inactivated with 5% sodium thiosulphate. A contamination control sample (CC) was taken using sterile paper points swabbed on the decontaminated outer surface of the target tooth for 30 seconds. Pulp vitality was confirmed clinically by bleeding upon access. The intracanal sample was collected (S1) with a paper point (Dentsply Sirona, Baillagues, Switzerland) inserted 1mm short of canal's working length and held in position for 30 seconds. In the case of multi-rooted teeth, all intracanal samples were obtained from the largest canal. Root canal treatment was then completed. For cases that were accessed in ADC (n=7), S1 samples were collected immediately at that emergency visit. After the initial intracanal samples, the initial file introduced to the canal before using any irrigation was collected (F-1). A second file used during treatment and after the use of NaOCl was also collected (F-2). Root canal treatment was carried out conventionally in one or two visits and

NaOCl was used as irrigant. At the time of obturation and after completion of the chemomechanical preparation, microbiological samples were collected using sterile swabs (Woodshaft swabs TS/8-A, Technical Service Ltd, Lancashire, UK). Samples were obtained after rubbing for 10 seconds the rubber dam surface around the tooth, the gloves worn by the dentist, endodontic rulers, and instruments (Tip of the tweezers, DG-16 endodontic explorer, plugger and flat plastic instrument).

Additionally, at time of obturation, the canal was dried and rinsed with physiological 0.9% saline. A final intracanal sample was then taken (S2) with paper points held in place for 30 seconds. Calcium hydroxide $Ca(OH)_2$ dressing (Hypocal., Ellman International, Oceanside, New York, USA) was applied into the root canal spaces as a standard interappointment medicament and a dry cotton pellet placed over the canal orifices followed by conventional glass-ionomer cement (GIC) (Fuji IX, GC Corporation, Tokyo, Japan). Additionally, negative control samples (NC) were collected from the sterile, unused paper point, files and burs as well as swabs from new, unused rubber dams, gloves and instruments.

Collection of all samples was done by the research investigator. All samples were placed in sterile Eppendorf microtubes containing 200μl of phosphate-buffered saline and immediately snap-frozen at -20^{o}C until DNA extraction and qPCR analysis were carried out.

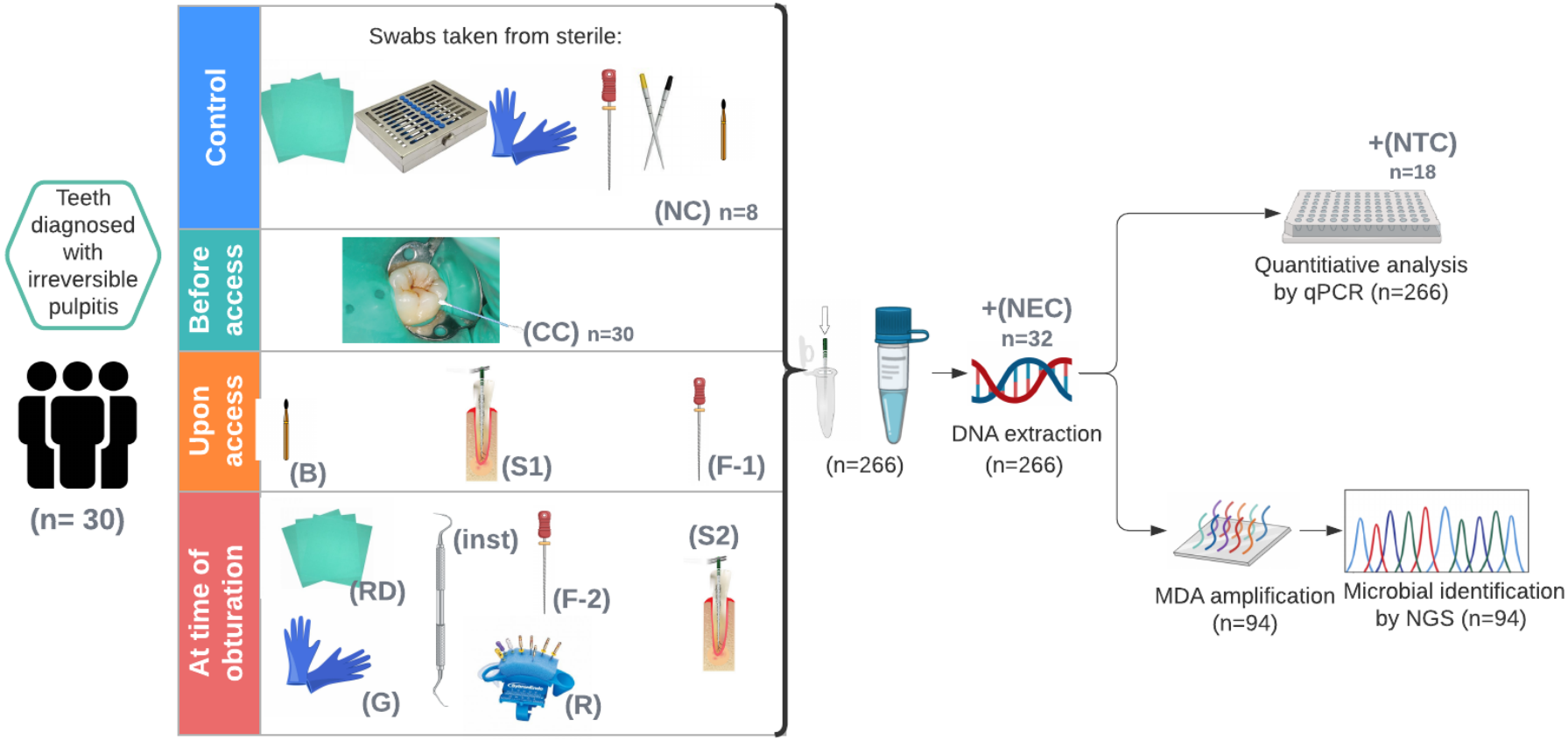


Figure 2.1: Illustration of different samples taken throughout the pilot study.

Total of 266 samples taken from 30 patients diagnosed with IP at different stages. (NC) samples were collected from sterile (new unused) burs, files, rubber dam, gloves, instruments and paper points. After RD application, tooth surface was decontaminated with 2.5% NaOCl and (CC) samples collected. Once teeth were accessed, access bur (B), initial intracanal samples (S1) and initial files (F-1) were collected. After chemomechanical preparation, pre-obturation samples were taken (S2) in addition to swabbed samples from rubber dam (RD), gloves (G), endodontic rulers (R), and instruments (inst) and files (F-2). DNA extraction of all collected samples (n=266) was carried out and (NEC) samples were included during each extraction. This was followed by qPCR and (NTC) were included. MDA amplification and NGS analysis was carried out on 94 selected samples.

Table 2.2: Description of samples taken during root canal treatment in addition to different control samples included throughout the experiment.

Sample	Abbreviation	Description and time of collection
Contamination control	CC	Samples from target tooth surface after rubber dam placement, OpalDam and decontamination with 2.5% NaOCl.
Access bur	B	Burs collected immediately after access cavity preparation.
Initial intracanal sample	S1	Paper points used to sample vital pulps once the chamber was accessed and before the use of any irrigant.
Pre-obturation intracanal sample	S2	At the end of chemomechanical preparation and irrigation, after drying the canals and rinsing with saline.
Initial file	F-1	The first #10 K file introduced to the canal before irrigation use.
Second file	F-2	Any patency file used during the course of treatment.
Endodontic ruler	R	At the time of obturation, the surface of endodontic ruler was swabbed.
Rubber dam	RD	At the time of obturation, the surface of rubber dam around the tooth was swabbed without touching the tooth.
Gloves	G	At the time of obturation, swabbing the gloves worn by the dentist.
Instruments	Inst	At the time of obturation, working tip of the tweezer, DG-16 endodontic explorer, plugger and flat plastic instrument were swabbed for 30 seconds.
Negative control	NC	Samples from sterile (new unused) burs, files, rubber dam, gloves, instruments and paper points were collected at the beginning of this trial and served as procedural control.
Negative extraction control	NEC	Ultra-pure water samples included for every batch of DNA extraction carried out.
No-template control	NTC	Ultra-pure water instead of DNA template added to each qPCR reaction as control for extraneous nucleic acid contamination.

2.3.3. DNA extraction

Following clinical sample collection, total genomic bacterial DNA was extracted from 266 samples using the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich, Irvine, UK) following the manufacturer's protocol for this sample type. All DNA extractions were performed by the same individual, and care was taken to minimise the risk of contamination. Initially, samples were subjected to mechanical disruption with tungsten carbide beads (Qiagen, Manchester, UK) and homogenised for 2x30 seconds at 6.5m/s (FastPrep®-24; MP Biomedicals, Solon, OH, USA), before the kit-based purification. With the exception of the file and burs, which were subjected to mechanical disruption by vortexing instead of FastPrep®-24. Negative extraction controls (NEC) were included for every batch carried out (n=32). DNA was quantified by fluorometry (Qubit, Invitrogen, Carlsbad, CA, USA). The integrity of extracted DNA was assessed by gel electrophoresis and confirmed by the predominance of >10 kb DNA bands. This was done in 2% TAE (Tris-acetate-EDTA) agarose gels by running the extracted products at 120V for approximate 45 minutes. The specificity and predicted size of the amplicon was confirmed by comparison against a 100 bp standard ladder (DNA Ladder, Norgen Biotek, ON, Canada). The staining was carried out using the intercalating dye GelRed™ Nucleic Acid Gel Stain (Biotium, CA, USA) at 0.01%, prior to visualisation of the bands under a UV transilluminator and image acquisition system ChemiDoc™ MP Imaging System (BioRad, Watford, UK). The gel electrophoresis revealed the correct specificity, as indicated by only one predominant band being present, and the correct size of the amplicon (>10 kb DNA bands).

2.3.4. Total bacterial enumeration by quantitative polymerase chain reaction (qPCR)

Amplification and detection of DNA was performed by qPCR on all DNA extracts as described elsewhere (Alm *et al.*, 1996). Validation of the assay performance and standard curves were carried out using an in-house *Enterococcus faecalis* DNA extracts. Triplicate samples were processed to estimate target 16S ribosomal DNA copy numbers in 96-well reaction plates on a Mx3000P Real-time PCR instrument (Agilent Technologies, Waldbronn, Germany). The qPCR assays were prepared using a SYBR intercalation probe (PowerUp™, Life Technologies, CA, USA). Universal primers were used at a concentration of 500nM; 16S rRNA gene BacUnivF (5'TCC TAC GGG AGG CAG CAG T-3') and BacUnivR (5'-GGA CTA CCA GGG TAT CTA ATC CTG TT-3'). Characteristics of primers used are shown in Table 2.3. Positive and negative control samples were included in each run of qPCR. Standardised dilutions of 2µl *Enterococcus faecalis* DNA extracts were used as positive control (1×10^5 copies/ml), while 2µl of nuclease-free water were used as negative no-template control (NTC). The amplifications were performed under the following conditions: initial activation at 50˚C for two minutes and Dual-Lock DNA polymerase at 95˚C for two minutes. This was followed by 45 cycles of denaturation at 95˚C for 15 sec, annealing at 55˚C for 50 sec and extending at 72˚C for 1 minute, prior to a final elongation step carried out at 72°C for two minutes.

Table 2.3: Primers used for bacterial quantification of samples by qPCR.

Primer used	Primer sequence (5'–3')	Annealing temperature (°C)	Primer length (base pairs)
BacUnivF	5'TCC TAC GGG AGG CAG CAG T-3'	60.8	19
BacUnivR	5'-GGA CTA CCA GGG TAT CTA ATC CTG TT-3'	57.2	26

2.3.5. Multiple displacement amplification (MDA)

The minimum concentration accepted for the NGS was 50 ng/ µl. The extracted DNA concentration was below the accepted input for NGS analysis. Since most samples had a concentration less than 50 ng/ µl, (average of 6 ng/ µl), and to allow for bacterial identification with NGS, an unbiased multiple displacement amplification and clean-up of the DNA was carried out using Qiagen REPLI-g (REPLI-g Mini Kit, Qiagen, USA) following the manufacturer's protocol on 94 selected samples. In brief, 5µl of Tris-HCL (pH of 8) was added to each extracted DNA microtube. Each reaction tube consisted of 17 µl of buffered DNA sample which was mixed with 29 µl of REPLI-g Mini Reaction Buffer and 1 µl of REPLI-g Mini DNA polymerase to obtain a final reaction volume of 47 µl. Each reaction volume was gently mixed and was then incubated in an AccuTherm™ Microtube Shaking Incubator (Labnet International, Edison, NJ, USA) for 16 hours at 30˚C. Inactivation of REPLI-g Mini DNA polymerase was followed by heating each sample for three minutes at 65˚C. Next, amplified DNA was purified precipitation induced by 100% and 70% ethanol following Qiagen REPLI-g Mini Kit supplementary protocol. Finally, the DNA was resuspended in 50 µl of 1xTE buffer (pH of 8).

2.3.6. 16S rRNA Gene next-generation sequencing (NGS)

Positive DNA amplification was confirmed by 1% horizontal agarose gel electrophoresis. In the presence of positive amplification, DNA extracts of 94 samples were selected. Samples were subjected to 2 x 300 bp paired-end, high-throughput sequencing of the V3-V5 hypervariable region of the bacterial 16S rRNA gene by Illumina MiSeq platform and the v3 chemistry (2x300 bp paired-end reads) according to the manufacturer's protocol (Eurofins Genomics, Cologne, Germany). Amplicons were generated using a two-step PCR protocol. Briefly, the V3-V5 region was PCR-amplified using 16S Amplicon PCR forward primer = 5'

CCTACGGGNGGCWGCAG and reverse primer 5' GGGTTGCGCTCGTTGCGGG (Sacchi *et al.*, 2002, Klindworth *et al.*, 2012). Amplicons were cleaned up and set up for the index PCR with specific primers directed to the universal overhangs. Final amplicon libraries were cleaned up, quantified and pooled equimolar.

2.3.7. Bioinformatic analysis, data processing and taxonomic classification

16S amplicon processing and bioinformatics analysis was carried out using Nephele, a cloud-based microbiome data analysis platform version 2.3.2 (Weber *et al.*, 2018) using Quantitative Insights into Microbial Ecology (QIIME) pipeline version 1.9.1 (Caporaso *et al.*, 2010). The Nephele quality check (QC) pipeline was used to run a quality control check (FastQC). Primers and adapters were trimmed, sequence filtered, dereplicated, and chimaeras removed. DNA sequences quality scores "Phred quality" was set at 16. In all samples, as paired-end sequence was not possible, the forward read was used for taxonomic classification. Sequences were aligned and clustered into operational taxonomic units (OTUs) using open-reference OTU picking and SortMeRNA tool for clustering (Kopylova *et al.*, 2012) in QIIME at 97% of similarity. Representative sequences from each cluster were used to assign taxonomy through matching against the Greengenes 16S rRNA gene database version 13_8, and the Human Oral Microbiome Database (HOMD) (Chen *et al.*, 2010).

Data obtained through QIIME as Biological Observation Matrix (BIOM) were imported in R software version 3.6.1. R environment was adopted to elaborate statistics as well as plotting. Read count and gene copy normalisations were carried out for differential abundance to a threshold of >6000 copies. A cut-off of 6000 reads was chosen for inclusion in the quantitative comparisons. The low reads were included only for microbial community description and analysis.

Alpha diversity was estimated to analyse the richness (chao1 and ACE) and diversity (Shannon index) of microbial species in the intracanal samples based on OTU and genus level within R

packages. Wilcoxon rank-sum test was used to perform differential analysis between different intracanal groups using a conventional $P<0.05$ statistical significance threshold.

2.4. Results

Initially, 50 patients were approached at the dental emergency department unit reporting a history of acute symptoms of IP. After clinical and radiographic examination, 34 patients were clinically diagnosed as IP. Four cases were subsequently excluded due to the absence of bleeding upon pulp chamber access (pulpal necrosis), and only intracanal samples were collected as positive control while no further sample collection was obtained from these four cases. The remaining 30 patients were included in this trial. The average age of patients was 44 years. Molars, premolars and anterior teeth were included. From the 30 patients who initially visited ADC, seven had emergency pulp extirpation in ADC, and were then referred to PG endodontic clinics. Treatment was initiated and completed in PG clinics in the remaining 23 patients. All treatment was carried out by one operator. Treatment was completed in one visit in 13 patients and two visits in 17 patients. Clinical details of included patients are given in Table 2.4.

Table 2.4: Overview of patients' demographic and clinical information (n=30).

	Number of patients (%)
Gender	
M	16 (53%)
F	14 (47%)
Tooth type	
Molar	18 (60%)
Premolar	8 (26%)
Anterior	4 (14%)
Treatment initiated in Acute Dental Care	
Yes	7 (23%)
No	23 (77%)
Number of visits	
One visit	13 (43%)
Two visits	17 (57%)*
Mean age	44 years (range 19-60)

*Out of 17 patients having two-visit treatment, seven had initial emergency pulp extirpation in ADC.

2.4.1. Bacterial enumeration by qPCR

Results of the qPCR assay were expressed as Ct (cycle threshold) values. These values were converted into categorical data because of the recognised difficulties in inferring absolute counts for PCR-amplified samples, as shown previously (Rôças and Siqueira, 2011a). *High* corresponded to Ct values < 24 (signal $\geq 10^5$ *E. faecalis* standard), *moderate*: Ct values of 25-28 (10^4 - 10^5), *low* was ascribed at Ct values values ranging from 29-30 (signal $\leq 10^4$), while Ct values above 31 were deemed *negative*. Serially diluted standard curves were generated using *Enterococcus faecalis* DNA extracts derived from known colony-forming units.

A total of 266 samples were collected and analysed; all control samples (NEC, NC and CC) samples had no identifiable PCR amplification at >2 cycles of the NTC with the exception of two CC samples. From the initial vital intracanal samples taken (S1) n=30, a third had high levels of bacteria (equal to or stronger than the signal of 10^5 *E. faecalis* standard), while 20% had moderate bacterial levels (signal between 10^4 and 10^5 standard). Collectively, 53% of samples had significant levels of bacteria within the vital pulp (10^4 to 10^5 16S rDNA copies). More than half (63.3%) of pre-obturation intracanal samples (S2) had high-moderate bacterial levels. The qPCR findings of initial and pre-obturation intracanal samples are shown in Table 2.5.

Sixteen cases had initial samples with high-moderate bacterial level, 75% of which maintained the same level at time of obturation (n=12). On the other hand, half of the initial intracanal samples with low bacterial levels (n=14) ended up with higher bacterial load at time of obturation (n=7). Figure 2.2 presents the transition from S1 to S2 with regard to bacterial counts.

Wilcoxon matched-pairs test was used to compare the bacterial load before the treatment (S1) and at time of obturation (S2). No significant difference between S1 and S2 was observed (P=0.366).

Table 2.5: Microbial load in initial (S1) and pre-obturation (S2) intracanal samples.

Bacterial load	S1 n(%)	S2 n(%)
High ($\geq 10^5$)	10 (33.3%)	11 (36.7%)
Moderate (10^4-10^5)	6 (20%)	8 (26.7%)
Low ($\leq 10^3$)	6 (20%)	3 (10%)
Negative (no signal)	8 (26.7%)	8 (26.7%)

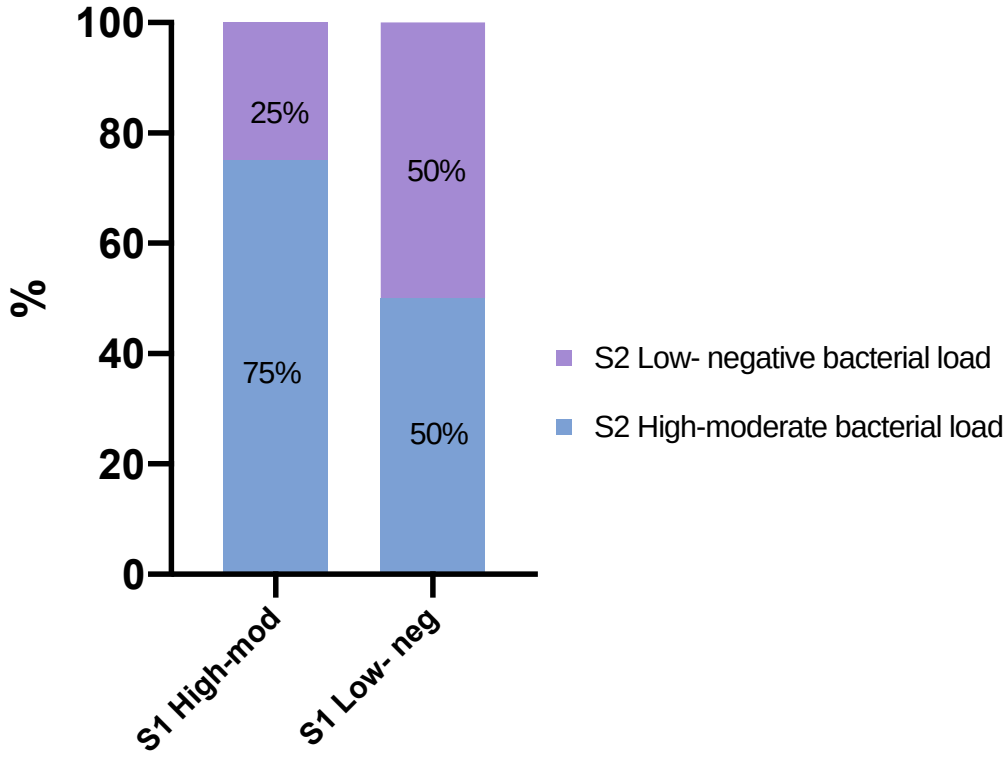


Figure 2.2: The transition from S1 to S2 with regard to bacterial levels.
Out of the 30 initial intracanal samples (S1), 16 had high-moderate bacterial load. 75% of cases which started with high bacterial load had high bacterial load in their corresponding pre-obturation samples. On the other hand, half of the cases with low bacterial load initially had high bacterial load before obturation.

When focusing on the cases completed in one visit (13 cases, 43% of the total), 77% yielded high-moderate intracanal bacterial levels just before obturation (10/13). On the other hand, of cases completed in two visits (17 cases, 56.7% of the total), 53% had high levels of bacteria before obturation. Differences between cases completed in one and two visits are demonstrated in Figure 2.3. There is no significant difference between the one and two visits group in the pre-obturation bacterial load (Fisher exact test, p=0.259).

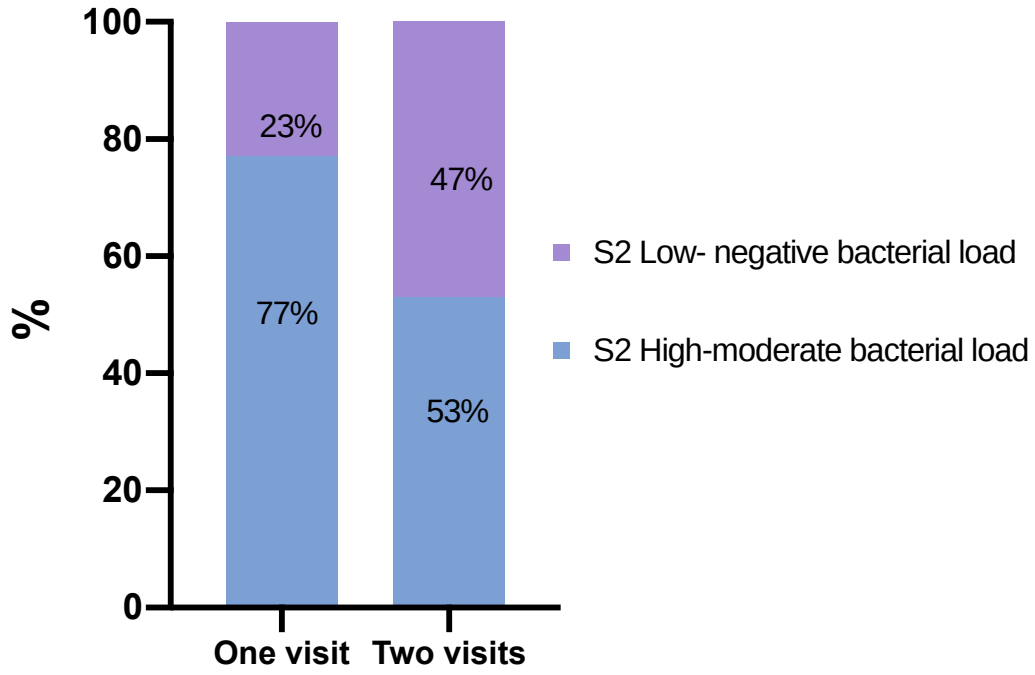


Figure 2.3: The differences in S2 bacterial load between cases completed in one and two visits. This highlights the increased percentage of samples having moderate-high bacterial load at S2 in cases completed in one visit, while almost equal distribution is noted in terms of high or low bacterial load at time of obturation in cases treated in two visits. Difference between the two groups was not significant (Fisher exact test, p=0.259).

Regarding the initial file introduced to the canal, 38% exhibited high-moderate levels of bacteria, while 29% of files used during treatment were considered contaminated. 21.3% of rulers and 44.7% of rubber dam surface showed moderate to high level of contamination at the time of obturation. Thirty-three percent and 26% of instruments and gloves, respectively, were contaminated at the time of obturation, as can be seen in Figure 2.4.

Fisher exact test was used to evaluate if any contaminated instrument or material (files, rubber dams, rulers, instruments and gloves) was related to bacterial load before obturation (S2).

There was a significant association between high-moderate bacterial load in S2 and contaminated rubber dam surface at time of obturation ($p=0.021$). The same association was observed with the initial file used during the treatment ($p=0.015$). On the other hand, a significant association was not noted between contaminated gloves, rulers or instruments and the S2 bacterial load.

All the contaminated instruments, materials, and surfaces were combined in one group (contaminated objects). Fisher exact test was used to check the association of contaminated objects to S2 bacterial load. Higher bacterial load (high-moderate) is more likely in S2 when any of the objects is contaminated ($p=0.008$). There were 18 cases with contaminated objects, and 83% of those ended up with high-moderate bacterial load at S2. On the contrary, only 33% of the cases with clean objects ended up with high-moderate bacterial load at S2. Table 2.6 and Figure 2.5 highlight the differences between the two groups. An overview of bacterial load of all samples is shown in Figure 2.6.

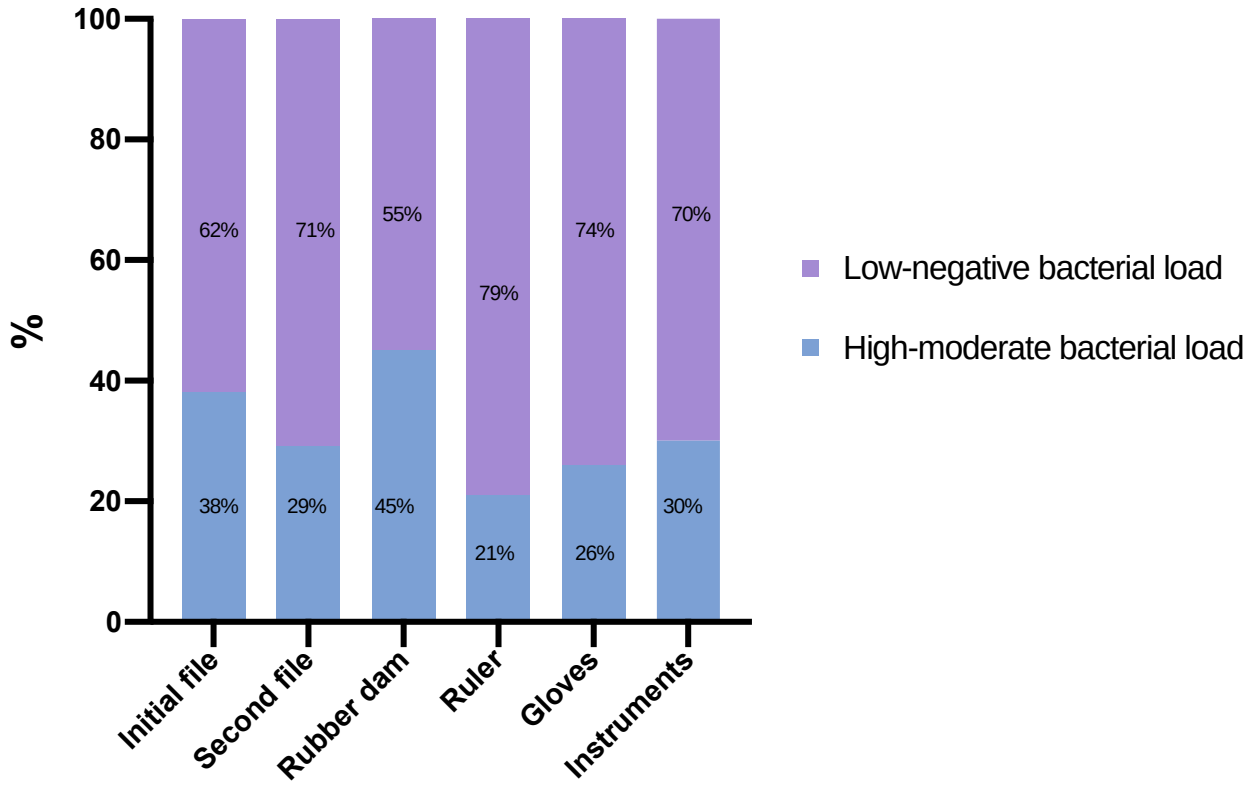


Figure 2.4: Levels of contamination in different materials and instruments during RCT.
Lowest level of contamination was shown in burs samples (all were below the detection threshold of qPCR). 20-25% of rulers and gloves were heavily contaminated with bacteria at time of obturation. The highest contamination level was seen in rubber dam surfaces (45%) and initial files used during the root canal treatment (38%).

Table 2.6: Pre-obturation bacterial status in association to contaminated objects found.
Higher bacterial load in S2 samples were associated with contaminated RD and files (p=0.021 and 0.015 respectively). Moreover, higher bacterial load (high-moderate) is more likely in S2 when any of the objects is contaminated (p=0.008).

Pre-obturation bacterial load	**Contaminated RD 13/29**	**Contaminated (F1)11/29**	**Contaminated objects (F,R,RD,G, inst)18/30**
	n(%)	n (%)	n (%)
S2 high-moderate	11 (84.6)	10 (91)	15 (83.3)
S2 low-negative	2 (15.4)	1 (9)	3 (16.7)

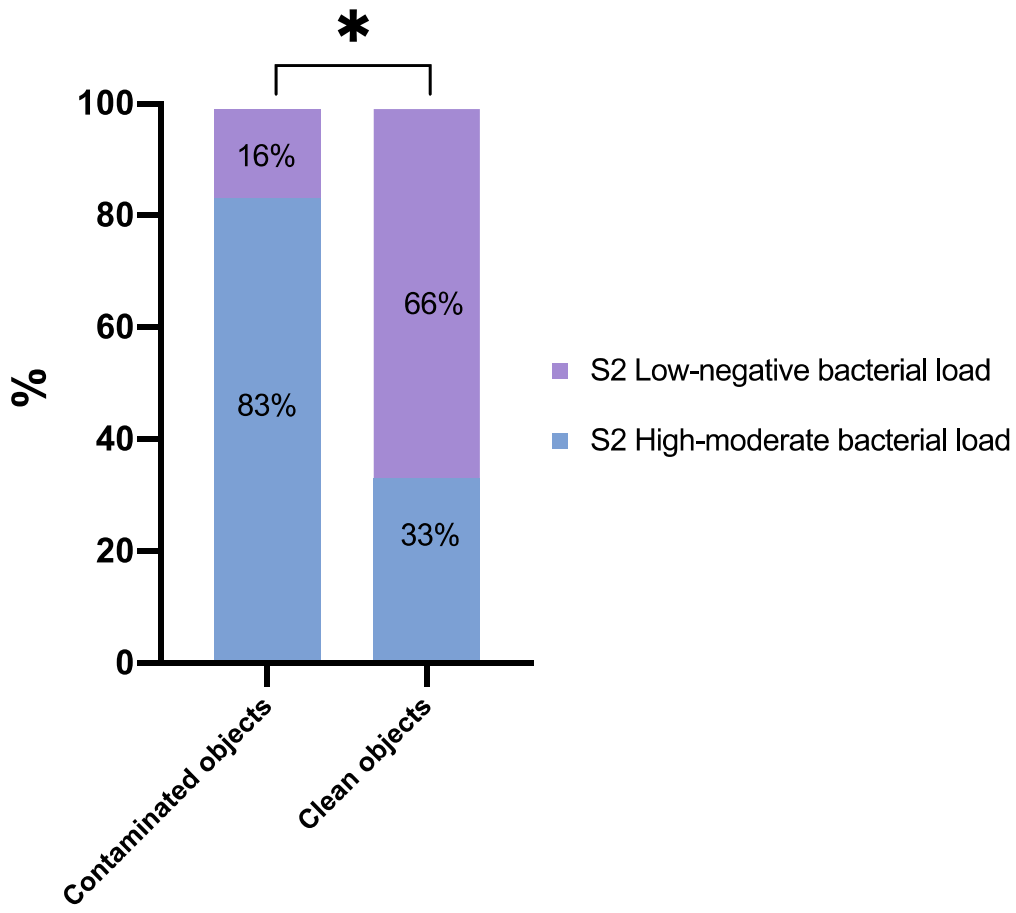


Figure 2.5: Pre-obturation bacterial status in association to contaminated/clean objects found.
Significant association between higher bacterial load at time of obturation (S2) and contaminated objects (Fisher exact test, P=0.008). Contaminated objects include rubber dam, gloves, ruler, files and instruments (tip of the tweezers, DG-16 endodontic explorers, pluggers and flat plastic instruments) with high-moderate bacterial load.

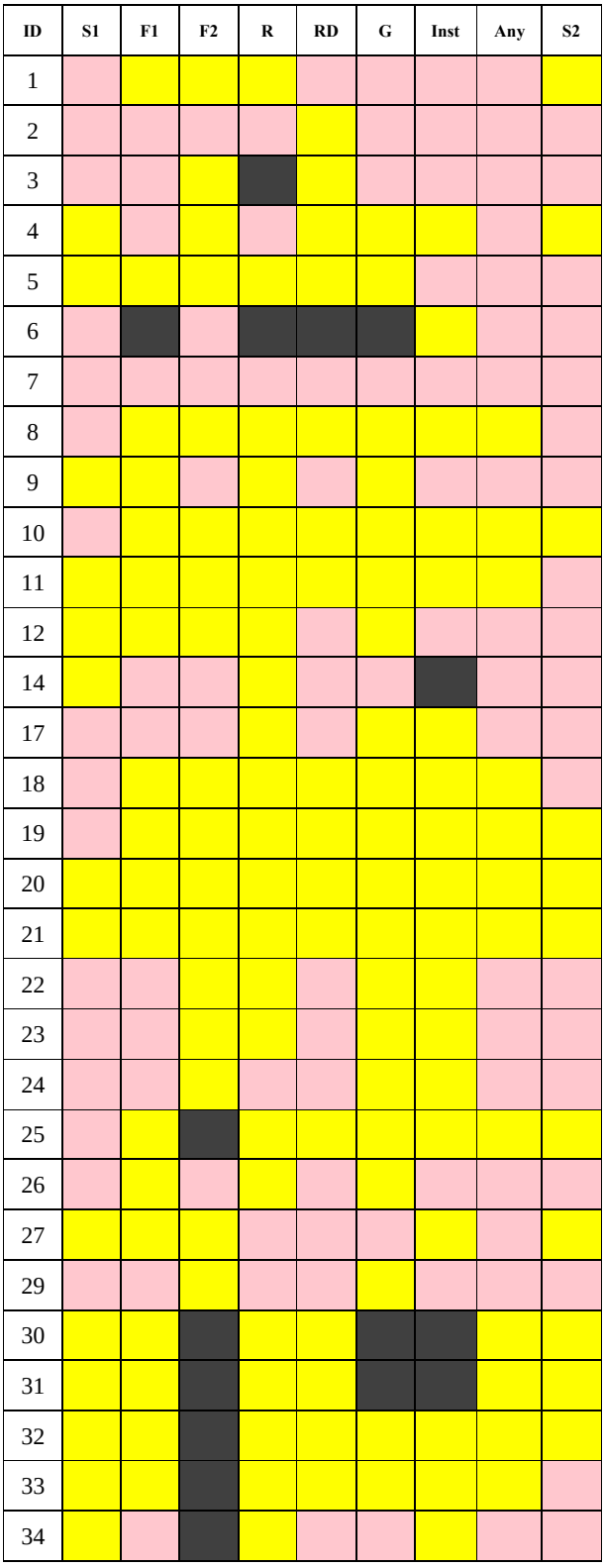


Figure 2.6: Summary of all samples bacterial load.

Out of 30 patients, 18 patients had at least two contaminated objects (rubber dam, files, gloves, rulers, or instruments). 15 out of those 18 patients (83%) had high bacterial load at time of obturation (S2), highlighting the risk of contamination from different operatory surfaces to intracanal space. *Red* indicates high-moderate bacterial load ($\geq 10^4$). *Yellow* indicates low-negative bacterial load ($\leq 10^3$). *Black* indicates sample was not collected/processed.

2.4.2. Bioinformatic analysis of the bacterial microbiome

Out of 94 samples selected for NGS, 58 (62%) successfully had positive PCR amplicons as shown in Figure 2.7. Due to low DNA quantity, amplification was not detected in 36 samples, which were all negative control samples (sterile paper points, gloves, RD, instruments and ruler surfaces) and non-template control as well as intracanal samples and 21 samples collected from gloves, rubber dams, rulers and files during treatment. All these un-amplified samples were considered sterile/negative. Out of the 58 successful samples, five samples had less than 200 reads and were not successfully classified, manual BLASTn searches revealed mitochondrial DNA amplification. Successful downstream analysis was achieved in the remaining 53 samples. Figure 2.7 illustrates the process of NGS selected samples.

Across all samples, the number of total valid reads from 16S rRNA sequencing was 2,151,207, ranging from 26 to 130,953 reads (median 31,456 / mean 37,089). The total number of OTUs detected in all samples was 2796. Regarding classification, 2726 (97.5%) of OTUs belonged to phyla, 2724 (97.42%) classes, 2677 (95.74%) orders, 2571 (91.95%) families, 2019 (72.21%) genera and 557 (19.92%) species.

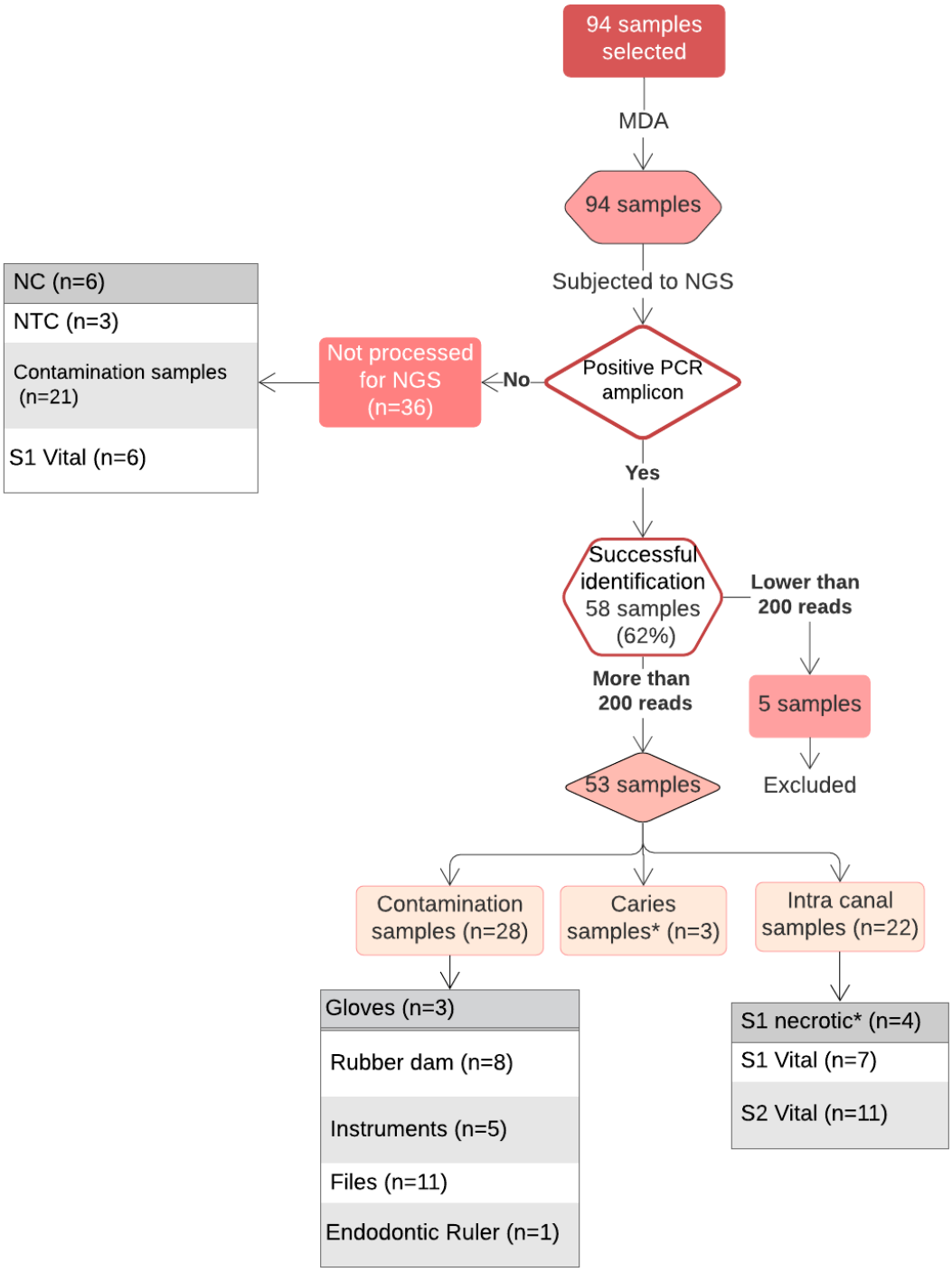


Figure 2.7 Diagrammatic representation of samples subjected to NGS procedures starting with 94 selected samples.

36 samples yielded weak amplification (all negative control samples, 21 contamination samples, and six S1 samples). Remaining 58 samples were successfully sequenced. After QC, five samples were excluded because of low reads (<200), while the remaining 53 samples were successfully analysed.

*Caries and necrotic samples were included as control samples for internal validation of methodology, but results not shown in this study.

Describing the bacterial community in pre-obturation samples

In pre-obturation samples, the most abundant phyla were Firmicutes (59.5%), followed by Proteobacteria (21.8%), Actinobacteria (17.2%), Bacteroidetes (0.9%) and Fusobacteria (0.2%). The remaining phyla (Cyanobacteria, Verrucomicrobia, TM7, and Spirochaetes) were of relatively low abundance (0.35% in total) as shown in Figure 2.8.

At the genus level, most genera occurred in relatively low abundance. In total, 63 genera were identified in S2. Half of the OTUs belonged to the genera *Streptococcus, Propionibacterium, Staphylococcus,* and *Bulleidia*. The top 12 genera ranked by their abundance are presented in Table 2.7 and Figure 2.8.

Details about the transition of microbial communities from S1 to S2 are shown in Appendix 2.

Table 2.7 : Most abundant genera in pre-obturation samples.

Genus	Relative abundance	Frequency in samples
Streptococcus	19.7%	8/9
Propionibacterium	11.5%	6/9
Staphylococcus	11%	1/9
Bulleidia	8.6%	3/9
Granulicatella	5.8%	3/9
Enterococcus	5.4%	7/9
Exiguobacterium	3.6%	3/9
Haemophilus	3.3%	5/9
Achromobacter	3.1%	1/9
Sphingomonas	2.3%	2/9
Mycobacterium	1.8%	3/9
Rothia	1.2%	5/9

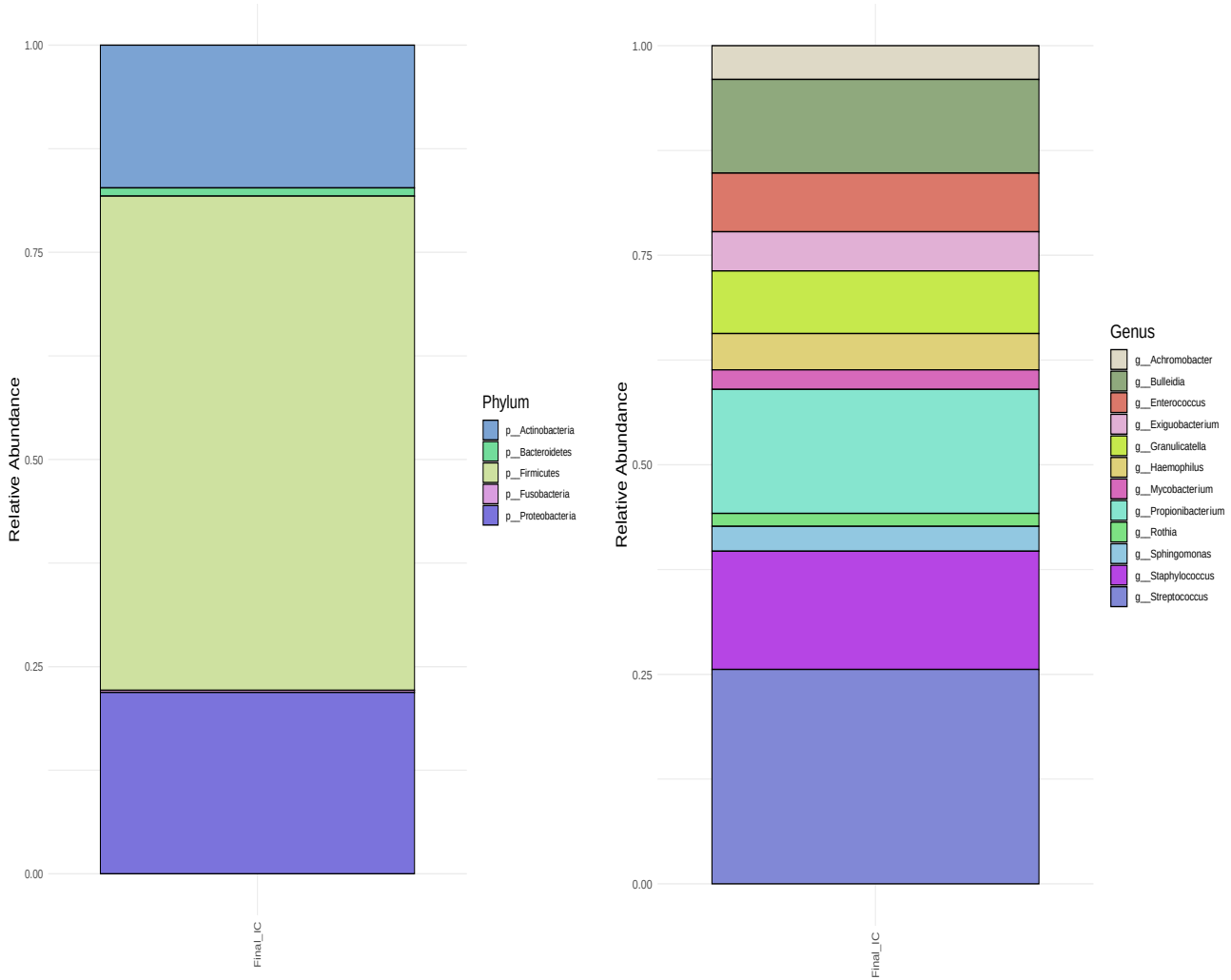


Figure 2.8: Most abundant phyla (left), and genera (right) in pre-obturation samples.
Highlighting the greatest abundance related to phyla Firmicutes (59%), Proteobacteria (21%), and Actinobacteria (17%). Members of the genera *Streptococcus* (19%), *Propionibacterium* (11%), *Staphylococcus* (11%), and *Bulleidia* (8%) dominated the S2 samples.

Describing the microbiota of contaminated surfaces and instruments

Microbiota of the sampled and examined surfaces/instruments were described and compared to the microbial findings of pre-obturation samples to assess the possible introduction and transmission of microorganisms from surfaces to the root canal space at point of obturation.

A) Gloves

The most abundant genera in gloves surfaces were *Streptococcus* (35% of all gloves samples in 3/3 samples), *Leptotrichia* (22.4% in 3/3), *Rothia* (21% in 3/3), and *Haemophilus* (4.5% in 3/3). The most abundant genera in all gloves samples are shown in Appendix 3.

When comparing gloves samples to pre-obturation samples, *Streptococci* were the most abundant genera in gloves samples (35%) and similarly expressed in S2 samples (20%), followed by *Rothia* in 21% and 1%. *Haemophilus* were commonly shared, accounting for 4% of the total OTUs in both groups at the genus level. The different taxa in S2 and gloves are highlighted in Figure 2.9.

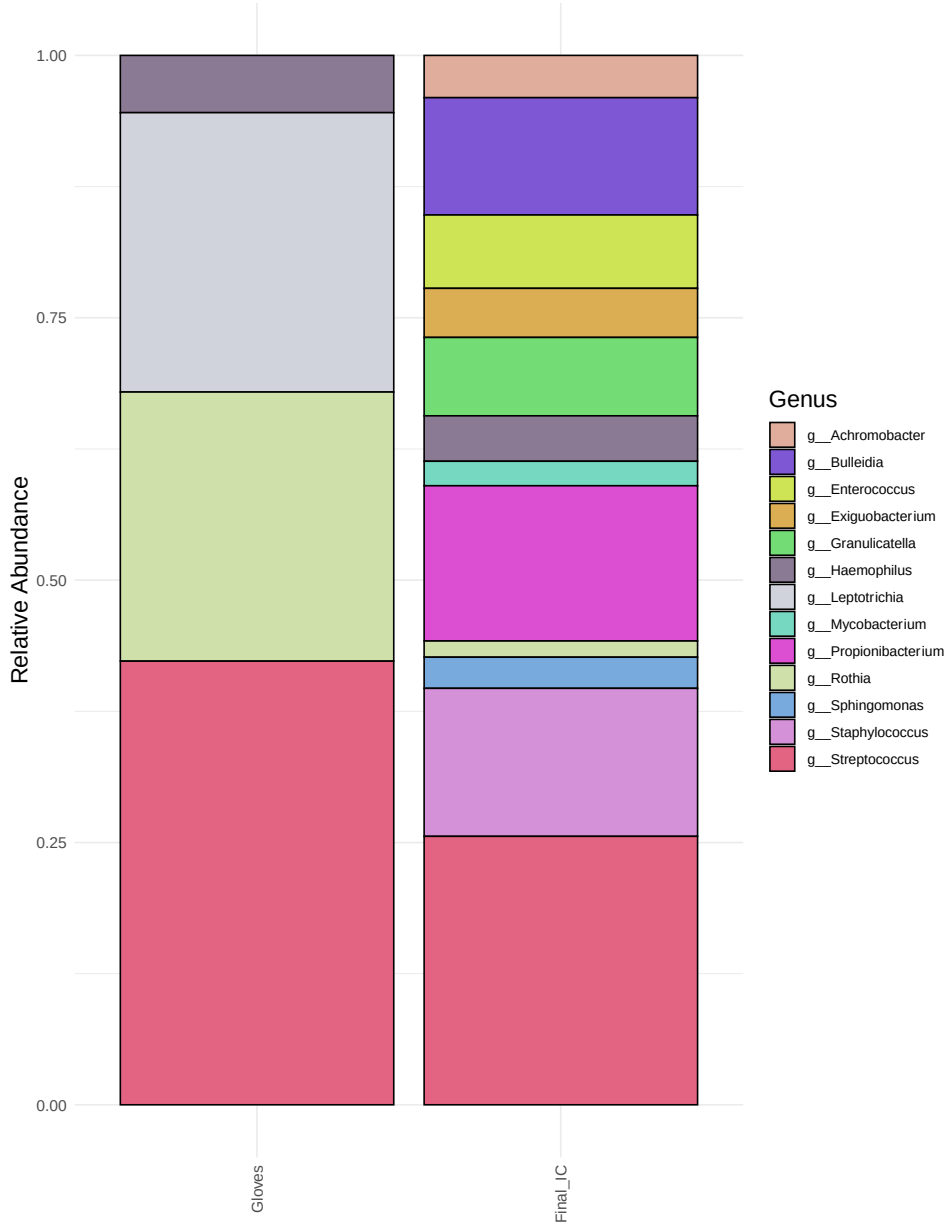


Figure 2.9: Different genera observed in gloves and S2 samples (cut-off 0.01).
Note the common genera expressed in gloves and pre-obturation samples. *Streptococci* with highest relative abundance in both gloves and S2 (35% and 20%), followed by *Rothia* and *Haemophilus* being expressed in both groups.

B) Rubber dam

The most abundant genera in rubber dam samples are expressed in Table 2.8 and Appendix 3. Phylum TM7 was found in 8/8 rubber dam samples ranging from .003% to 2% relative abundance (0.5% on average in all samples).

The rubber dam surface carried out the more common microbial community with S2 samples compared to other tested surfaces. The commonly presented genera in both groups were *Streptococcus* (18% in RD, 19% in S2), *Granulicatella* (17%, 5%), *Rothia* (15%, 1.1%), *Sphingomonas* (13%, 2.3%), *Propionibacterium* (2% ,11%), and *Haemophilus* (1.5%, 3.3%) as presented in Figure 2.10.

Table 2.8: Most abundant genera in rubber dam samples (cut-off 0.01).

Genera	Relative abundance	Frequency in samples (n=8)
Streptococcus	18.0%	8
Granulicatella	18.0%	8
Rothia	15.9%	8
Sphingomonas	13.3%	6
Actinomyces	3.5%	7
Dialister	3.4%	4
Leptotrichia	2.9%	7
Propionibacterium	2.0%	8
Fusobacterium	1.8%	5
Haemophilus	1.6%	4
Corynebacterium	1.3%	8
Finegoldia	1.3%	1

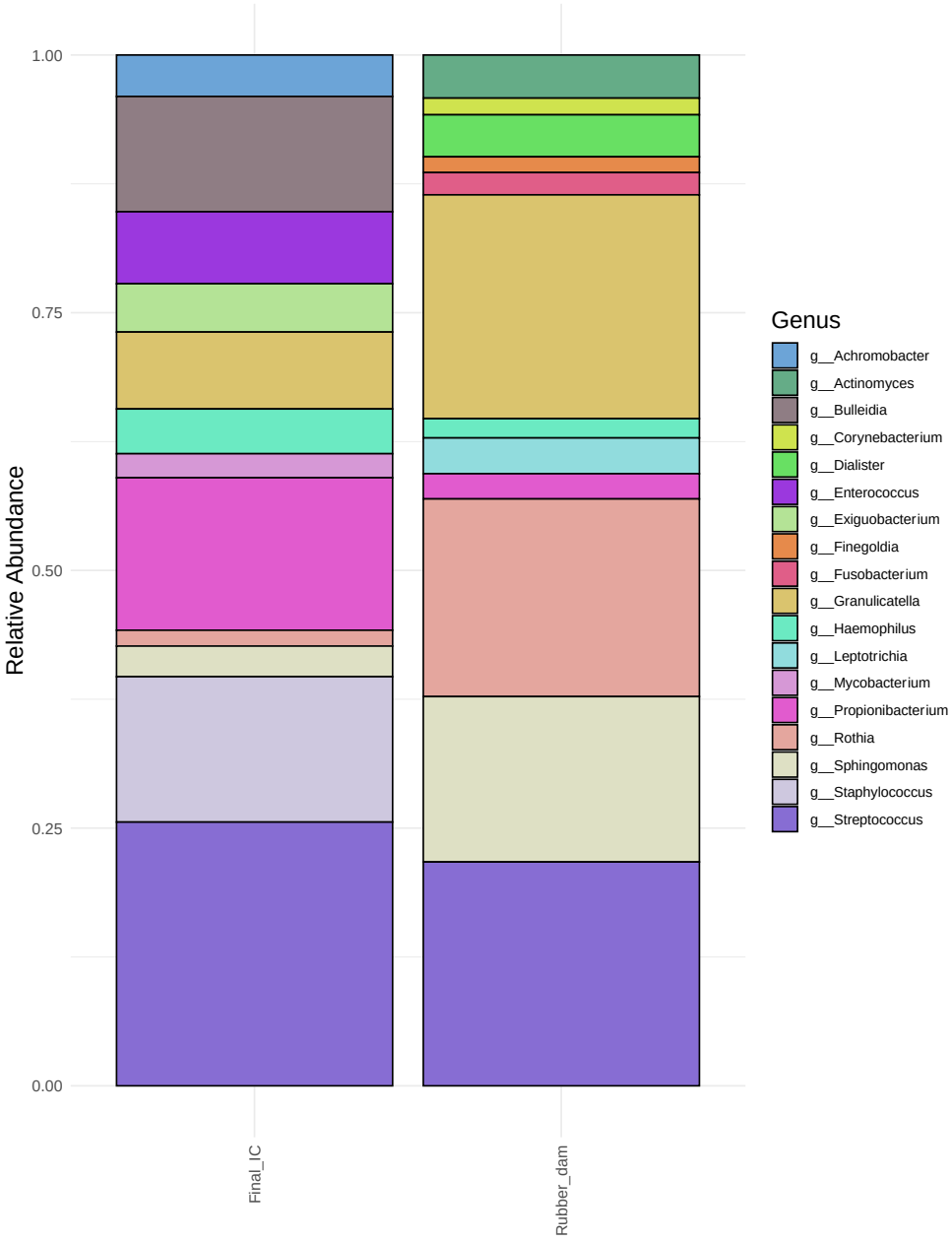


Figure 2.10: Genera observed in rubber dam and S2 samples (cut-off 0.01).
Similar genera expressed in rubber dam and pre-obturation samples can be recognised. Commonly shared genera such as *Streptococcus*, *Granulicatella*, *Rothia*, *Sphingomonas*, *Propionibacterium*, and *Haemophilus* were noted.

C) Instruments (tip of the tweezers, DG-16 endodontic explorers, pluggers and flat plastic instruments)

The most abundant genera detected on instrument surfaces are shown in Table 2.9. At the time of obturation, the tip of the explorer, tweezers, pluggers, and plastic instruments carried most abundantly *Streptococci* (22%) which were similarly expressed in the pre-obturation samples as 19%. *Rothia* was also expressed in both sample groups (19% in instruments and 1% in S2), as shown in Figure 2.11. Details of genera in all instrument surfaces are shown in Appendix 3.

Table 2.9: Relative abundance and frequency of different genera observed in instruments at the time of obturation.

Genera	**Relative abundance**	**Frequency in samples (n=5)**
Streptococcus	22.8%	5
Rothia	19.1%	4
Lactobacillus	14.7%	3
Fusobacterium	8.5%	1
Selenomonas	4.6%	2
Eikenella	1.4%	2
Oribacterium	1.3%	1
Peptostreptococcus	1.2%	2

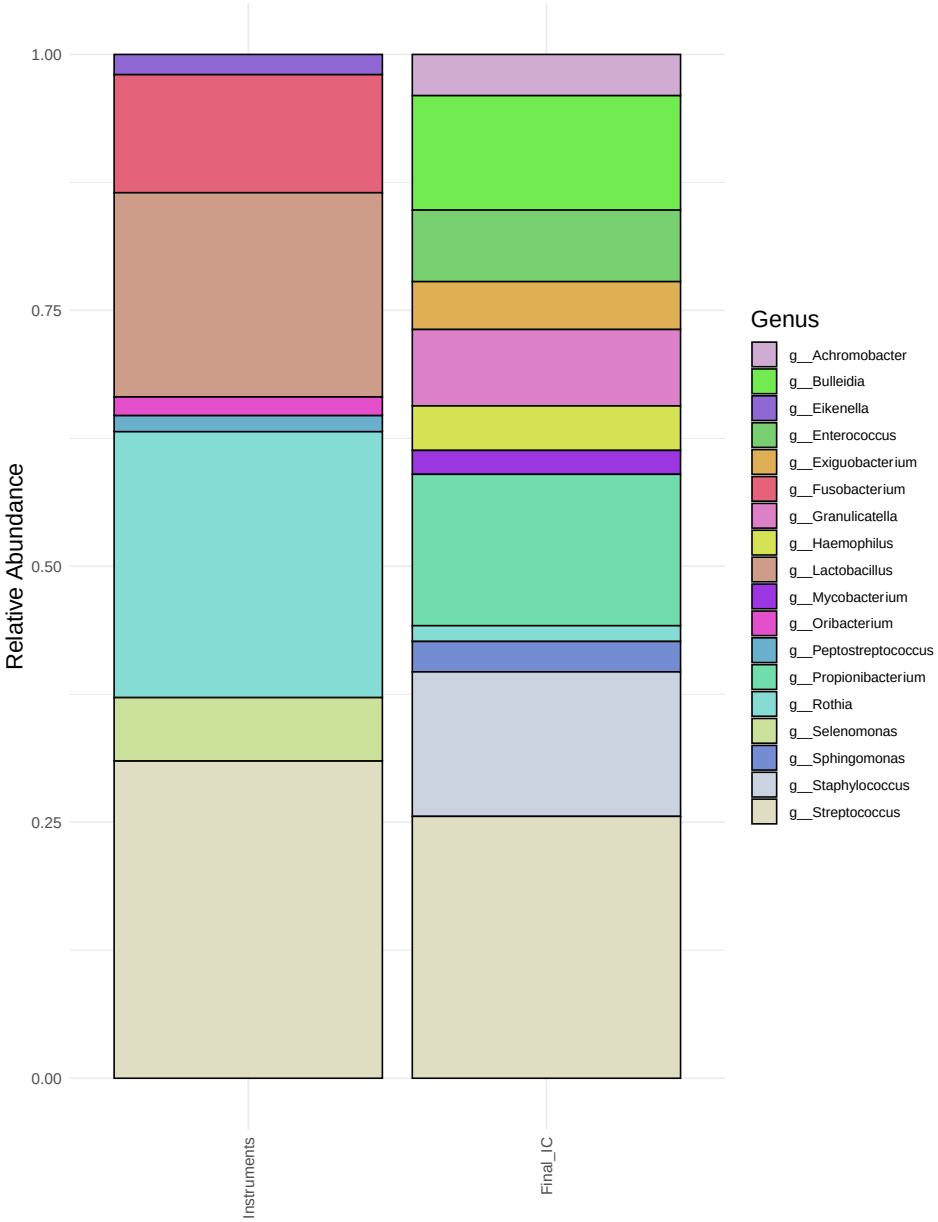


Figure 2.11**:** Comparison of genera detected in S2 and instruments at time of obturation (cut-off 0.01).

Similar expression of *Streptococci* is noted in both S2 and instrument surfaces. *Rothia* was also among the commonly found genera, but to a lesser extent in the pre-obturation samples.

D) Files

The most abundant genera in files are highlighted in Table 2.10 and Figure 2.12, while details of all file samples are given in Appendix 3.

Similarly expressed genera were in files and S2 samples were *Streptococcus* (12% in file, 20% in S2), *Propionibacterium* (17%, 11%), and *Rothia* (16%, 1%).

Table 2.10: Top genera identified in files samples.

Genera	Relative abundance	Frequency (n=11)
Propionibacterium	17.5%	9
Rothia	16.0%	5
Streptococcus	12.3%	10
Corynebacterium	8.1%	6
Veillonella	7.2%	3
Prosthecobacter	5.8%	1
Lactobacillus	2.3%	3
Actinomyces	2.1%	8
Renibacterium	1.3%	11
Acinetobacter	1.1%	6

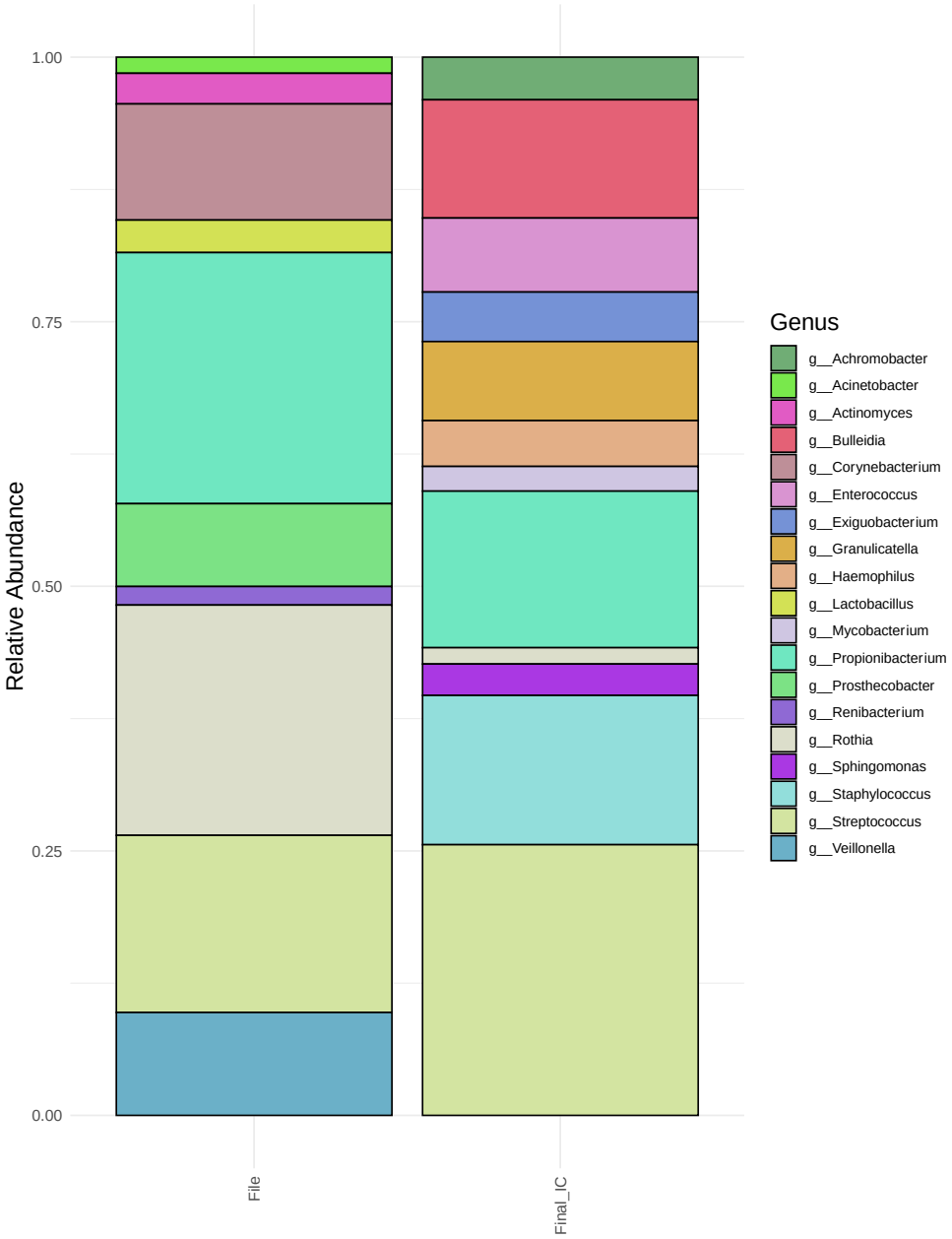


Figure 2.12: Different genera expressed in files and S2 samples (cut-off 0.01).
Showing the expression of *Streptococcus*, *Propionibacterium*, and *Rothia* to be common among the gloves and pre-obturation intracanal samples.

E) The microbial profile of all contaminated objects (gloves, rubber dam, instruments and files)

When contaminated instruments, files and surfaces were combined together, the most abundant genera were; *Streptococcus* 18%, *Rothia* 16%, *Propionibacterium* 8%, *Granulicatella* 5%. While *Corynebacterium*, *Sphingomonas*, *Lactobacillus* and *Veillonella* were expressed less, at 3%. Other less abundant genera included *Leptotrichia*, *Fusobacterium*, *Neisseria*, *Actinomyces*, *Selenomonas*, *Cutibacterium*, *Dialister*, *Haemophilus* and *Pseudopropionibacterium*. These genera are summarised in Table 2.11.

When comparing S2 microbiota to all contaminated objects (gloves, RD, files, ruler and instruments), Proteobacteria, Actinobacteria and Firmicutes were the most abundant phyla in both groups, as presented in Figure 2.13.

At the genus level, *Streptococcus* accounted for the most abundant genera in contaminated objects and S2 samples with a relative abundance of 18% and 20%, respectively, followed by *Rothia* (16%, 1%), *Propionibacterium* (8%, 11%), *Granulicatella* (5% in both), and *Sphingomonas* (3% in both) as shown in Figure 2.13.

Table 2.11: Relative abundance of most abundant genera in all contaminated objects (gloves, RD, instruments and files).

Genus	Relative abundance (%)
Streptococcus	18.3
Rothia	16.7
Propionibacterium	8.0
Granulicatella	5.2
Corynebacterium	4.5
Lactobacillus	3.9
Sphingomonas	3.9
Veillonella	3.3
Leptotrichia	3.2
Fusobacterium	2.3
Prosthecobacter	2.3
Actinomyces	2.1
Renibacterium	1.6

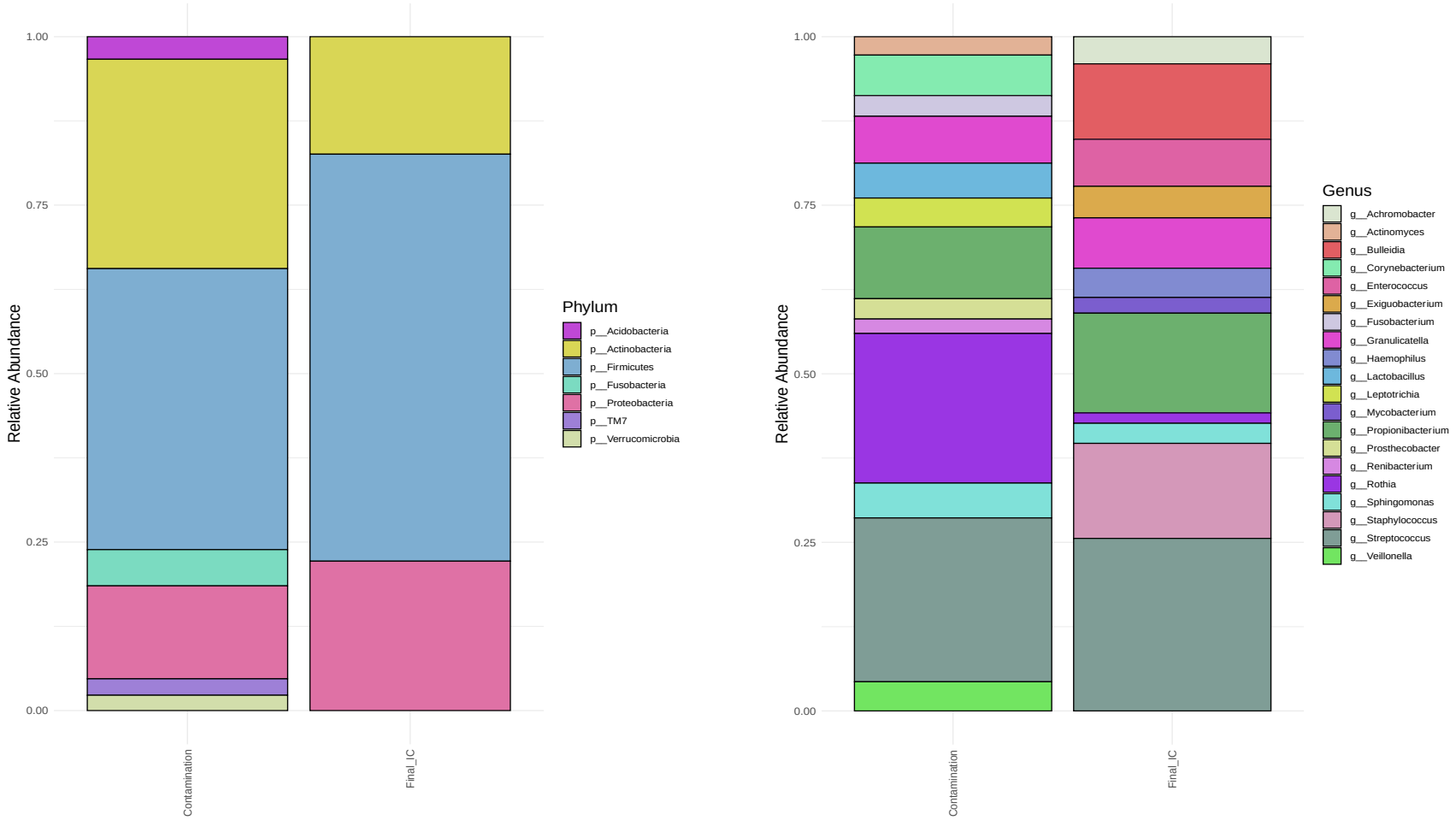


Figure 2.13**:** Common phyla and genera in S2 and all contaminated objects (cut-off 0.01).
Contaminated objects include rubber dam, gloves, ruler, files and instruments (tip of the tweezers, DG-16 endodontic explorers, pluggers and flat plastic instruments). *Left*: *Proteobacteria*, *Actinobacteria* and *Firmicutes* were the most abundant phyla in both groups. *Right*: *Streptococcus* was the most abundant genus in contaminated objects and S2 followed by *Rothia*, *Propionibacterium*, *Granulicatella*, and Sphingomonas.

Summary of microbiota in all contaminated objects and pre-obturation samples

As shown in Tables 2.12 and 2.13, the most abundant microbial taxa detected in S2 samples and the possible contamination sources are presented.

Table 2.12: Overall relative abundance of taxa in S2 as well as their possible contamination sources (cut-off 0.01).

Taxa	**Relative abundance (within the group)**				
	S1	**RD**	**G**	**Inst**	**S2**
Propionibacterium	9.3%	2%	Less than 0.01%	Less than 0.01%	11.4%
Staphylococcus	Less than 0.01%	0.17%	Less than 0.01%	Less than 0.01%	10.9%
Granulicatella	1.8%	17%	Less than 0.01%	Less than 0.01%	5.7%
Enterococcus	2.8%	0.06%	Less than 0.01%	Less than 0.01%	5.4%
Bulleidia	Less than 0.01%	0.03%	Less than 0.01%	Less than 0.01%	8.6%
Exiguobacterium	2.8%	Less than 0.01%	Less than 0.01%	Less than 0.01%	3.6%
Streptococcus	13%	18%	35.4%	22.7%	19.7%
Rothia	2%	15%	21.4%	19%	1.1%
Haemophilus	Less than 0.01%	1.5%	4.5%	Less than 0.01%	3.3%

Table 2.13: Taxa detected in S2 samples and their possible sources of contamination (presence/absence) (cut-off 0.01).

Taxa	S2	RD	Inst	Gloves	S1
Staphylococcus	✓	✓	✓	✓	✓
Sphingomonas	✓	✓	✓	X	✓
Bulleidia	✓	✓	✓	✓	✓
Haemophilus	✓	✓	X	✓	X
Streptococcus	✓	✓	✓	✓	✓
Rothia	✓	✓	✓	✓	✓
Propionibacterium	✓	✓	✓	✓	✓
Exiguobacterium	✓	X	X	X	✓
Enterococcus	✓	✓	X	X	✓
Microbacterium	✓	X	X	X	✓
Granutecella	✓	✓	✓	✓	✓
Actinomyces	✓	✓	✓	✓	✓

2.5. Discussion

This pilot study was designed and conducted to generate proof-of-concept data to determine the feasibility of implementing an 'enhanced infection control' protocol in root canal treatments. The main objective was to determine the potential sources of contamination, which will lead to the establishment of a treatment protocol to be followed in a clinical study, aiming eventually to improve the best current practice and root canal treatment outcomes.

Choice of vital teeth

In the present study, the choice was to include only vital pulps in which teeth were diagnosed as IP. This was confirmed clinically with bleeding upon access. The reason was to examine the setting of reversible/irreversible pulpitis, where bacterial presence within the canal system at time of obturation should be predominantly iatrogenic, rather than originating from the canal as in necrotic or retreatment cases. Furthermore, as highlighted previously, the endodontic microbiome contains bacteria also found in saliva, and thus contamination cannot be categorically ruled out.

Because the dental pulp is equipped with immune response aimed to limit bacterial infection (Hahn and Liewehr, 2007b, Hahn and Liewehr, 2007a, Duncan and Cooper, 2020) and less bacterial invasion and penetration is present in vital cases (Nagaoka *et al.*, 1995), it was assumed that iatrogenic contamination would be more easily detectable. Subsequently, it was sensed that this would lead to better detection of contamination and bacterial transmission from instruments or surfaces during the treatment.

Initially, we aimed to examine bacteria typically not described as predominantly associated with endodontic lesion. But, in our study, vital cases revealed typical, but reduced diversity,

endodontic microflora. Thus, instead of targeting only microbial introduction, we shifted on exploring enrichment of bacteria from initial to pre-obturation intracanal samples. Although half of the vital cases had bacteria initially in our study, still samples from necrotic teeth will no doubt show greater microbial enrichment at baseline (Siqueira and Rôças, 2005a). Details about microbial findings in IP cases will be discussed in detail in Chapter 3.

Microbial findings of contaminated surfaces: rubber dams, gloves, endodontic rulers and instruments

Since the main question of this pilot study was to detect the origin of contamination, instruments, gloves, files, burs, rubber dam, and ruler surfaces were sampled just before obturation. This timing is critical to avoid re-contaminating the canals after chemomechanical preparation completion and before definitive restoration placement.

Almost half of the rubber dam surfaces had a significant bacterial load at the time of obturation. These results are consistent with those of other studies suggesting that complete asepsis of the rubber dam surface was not feasible even after disinfection, as pointed out in the introduction of this chapter (Ng *et al.*, 2003, Malmberg *et al.*, 2016, Rorslett Hardersen *et al.*, 2019). Although the resin-based sealant, OpalDam, was used around all teeth to prevent leakage (Fors *et al.*, 1986), a leakage-proof environment was yet not achieved. The findings indicate how rubber dam surface acts as a reservoir for microorganisms being in close contact to tooth and other instruments.

The current study reported a higher risk of contaminated rubber dams at the time of obturation when compared to another qPCR study (Rorslett Hardersen *et al.*, 2019). Such a difference possibly contributed to the fact that Hardersen *et al*. implemented rubber dam disinfection protocol using chlorhexidine in alcohol and NaOCl. Also, in their study, in some cases, the

rubber dams were intentionally disinfected after radiographs were taken. On the contrary, the current study was designed to follow the common clinical practice and did not implement any disinfection protocol. Our study was directed towards reflecting the standard clinical situation and thus allowing the detection of any contamination throughout the process. NGS findings revealed that the rubber dam surfaces were contaminated mainly with *Streptococcus*, *Granulicatella*, *Rothia*, *Sphingomonas*, *Actinomyces*, *Propionibacterium*, *Fusobacterium*, and *Haemophilus*. Some of those were reported previously on rubber dams using Sanger sequencing, such as *Propionibacterium*, *Fusobacterium*, *Haemophilus*, and *Streptococcus (Rorslett Hardersen et al., 2019)*.

Almost forty percent of the initial files used were contaminated mainly with *Propionibacterium*, *Rothia*, *Streptococcus*, and *Corynebacterium*. This finding highlights the risk of using the same initial contaminated file until the end of the treatment session, especially for patency, possibly resulting in introducing microorganisms to the root canal space. Unlike initial files, fewer second files were contaminated which is due to the continuous use of NaOCl during the treatment in contact with the second files. Also, the initial files were the very first files introduced to the canals and carried out the existing intracanal microbiota. Unlike files, all access burs did not show detectable microbial load with qPCR. Disruption and dislodgment of all bacteria from bur surfaces due to higher speed of rotating burs (300,000 rpm) is a potential reason for the low bacterial count findings. Moreover, burs used for caries or defective restoration removal were discarded and the sampled burs were new sterile burs used only for accessing vital teeth.

Regarding the gloves worn by the dentist, 26% showed a detectable bacterial level at the time of obturation, which is in agreement with previous studies (Fiehn and Westergaard, 1993,

Berthelot *et al.*, 2006, Luckey *et al.*, 2006). Such contamination risk might arise through transmitting bacteria from operatory surfaces to the operator's gloves (Monarca *et al.*, 2000, Williams *et al.*, 2003). Also, a previous report pointed towards the risk of microbial growth on the GP point surfaces after glove manipulations (Gomes *et al.*, 2005). Our findings also highlight the risk of jeopardising the canal's cleanness with such contaminated gloves. Taxa detected in glove surfaces in the current study are consistent with detectable taxa reported previously on gloves, such as *Streptococcus*, *Propionibacterium*, and *Haemophilus* (Gomes *et al.*, 2005, Niazi *et al.*, 2016).

Moreover, 20-30% of ruler surfaces and the instruments used showed a detectable bacterial level at the time of obturation. These surfaces were dominated by *Streptococcus*, *Rothia*, *Haemophilus*, *Lactobacillus*, *Peptostreptococcus*, and *Fusobacterium*.

Origin of microbiome detected on contaminated surfaces and their association to endodontic infection

Overall, the most abundant genera on any contaminated objects were *Streptococcus*, *Rothia*, *Granulicatella*, *Propionibacterium*, *Lactobacillus*, *Sphingomonas*, *Veillonella*, and *Fusobacterium*, which are well known to be salivary organisms. The most likely route of transmission is through the saliva to the surfaces and instruments. Some are known plaque taxa such as *Streptococcus*, *Granulicatella*, *Veillonella*, and *Fusobacterium*. Some taxa were reported to be very low in saliva but present in our contaminated samples, indicating possible iatrogenic contamination such as *Propionibacterium* (Peterson *et al.*, 2013). Some skin microorganisms were evident in gloves, rubber dams and file surfaces such as *Corynebacterium* and *Propionibacterium*, indicating transmission from the operator's or patient's skin (Grice and Segre, 2011).

Just over half of the pre-obturation (S2) samples revealed high-moderate levels of bacteria. Higher bacterial load in S2 was significantly more likely when any of the instruments used or surfaces were contaminated for the same patient (instruments, rubber dams, gloves or endodontic rulers). Such an association was not present in cases with less contaminated instruments. These findings provide more evidence of the risk of iatrogenic contamination of the root canal space during the treatment. Yet, such a statement cannot be absolute since many studies showed bacteria persisting after chemomechanical and/or calcium hydroxide intracanal medication (Siqueira and Rôças, 2008).

Microbial communities in contaminated surfaces and pre-obturation intracanal samples

Metagenomic findings of S2 samples showed that half of the OTUs belonged to *Streptococcus*, *Propionibacterium*, *Staphylococcus*, and *Bulleidia*. Although some of the taxa were present in S1 and might persist in S2, their presence in almost all contaminated surfaces should not be disregarded. For example, *Streptococci* and *Propionibacterium* accounted for 30% of S2 samples which were detected within the most frequent taxa in the RD, files and, to a lesser extent, in gloves and instruments. This suggests a possible transmission from these different surfaces during the treatment, even after root canal disinfection with NaOCl.

Greater chance of transmission is speculated when the same taxa are isolated in S2 and contaminated surfaces but not present in S1. For instance, *Haemophilus* and *Staphylococcus* were among those taxa present in gloves, RD and files, and also present in S2. Although these taxa were detected less abundantly in S2 compared to the contaminated surfaces, their absence or very low abundance in S1 suggests the potential risk of contamination. A similar scenario was also observed with *Bulleidia* (present in RD, inst, G), and *Sphingomonas* (present in RD, G).

This risk of contamination is concerning, especially with some of the microorganisms that have been reported to be associated with secondary or extra-radicular endodontic infection, such as *Staphylococcus*, *Enterococcus*, *Sphingomonas*, *Streptococcus*, *Actinomyces*, *Fusobacterium*, *Porphyromonas*, *Prevotella*, *Propionibacterium*, *Veillonella*, and *Peptostreptococcus* (Tronstad *et al.*, 1987, Gatti *et al.*, 2000, Sunde *et al.*, 2000a, Sunde *et al.*, 2000b, Chávez De Paz *et al.*, 2003, Niazi *et al.*, 2010, Dioguardi *et al.*, 2020). Those taxa were all present in our study among the contaminated surfaces or instruments, and some were detected in high abundance: *Streptococcus* (18% in RD, 22% in inst), *Propionibacterium* (17.5% in files), *Sphingomonas* (13% in RD). Others were moderately expressed: *Veillonella* (7.2% in files), *Haemophilus* (4.5% in gloves, 1.6% in RD), *Actinomyces* (3.5% in RD, 2.1% in files), *Fusobacterium* (1.8% RD, 8.5% in inst). Others were expressed in low abundance: *Peptostreptococcus* in instruments or *Staphylococcus* in rubber dams, gloves and files, and *Enterococcus* in rubber dams.

Rothia is a Gram-positive facultative aerobic, rod-shaped bacterium from the family of *Micrococcaceae*. It is a member of the human saliva, part of caries microbiota in irreversible pulpitis teeth (Hahn *et al.*, 1991, Munson *et al.*, 2004). It has also been reported to be commonly present in endodontic secondary infections (Rolph *et al.*, 2001, Anderson *et al.*, 2013, Tzanetakis *et al.*, 2015, Keskin *et al.*, 2017). Additionally, it was reported to persist even after $Ca(OH)_2$ intracanal medication (Sakamoto *et al.*, 2007). *Rothia* was among the highest detected taxa in our trial. It was expressed in 20% of gloves, instruments and files as well as in 16% of rubber dams.

Granulicatella was among the highly expressed taxa present in all rubber dam surfaces, accounting for 18% of the OTUs. It was also present but less abundantly in all other contaminated instruments and surfaces. *Granulicatella* has been reported to be one of the most abundant genera in endodontic infections using sequencing analysis (Keskin *et al.*, 2017) and other molecular studies (Siqueira and Rôças, 2006, Siqueira and Rôças, 2009c). It was also reported to be one of the top genera in acute periradicular abscess (Hsiao *et al.*, 2012) and acute periapical periodontitis (Rôças and Siqueira, 2005).

The clinical relevance of residual microorganisms in endodontics

NGS was employed in this trial to qualitatively investigate all possible members contributing to contamination rather than targeted microbiota such as in PCR or DNA-DNA hybridisation. When using such a highly sensitive detection method, even very low microorganisms in situ can be detected, which might not have significant clinical importance (Siqueira and Rôças, 2009c). On the other hand, however, these species might play a vital role in pathology or might undergo environmental changes and become more abundant in later disease stages (Siqueira and Rôças, 2009a). As endodontic diseases are polymicrobial, identifying and understanding every contributing microbial member of the community is essential to explore the pathological process and the whole community (Siqueira and Rôças, 2009a). This can be achieved through advanced microbial methods such as NGS.

One can argue that the remaining bacteria in S2 might not reach the threshold to cause any pathological disease. Since such a threshold is as yet unknown, it is still important to investigate all bacteria and their possible contribution and pathogenic implications (Siqueira and Rôças, 2008). Environmental changes in the future might potentially lead to dominating those low numbered bacteria resulting in secondary endodontic infections (stochastic effect rather than

deterministic). Although presence of bacteria prior to obturation does not predict outcomes, the complete absence of bacteria has been reported to predict clinical success (Sjögren *et al.*, 1997, Molander *et al.*, 1998).

One- vs two-visits treatment

Our findings revealed that more cases with moderate to high bacterial levels at the time of obturation had been treated in one visit. However, the difference in bacterial load between one or two visits did not reach a statistically significant level. The low number of cases in this pilot study does not allow for a definitive conclusion regarding the effectiveness of one- versus multiple-visit appointments. This is a well-known debatable issue in the endodontic literature. Meta-analysis of three randomised clinical trials did not find a significant difference between single- and multiple-visits treatments regarding healing rate (Sathorn *et al.*, 2005) and evidence is lacking to support one regimen (Manfredi *et al.*, 2016). Some *in vivo* microbiological studies, on the other hand, support added antimicrobial benefits of using calcium hydroxide dressing in between visits (Byström *et al.*, 1985, Sjögren *et al.*, 1991). It should be noted that these studies were culture-based, and similar definitive effect was not reached using molecular methods (Rôças and Siqueira, 2010, Rôças and Siqueira, 2011b).

Among the plausible explanations for our finding, besides the uncertain effect of intracanal medication with $Ca(OH)_2$, the use of new sterile instruments in the two-visits group might attribute to less microbial load at the time of obturation. Additionally, cases finished in one visit with higher bacterial load at the time of obturation were associated with either contaminated files, instruments, rubber dams, or gloves.

Sodium thiosulfate was not used to deactivate NaOCl to avoid altering the clinical situation

When taking the pre-obturation intracanal samples, sodium thiosulfate was not used. This can be justified by the fact that the goal of the current trial was to accurately represent the clinical situation without breaking the conventional clinical protocol. Paper points were used to dry the canal and then flushed with copious physiological saline before sampling to remove any residue of NaOCl and lower its effect and, eventually, have less false-positive results.

The choice of "taxa-specific" variable region of the 16S rRNA gene and the employment of MDA amplification

The bacterial hypervariable V3-V5 region was targeted in this study with the aim of achieving best spread and resolution for oral microbiota and, in particular, *Streptococci*. This region was previously used in endodontic microbial investigations (Baker *et al.*, 2003, Wang and Qian, 2009, Zandi *et al.*, 2018). One of the main limitations of such a long region is that merging of 2 x 300 bp reads is not always guaranteed. In our experiment, very poor merging was noted and eventually only the forward sequence, covering V3 and V4 hypervariable regions, was used for all sequencing analysis.

Multiple displacement amplification (MDA) is a non-PCR type of DNA amplification. It has been reported to have the lowest amplification bias, and a relatively uniform amplification of the genomic DNA template has been confirmed (Hosono *et al.*, 2003). This method uses exonuclease-resistant thiophosphate-modified degenerate hexamers as primers bacteriophage Phi29 DNA polymerase to amplify the DNA (Dean *et al.*, 2001). This method was proven to generate large amounts of whole-genome DNA up to 10,000-fold (Dean *et al.*, 2001, Hosono *et al.*, 2003), and was shown to be very beneficial in cases were DNA concentrations, as in our study, are expected to be minimal (Rôças *et al.*, 2010, Henriques *et al.*, 2016, Hoy, 2019, de

Brito *et al.*, 2020). This trial has proven the implementation of this method in endodontic samples by amplifying different bacteria targeted with universal primers. Due to known sampling limitations in endodontics, this MDA method allowed proper molecular identification even in samples with very low yields of DNA. Nonetheless, false amplification cannot be completely disregarded, however negative controls gave few positive reads not covered by genera present for clinical samples.

The use of molecular methods to detect microorganisms present

One of the main limitations of non-culture approaches is their inability to differentiate between live and dead bacteria (Siqueira, 2008). Dead bacterial DNA was still detectable with molecular methods even after years of inoculation. *In vitro* studies showed that bacterial DNA had high affinity to bind to dentine. While free DNA readily underwent enzymetic decomposition, hydroxyapatite-bound DNA was clearly more resistant (Brundin *et al.*, 2010, Brundin *et al.*, 2013, Brundin *et al.*, 2014).

Such a limitation is yet considered an advantage and allows the detection of bacteria which are challenging to cultivate and cannot survive the sampling, transportation and isolation process (Siqueira and Rôças, 2005b). The findings obtained with molecular methods should be interpreted with caution. Several validations can be given. First, the dead cell issue can be possibly overcome with the routine use of NaOCl in endodontics. Hypochlorous acid (HOCl) is the active ingredient released from NaOCl. Direct reaction of HOCl with plasmid DNA gives rise to single- and double-strand breaks via chloramine-mediated reactions. The resulting DNA, RNA damage should not eventually be detectable in PCR and other non-cultural approaches (Hawkins and Davies, 2002). One study evaluated the effectiveness of NaOCl by detecting nucleic acids, which is commonly used to assess cells' viability and compared it to DNA findings. Only three out of 45 samples showed disagreement between rRNA and DNA

bacterial PCR (Rôças and Siqueira, 2010). This figure suggests that the high copy number of 16S rDNA detected with such sensitive methods is unlikely to originate from dead cells alone (Zandi *et al.*, 2016). Moreover, many clinical studies with DNA-based PCR assays showed significant bacterial reduction after NaOCl irrigation and calcium hydroxide medication, supporting the possibility of DNA being destroyed and thus not detected in PCR (Sakamoto *et al.*, 2007, Rôças and Siqueira, 2011b). Other investigations also confirmed that NaOCl can be used to remove and destroy DNA surface contamination (Kemp and Smith, 2005) as well as in our findings where CC samples did not show bacterial DNA after the surface decontamination with NaOCl. Evidence also suggests that the free DNA from dead cells has a very short half-life and degrades rapidly in a living environment such as that of our study, where only vital cases were included (Siqueira, 2008). This degradation is due to the presence of live endodontic pathogens releasing DNases such as *Porphyromonas endodontalis*, *Porphyromonas gingivalis*, *Tannerella forsythia, Fusobacterium* species, *Prevotella intermedia*, and *Prevotella nigrescens* (Leduc *et al.*, 1995, Siqueira and Rôças, 2005b, Brundin *et al.*, 2010). Recent evidence suggests that no difference was found in the bacterial richness and diversity, and the relative abundance and microbial composition when DNA- and rRNA-based NGS analysis was carried out (Nardello *et al.*, 2020a).

Although the fate of DNA of dead bacteria in the root canal is not yet determined, their presence does not indicate the absence of their pathogenicity or disease contribution (Siqueira, 2008). So even if these dead cells were detected, still, their participation in the disease process cannot be disregarded completely. Finally, some methods have been suggested to overcome this issue. One example is the analysis of rRNA or mRNA, which has a shorter half-life and is readily degradable after cell death, unlike DNA, thus allowing good correlation to cellular activity (Rôças and Siqueira, 2010). Another method is the degradation of the free DNA before

extraction using DNase, or the use of propidium monoazide or ethidium monoazide for selective removal of dead cells' DNA (Nogva *et al.*, 2003, Nocker *et al.*, 2006).

In this study, we did not use any of these methods to degrade DNA. Since each and every method still has its drawbacks, our interest in this study was not directed towards living bacteria only.

The meticulous inclusion of different control samples

Positive responses to the universal primers highlighted the need for meticulous negative controls, especially in our case in which low DNA copies were found, and to exclude the possible contamination from qPCR component (Espy *et al.*, 2006). Negative control samples were collected from sterile file, burs and paper points, as well as swabs of the sterile endodontic ruler, sterile instruments, new gloves and rubber dam. All these negative control samples showed very low to no contamination and were used as a negative control in the analysis of the qPCR results. However, the presence of PCR inhibitors cannot be ruled out. Due to very low reads, those negative control samples were not qualified for sequencing analysis. Procedural contamination was ruled out from these negative controls, contamination control, and non-template controls. Moreover, absence of bacteria in all our negative control samples confirm the absence of carry-over of dead DNA after instrument sterilisation.

Besides the negative control samples, 21 samples collected from gloves, rubber dams, rulers and files were below the NGS detection threshold. Although these samples were amplified in our qPCR experiments, technical challenges such as lost DNA palette during MDA might be a possible explanation of their absence of amplification prior to sequencing.

Another primary source of uncertainty is the contamination with molecular methods. Despite thorough field decontamination and control samples through the processes, the possibility of partial environmental or PCR/kit reagents contamination could not be categorically excluded (de Goffau *et al.*, 2018). Those might originate during the process from reagents, kits, or disposables. One negative extraction control sample was processed for sequencing, although there was a very low read number (76 reads) after QC. The detected genera were *Granulicatella, Haemophilus, Mogibacterium, Parvimonas, Peptostreptococcus, Propionibacterium, Rothia, Sphingomonas,* and *Streptococcus.*

A comparison between known endodontic bacteria and our findings supported the endodontic, rather than the environmental origin of these bacteria. Examples of known contaminants are; *Cutibacterium*, *Acinetobacter*, *Methylobacterium*, *Microbacterium*, and *Sphingomonas* (Strong *et al.*, 2014). It is worth mentioning that there is no standard method in removing kit-originating contaminants (de Goffau *et al.*, 2018, Boers *et al.*, 2019).

2.6. Key findings and conclusion

Key findings:

Around half of the rubber dam surfaces were contaminated with bacteria at time of obturation and 38% of initial files introduced into the canal had significant levels of bacteria. Bacteria were also detected in 20-30% of gloves, instruments and rulers prior to obturation. This study also provided additional evidence on types of bacteria found in such contaminated materials and surfaces, mainly *Streptococcus*, *Rothia*, *Granulicatella*, *Propionibacterium*, *Lactobacillus*, *Sphingomonas*, *Veillonella*, and *Fusobacterium*. The present findings suggested the risk of introducing bacteria into the root canal space after chemomechanical preparations, higher bacterial loads were more frequently present in intracanal samples before root canal filling when instruments and surfaces were found to be contaminated.

Conclusion:

The evidence from this study demonstrated that aseptic field during root canal treatment was not maintained and is strongly suggestive of iatrogenic contamination, mainly from repetitive use of the same patency file, from rubber dam surfaces, and from gloves and instruments used at time of obturation. The findings suggested that even in best-practice conditions, substantial levels of contamination occurred, and 18/30 patients were at risk of contamination, having at least two contaminated surfaces or instruments. Data from this pilot study justified a full clinical trial to provide more definitive evidence. A randomised clinical trial was designed to investigate the impact of implementing an enhanced infection control protocol in reducing bacterial levels, as well as improving the outcomes of root canal treatments.

Chapter Three: Characterisation of Root Canal Microbiota in Teeth Diagnosed with Irreversible Pulpitis

3.1. Introduction

As discussed in the previous Chapter, only vital teeth were included to examine the iatrogenic microbial introduction in our pilot study, since bacteria presence within the root canal system of vital teeth is believed to be minimal due to the pulpal immune protective response. This Chapter will discuss in detail the quantitative findings, characterisation, and diversity of endodontic microflora in cases diagnosed with irreversible pulpitis.

Irreversible pulpitis (IP) is characterised by a severe inflammatory reaction developing within the confined pulpal space, commonly as a sequelae of microbial products arising from deep carious lesions (Bergenholtz, 1981). Unlike *reversible* pulpitis, where removal of the stimulus (carious tissue) is expected to allow the pulp to repair and heal, healing of inflamed pulp in IP is unlikely (Levin *et al.*, 2009).

Classically, the diagnosis of IP is based on clinical and radiographic examinations. A history of pain, pain nature and quality, and tooth response to thermal or electrical stimuli are among the standard methods to evaluate the pulpal inflammatory condition (Bender, 2000). According to the AAE, reversible pulpitis is characterised by sharp, non-lingering pain to thermal stimulus, while teeth with IP will respond with spontaneous, lingering and exaggerated pain (Levin *et al.*, 2009).

As previously demonstrated in histological studies, caries pulpal exposure will result in direct bacterial invasion to the pulp tissue leading to pathological response (Reeves and Stanley, 1966). Yet, pulpal tissue can be irreversibly inflamed even without a frank perforation (Ricucci *et al.*, 2014). This is attributed to the diffusion of bacterial by-products and endotoxins through dentinal tubules and the interstitial reactive dentine barrier thus triggering pulpal inflammation

(Reeves and Stanley, 1966, Ricucci *et al.*, 2014). When pulpal exposure cannot be clinically ascertained, differentiating between reversible and irreversible pulpitis becomes challenging, and the diagnosis is based on other clinical and radiographic criteria. Although a good agreement was shown between clinical and histopathological findings for pulpal diagnosis (Ricucci *et al.*, 2014).

Teeth presenting with symptoms of IP, when examined histologically, demonstrate acute inflammation with neutrophilic infiltration as a response to the proximity of bacteria and diffusion of their by-products from the caries-pulpal interface (Ricucci *et al.*, 2014). The inflammation and bacteria are confined within the coronal pulp tissue, and the radicular pulp is usually free of bacteria (Ricucci *et al.*, 2014).

Over the past two decades, non-culture approaches have overcome the limitations of previous culture methods. Next-generation sequencing (NGS) is a high-throughput massive parallel DNA sequencing technology developed and used to investigate the human oral microbiome, including in the field of endodontics (Manoil *et al.*, 2020). It did allow not only the identification of uncultured taxa but also the taxa that do not belong to phylogenetically validly described taxa (Manoil *et al.*, 2020).

The rate of microbial penetration in vital and necrotic pulp is believed to differ significantly; within healthy teeth this may be slow or impossible while it is rapid in the necrotic pulp (Nagaoka *et al.*, 1995). In vital teeth, the presence of odontoblastic processes, collagen fibres, and dentinal fluid resulting in reduced dentinal tubular diameter, alongside the intra-pulpal

pressure are among the reasons contributing to this permeability difference (Nagaoka *et al.*, 1995).

Previous endodontic microbiological investigations focused on primary and persistent/secondary infected root canals, while the microbiome of the relatively short-lived clinical stage of IP was not fully explored and poorly described. Also, none of the advanced sequencing studies reported the intracanal microbiota composition of such diagnosed cases (Manoil *et al.*, 2020). Mechanistically, we hypothesised that at the stage of IP, initial colonization of the pulpal space had already taken place, with phylogenetic reductions mirroring transition of caries to endodontic pathology.

3.2. Materials and Methods

Samples were obtained from the same 34 adult patients included in Chapter two. The clinical and radiographic conditions of included teeth were as follow: four teeth were recently prepared for full coverage crowns and presented with symptoms of IP developed after temporary crowns cementation. Based on ESE recent terminologies (Duncan *et al.*, 2019), extremely deep caries (caries penetrating the entire thickness of the dentine) with pulpal exposure was clinically and radiographically noted in 6 teeth. Four teeth had pre-operative cracks diagnosed clinically but not extending to the pulpal floor. Sixteen teeth had extensive and defective coronal restorations (seven amalgam and ten composite restorations). Of these 16 teeth with defective restorations, 10 had extremely deep recurrent caries causing pulpal exposure while in 6 teeth had recurrent deep caries extended >3/4 of the dentine thickness but did not cause pulpal exposure.

Sample collection was carried out as described in Section 2.3.2. Following clinical sample collection, total genomic bacterial DNA was extracted from 62 samples (30 S1, 30 CC, and 2 NC samples) using the GenElute Bacterial Genomic DNA Kit as described in Chapter two. Total bacterial enumeration by quantitative polymerase chain reaction (qPCR), Multiple Displacement Amplification (MDA) and 16S rDNA gene next-generation sequencing (NGS) as well as data analysis were followed as described from Section 2.3.3 to 2.3.7.

3.3. Results

A total of 34 patients attending a dental emergency department with acute symptoms of IP and fulfilling criteria set out in Table 2.1 were initially recruited. Four cases were subsequently excluded due to the absence of bleeding upon pulp chamber access (pulpal necrosis). The remaining 30 teeth with IP comprised 18 molars, eight premolars and four anterior teeth. The average age of patients was 44 years (range from 19 to 56).

3.3.1. Bacterial enumeration by qPCR

Twenty two out of thirty S1 samples provided positive amplification with the universal primer used. All the negative controls (sterile paper points) did not have identifiable qPCR amplification at two cycles below the non-specific amplification of the negative no-template control samples. Such finding does not indicate the complete absence of microbial DNA but it's presence below the background noise cut-off in our study.

From the initial vital intracanal samples taken (S1) n=30, a third had high levels of bacteria, while 20% had moderate bacterial levels. Collectively, 53% of samples had significant levels of bacteria within the vital pulp ($\geq 10^4$ 16S rRNA copies). For contamination control samples, 2/30 samples amplified, leading to rejection of the corresponding clinical samples. For those intracanal samples with a negative contamination control (n=28), 52% had high-moderate bacterial load.

3.3.2. Bioinformatic analysis of the bacterial microbiome in root canal space of IP teeth

Out of the sixteen selected samples for MDA amplification, three did not have a positive band on the agarose gel electrophoresis and were excluded, and four provided no specific amplicon during NGS amplification. Amplicon sequencing of the V3-V5 hypervariable region of the 16S rRNA gene resulted in successful amplification from all remaining samples selected, with

details about samples provided in Figure 3.1. Procedural contamination was ruled out from negative control paper points and contamination control as well as non-template controls. Two samples had very low reads and were not successfully classified, and manual BLASTN searches revealed significant human mitochondrial 16S amplification and were discarded. Overall, >90% of reads passed initial QC for quality and length. The remaining 7 samples had a successful microbial identification with NGS. Across these samples, the number of total valid reads from 16S rRNA sequencing was 260,000 after quality filtering, ranged from 3074 to 78,801 reads (median 31,083 / mean 32,512). The total number of OTUs detected in all IP samples was 451, of which 187 contained more than 2 reads in more than 2 samples.

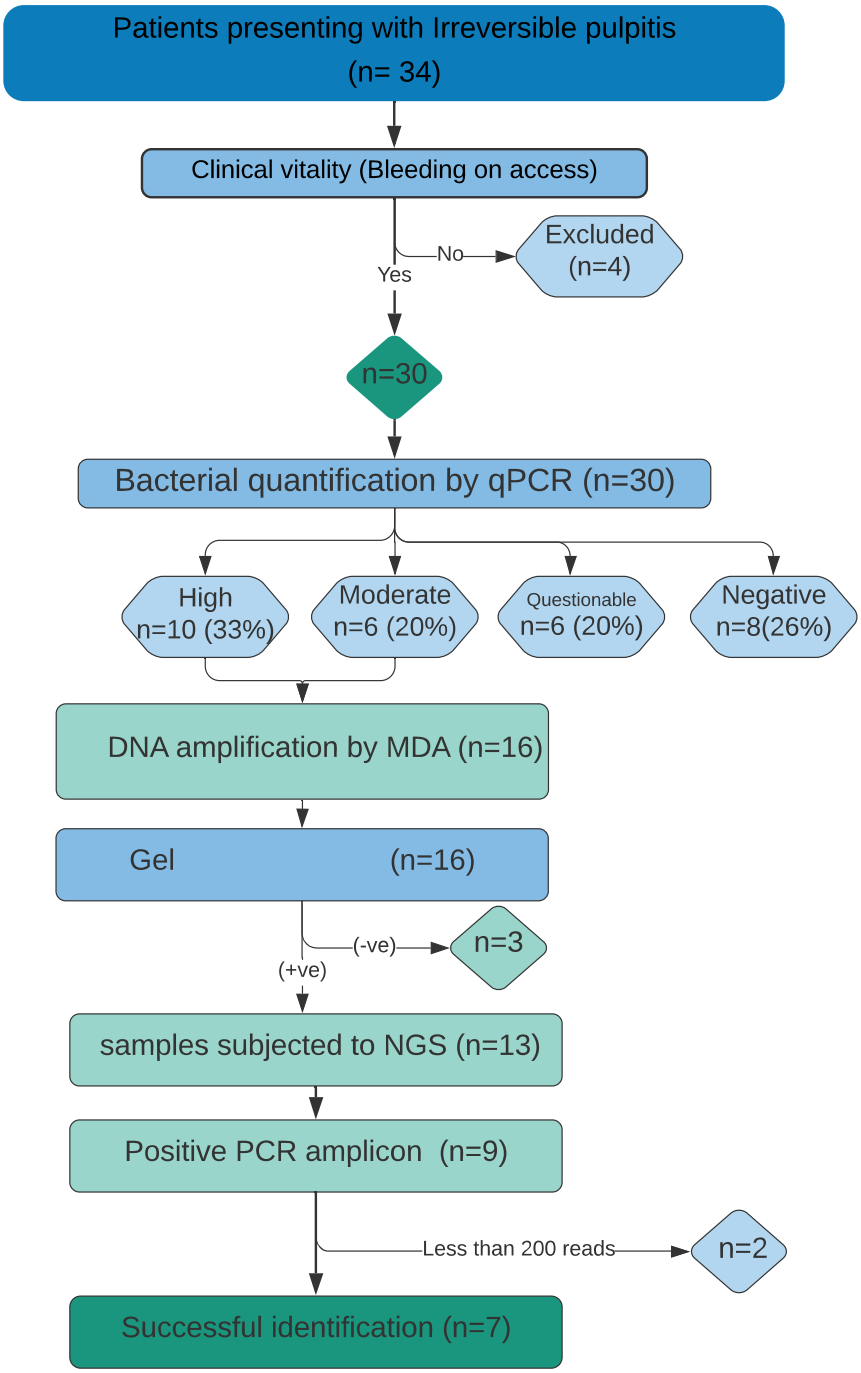


Figure 3.1 Diagrammatic representation of experimental procedures carried out in this study and samples subjected to NGS.

For alpha diversity indices, the minimum number of total valid reads for rarefication was set at 11,400 reads. The mean of Chao1 and ACE nonparametric measures of richness were 132.8 and 72.7, respectively. The mean Shannon index, which takes into account the species richness and evenness, was averaged as 1.8.

Overall, a total of 12 phyla were identified within IP samples (Figure 3.2, 3.4). The predominant taxa by abundance were Firmicutes (constituting 48% of reads and present in 100% of specimens examined at a relative abundance of > 0.01%), Actinobacteria (25% of reads, present in 100% of cases), Fusobacteria (7% reads, 71% of cases), Bacteroidetes (6% of reads, 30% presence), Proteobacteria (5% of reads, 85% presence) and Saccharibacterium (formerly TM7; 7% of reads, 28% presence). At low relative abundance, candidate phylum Synergistetes (3/7 samples), Verrucomicrobia (3/7), Acidobacteria (2/7) and Spirochaetes (2/7) were identified within samples.

At the genus level, a total of 147 genera were identified in vital intracanal samples (Table 3.1 & Figures 3.3, 3.4). The most selectively enriched abundant taxa were *Veillonella* (relative abundance 16%), *Streptococcus* (13%), *Corynebacterium (10%)*, *Cutibacterium* (formerly *Propionibacterium*) (9.3%) and *Porphyromonas* (5.7%).

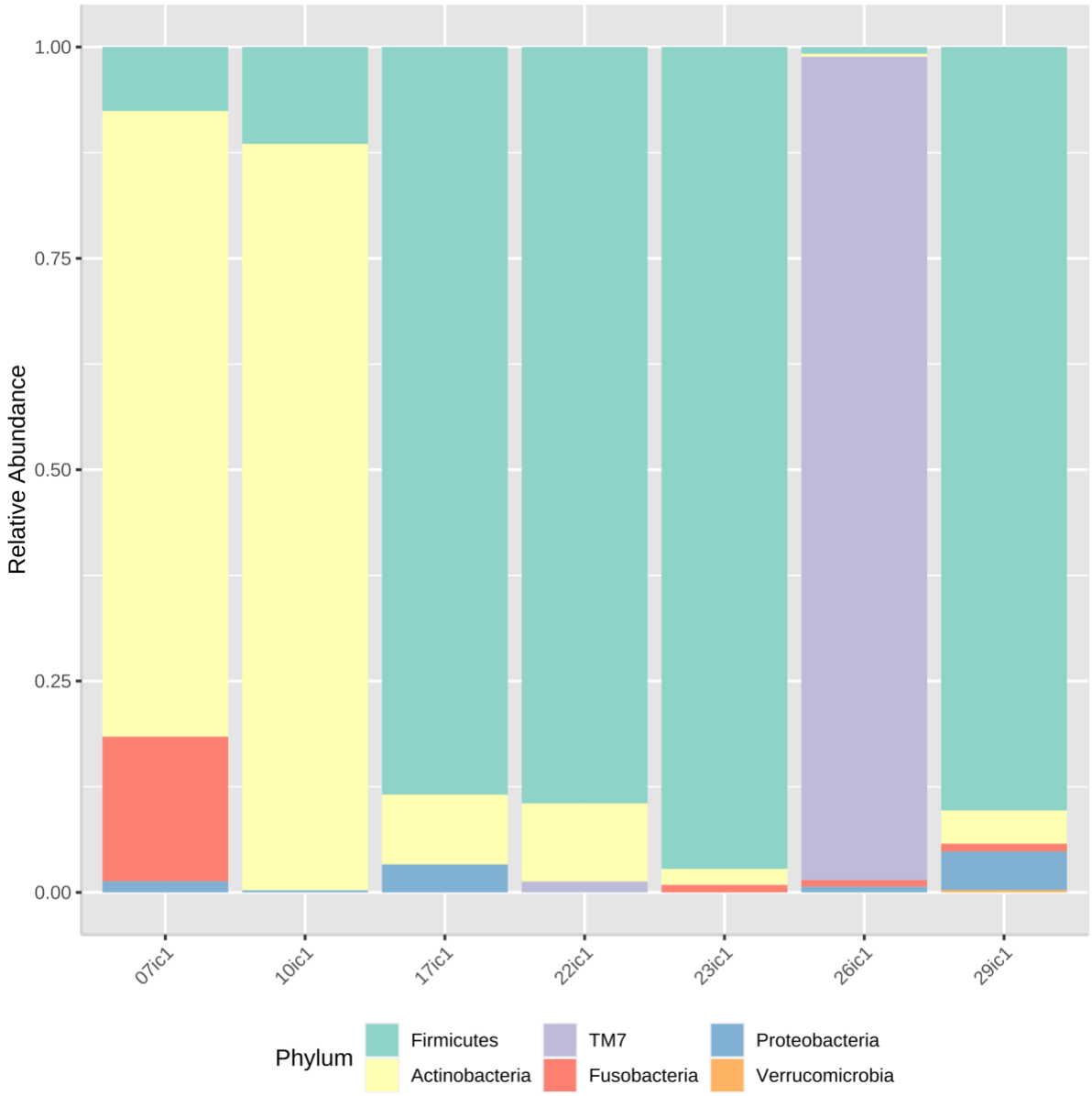


Figure 3.2: Relative abundance of bacterial taxa at phylum-level present in IP endodontic samples

Demonstrating a presence of Firmicutes constituting 48% of reads, Actinobacteria (25%), Fusobacteria (7%), Bacteroidetes (6%), Proteobacteria (5%), and Saccharibacterium (7%).

Table 3.1: Microbial composition of vital intracanal samples at abundances >0.1%

Genus	Relative abundance	Frequency in samples
Veillonella	16%	4/7
Streptococcus	13%	6/7
Corynebacterium	10%	4/7
Cutibacterium*	9.3%	7/7
Porphyromonas	5.7%	1/7
Fusobacterium	4.4%	4/7
Alkalibacterium	4.4%	3/7
Exiguobacterium	2.9%	2/7
Enterococcus	2.8%	4/7
Leptotrichia	2.7%	4/7
Rothia	2%	6/7
Paenibacillus	2%	2/7
Granulicatella	2%	3/7

*Reclassified from *Propionibacterium.*

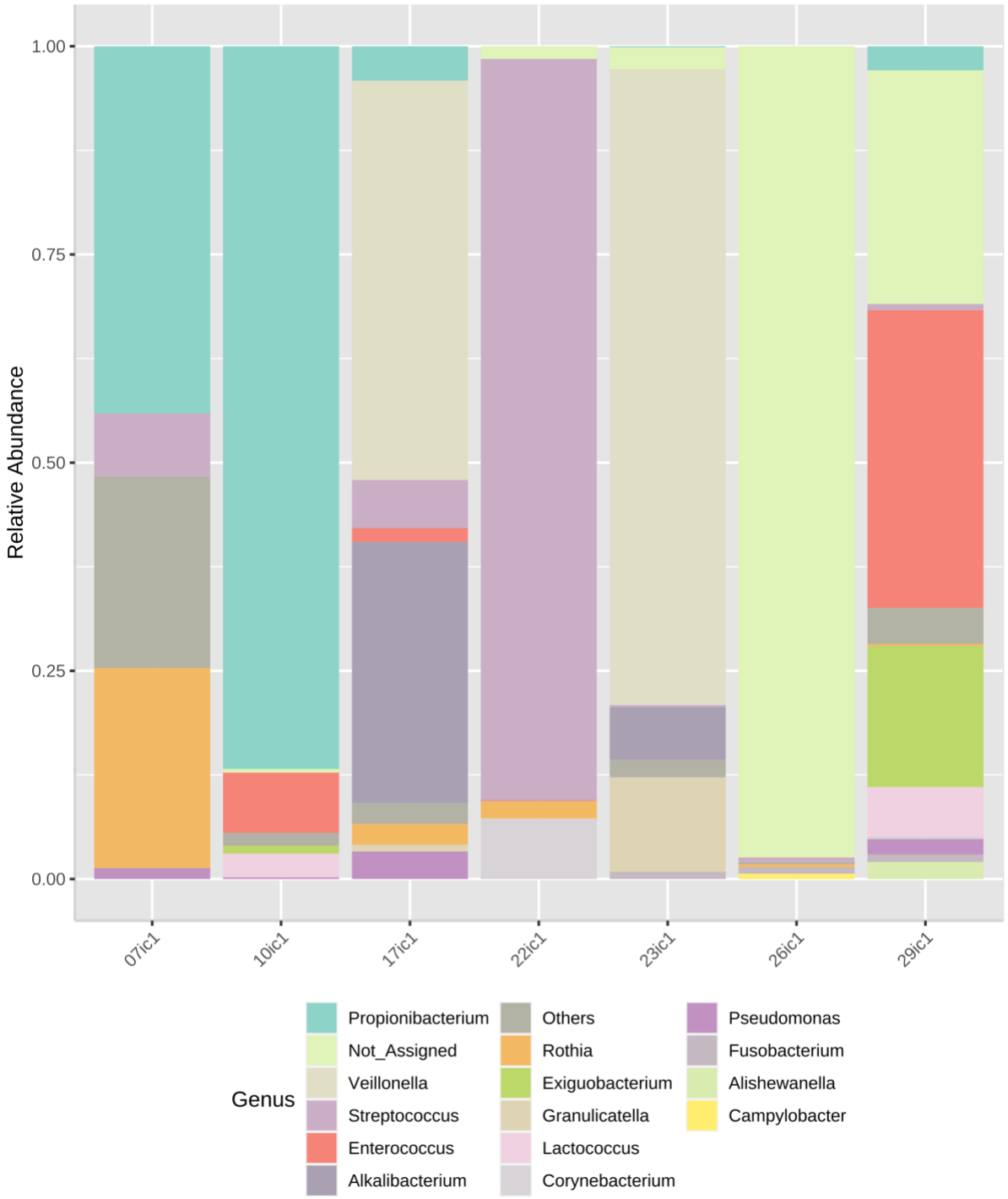


Figure 3.3: The taxonomic composition from all samples showing the most abundance genera across present in IP endodontic samples at >0.1%.
Demonstrating a presence of *Veillonella* constituting 16% of reads, *Streptococcus* (13%), *Corynebacterium* (10%), *Cutibacterium* "formerly *Propionibacterium*" (9.3%), *Porphyromonas* (5.7%), and *Fusobacterium* (4.4%).

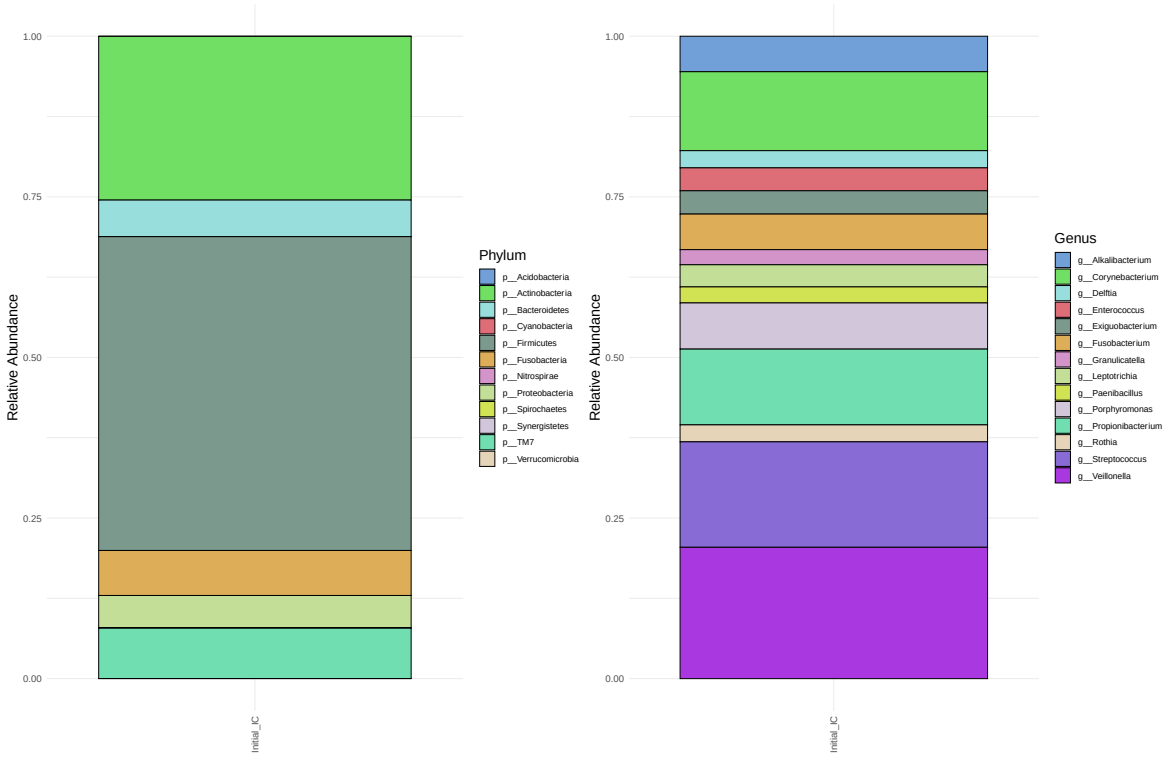


Figure 3.4: Relative abundance of bacterial taxa at phylum and genus level present in IP endodontic samples collectively at >0.1%.
Left: Demonstrating a presence of Firmicutes constituting 48% of reads, Actinobacteria (25%), Fusobacteria (7%), Bacteroidetes (6%), Proteobacteria (5%), and Saccharibacterium (7%).
Right: Grouping of bacterial taxa at genus level present in IP endodontic samples, demonstrating a presence of *Veillonella* constituting 16% of reads, *Streptococcus* (13%), *Corynebacterium* (10%), *Cutibacterium* (9.3%), *Porphyromonas* (5.7%), and *Fusobacterium* (4.4%).

3.4. Discussion

To our knowledge, this is the first study in which NGS has been used to investigate the microbiome of the pulp space of teeth presenting with signs and symptoms of irreversible pulpitis in the absence of apical radiolucency.

The microbiologic status of the sampled teeth ranged from no detection of bacteria to a substantial bacterial load of limited diversity, suggesting a selective ingress of bacteria into vital pulp tissue. Despite the intrinsic variability in bacteria and sampling efficiency, the pulp of almost half of the cases presenting clinically as IP harboured a significant number of bacteria. These ranged in gene copy number from 10^4-10^5 16S rRNA gene copies. This is within the lower range of what has been typically reported in primary infected root canals from 10^3-10^8 cells (Siqueira and Rôças, 2009c).

In support of our finding, previous animal studies demonstrated a lower bacterial penetration rate in vital pulps when compared to the necrotic pulp (Nagaoka *et al.*, 1995). Moreover, histologically, beside localised areas of coagulation or liquefaction necrosis, bacterial colonies were evident in approximately 85% of the coronal pulps of such cases (Ricucci *et al.*, 2014). Furthermore, bacteria were identified within the dentinal tubules of caries affected, vital teeth after extraction (Hoshino *et al.*, 1992). Our findings further confirmed histopathological studies (Ricucci *et al.*, 2014) and added details on bacterial identification of in some cases of IP.

The present study identified approximately 190 bacterial OTUs across all samples, in keeping with previous reports of 190 to 600 OTUs using similar molecular approaches in primary endodontic infections (Siqueira *et al.*, 2011, Özok *et al.*, 2012, Persoon *et al.*, 2017, Bouillaguet *et al.*, 2018). The low OTU number in our study is not surprising, considering that vital as opposed to necrotic pulp was sampled (Siqueira *et al.*, 2011, Özok *et al.*, 2012, Persoon *et al.*,

2017, Bouillaguet *et al.*, 2018) or caries (Rôças *et al.*, 2016, Zheng *et al.*, 2019). Moreover, diversity and richness measures were all shown to be lower than what has been previously reported in primary endodontic infections (mean of 193 and 198 of Chao and ACE respectively) or caries samples (mean of 333 and 361 of Chao and ACE respectively) (Siqueira *et al.*, 2011, Rôças *et al.*, 2016, Bouillaguet *et al.*, 2018), supporting the notion of early colonisers within IP teeth.

Although teeth diagnosed with IP included in this study presented with different clinical presentations, (carious exposure or absence of exposure, recent crown preparation, and cracks), the findings of qPCR amplification and positive NGS reads did not, in our hands, align with any specific clinical presentation. Due to the meticulous removal of potentially confounding samples, 7 samples remained for the successful microbial identification with NGS from 30 initial samples included.

The predominant phyla identified were Firmicutes and Actinobacteria, followed by Fusobacteria and Bacteroidetes. The same abundant phyla were generally reported in previous studies investigating primary infected root canals (Santos *et al.*, 2011, Özok *et al.*, 2012) as well as caries samples of teeth diagnosed with IP (Rôças *et al.*, 2016, Zheng *et al.*, 2019) with a lower abundance in our study. Remarkably, phylum Saccharibacterium (formerly TM7) was commonly encountered and comprised 7% of the total reads. This phylum has been only described through molecular methods in infected root canal spaces with very low abundances <0.5% (Hsiao *et al.*, 2012).

At the genus level, many of the most prevalent genera in the present study are known endodontic pathogens in primary and persistent/secondary endodontic infections as well as

extraradicular infections such as *Veillonella, Streptococcus, Rothia, Cutibacterium, Porphyromonas, Enterococcus* and *Fusobacterium* (Sunde *et al.*, 2000b, Siqueira and Rôças, 2009c). Sequencing studies have also shown that taxa belonging to the genera *Streptococcus* (Persoon *et al.*, 2017), *Veillonella* (Özok *et al.*, 2012), *Cutibacterium* and *Corynebacterium* dominated in primary and persistent/secondary endodontic infections (Anderson *et al.*, 2013, Persoon *et al.*, 2017). This further supported our hypothesis that these organisms may constitute early colonisers in vital cases and contribute to the loss of tooth vitality. On the other hand, some genera commonly found in endodontic infections were present, if at all, at very low abundance in our IP intracanal samples. Examples of those genera not found in our samples are *Prevotella, Bacteroidetes,* and *Parvimonas* (Santos *et al.*, 2011, Siqueira *et al.*, 2011, Özok *et al.*, 2012, Persoon *et al.*, 2017, Bouillaguet *et al.*, 2018).

Culture and molecular based studies showed that caries samples of cases diagnosed with IP typically comprise high reads of *Lactobacilli* (Hoshino, 1985, Hahn *et al.*, 1991, Massey *et al.*, 1993, Rôças *et al.*, 2016, Zheng *et al.*, 2019). An increase of *Lactobacilli* numbers was noted in advanced carious lesions (Hahn *et al.*, 1991). Beside the role *Lactobacilli* play in caries progression, it was suggested that this genus directly causes IP (Zheng *et al.*, 2019). In our study, *Lactobacilli* were not present among the most abundant taxa in IP cases confirming the transition from caries to IP as *Lactobacilli* diminish and enrichment of other taxa was noted. This is keeping with the paucity of this genus described in endodontic lesions (Siqueira and Rôças, 2009c), and supporting the absence of contamination from carious tissue during sampling. On the other hand, some taxa as *Streptococcus, Fusobacterium* and *Veillonella* which are commonly found in deep caries samples (Hahn *et al.*, 1991, Massey *et al.*, 1993, Rôças *et al.*, 2016, Zheng *et al.*, 2019), were present within the pulpal space of IP cases. *Streptococcus* and *Fusobacterium* were correlated to thermal sensitivity when recovered from

deep carious lesions (Hahn *et al.*, 1993). Changes in environmental conditions and pulp innate and adaptive immune response are among the factors to be influencing the suggested shift in microbiota from caries to pulpitis, necrosis and ultimately to established endodontic infection.

Porphyromonas and *Fusobacterium* are Gram-negative anaerobic bacteria that are associated with endodontic infections (Sundqvist *et al.*, 1989), as well as being reported in periapical abscess and extra radicular infections (Sunde *et al.*, 2000b). These two genera were associated with symptoms in persistent/secondary endodontic infections (Anderson *et al.*, 2013). In sequencing studies, these taxa were not among the most abundant genera in carious samples of IP cases (Rôças *et al.*, 2016, Zheng *et al.*, 2019). In the current study, *Porphyromonas* and *Fusobacterium* were detected in 5.7% and 4.4% of the reads, respectively. A transitional enrichment of these two genera is proposed when shifting from caries to IP and necrosis.

Cutibacterium was the only genera present in all samples, accounting for 9.3% of the total reads. It was reported to be more predominant in carious samples of reversible pulpitis and normal pulp when compared to IP (Zheng *et al.*, 2019). The reduced abundance of *Cutibacterium* in carious samples of IP (Zheng *et al.*, 2019), and its association with endodontic and extra radicular infections (Siqueira and Rôças, 2009c) again suggests it to be among the taxa gradually enriched when transitioning from caries to IP and endodontic infection.

The prototypical endodontic pathogenic genus *Enterococcus* is not commonly observed within carious lesions (Rôças *et al.*, 2016), but certainly is associated with endodontic infections (Siqueira and Rôças, 2009c). Being detected in IP indicates its pathogenic role in endodontic infections starting at an early stage where pulpal devitalization not reached yet.

While the present study offers insights into early microbial presence within IP teeth, several significant limitations need to be considered. First, the limited suitable case number arose from the relatively short duration of the pulpitis response before progression to pulpal necrosis, thereby affecting the power of the present study. Moreover, although the response to thermal stimulus and the patient's symptoms are acceptable indicators of the pulpal inflammatory condition, this method is beset with limitations regarding its sensitivity and specificity (Levin *et al.*, 2009). A possibility of having teeth with partial necrosis cannot be disregarded. Moreover, the current clinical diagnostic methods are not error free to precisely identify the inflammatory status of the pulp, especially when it comes to differentiating between reversible and irreversible pulpitis (Mejàre *et al.*, 2012, Ricucci *et al.*, 2014). This unreliability might also affect the diagnosis of our selected cases. Another limitation is the inability to assess the viability of bacteria found, such as through culture-based methods or the use of propidium monoazide (Nocker *et al.*, 2006). Furthermore, the use of MDA demonstrated adequate first-round amplification before next-generation sequencing but introduced concerns regarding contamination. Given these multiple amplifications, stringent contamination controls were essential, and a low threshold of sample rejection was applied to reduce potential contamination bias. This was supported by all sequences being assessed against the HOMD database (Escapa *et al.*, 2018) to ascertain the oral origin (as opposed to procedural contamination). Previous studies reported difficulties in obtaining DNA-free tooth surfaces despite meticulous field decontamination and control samples through the processes (Figdor and Brundin, 2016). Given that, the possibility of partial environmental or reagent contamination could not be categorically excluded (de Goffau *et al.*, 2018), as there is an overlap between some endodontic taxa and common environmental contaminants such as *Enterococcus*. However, comparison between known endodontic bacteria and our findings supported the endodontic, rather than environmental, origin of these bacteria.

Newer bioactive treatments have improved outcomes in deep carious lesions with reversible pulpitis (Ali *et al.*, 2018). However, even if well-sealing restorations are placed, teeth diagnosed with irreversible pulpitis are occasionally recalcitrant to interventions aimed at maintaining vitality (e.g. pulpotomy), and only temporary symptomatic relief is attained (McDougal *et al.*, 2004). The present study offers initial insights into the disease process and the substantial bacterial load detected in many cases may help understanding the causes of vital pulp treatment failures.

Chapter Four: The Impact of Implementing an Enhanced Infection Control Protocol on Root Canal Treatment Outcomes of Molars: A Randomised Clinical Trial

4.1. Introduction

Inadvertent introduction of bacteria into the root canal system may occur when the aseptic chain is breached during treatment. Thus the risk of secondary infection might arise when root canal space is being exposed and manipulated by the clinician (Hargreaves *et al.*, 2016, Rotstein and Ingle, 2019), as detailed in Section 1.8.

Sources of contamination include leakage from rubber dam, non-sterile materials, contamination from operating surfaces, caries or saliva (Bergenholtz *et al.*, 2013, Hargreaves *et al.*, 2016). Therefore, measures and efforts should be taken towards the prevention of such microbial access and establishing an aseptic environment (Sathorn *et al.*, 2007, Bergenholtz *et al.*, 2013, Ørstavik, 2020). These measures include the use of rubber dam, scaling and polishing of tooth surfaces, caries removal of target tooth, chemical disinfection of the operative field, and the use of sterile instruments (Ørstavik, 2020). It was advised to remove all instruments used for rubber dam application and access preparation after access and to use a new sterile tray for root canal instrumentation (Tronstad, 2003, Ørstavik, 2020). Clinical evidence supporting the effectiveness of these measures or other sterility protocols on root canal treatment outcomes is lacking.

As described in Chapter 2, findings from our trial study showed that asepsis of the operative field was not maintained during standard root canal treatment, despite stringent adherence to best-practice standards. Different sources of contamination were determined including rubber dam, gloves, instruments, and files. Eighteen out of 30 patients were at risk of potential contamination by having at least one of the surfaces sampled with significant bacterial load. Moreover, previous studies demonstrated significant clinical and preclinical contamination, from gloves, rubber dam, or dental materials (Williams *et al.*, 2003, Niazi *et al.*, 2016, Saeed *et al.*, 2017), however, the clinical significance of these remained to be shown.

Although most clinical outcome studies mentioned following asepsis protocol during treatment (Friedman *et al.*, 2003, de Chevigny *et al.*, 2008), or following the ESE guidelines for asepsis (Ng *et al.*, 2011), description of operative surface disinfection or details about asepsis protocols were not provided. With the exception of outcome studies involving microbiological sampling where Möller's disinfection protocol was followed (Byström *et al.*, 1987, Sjögren *et al.*, 1990, Kvist *et al.*, 2004, Molander *et al.*, 2007). Moreover, a recent survey on infection control procedures undertaken by general dentists and endodontists showed that only a very small proportion of the operators changed instruments (12%) after caries removal and only 17% change their gloves before obturation. Similarly, only half of the operators reported disinfecting the operative surface (Shuen *et al.*, in press).

Cone-beam computed tomography (CBCT) showed an improved accuracy in the detection of periapical radiolucencies when compared to PA radiographs (Patel *et al.*, 2009, Patel *et al.*, 2012b, Leonardi Dutra *et al.*, 2016). Ex-vivo human cadaver and *in vivo* animal studies using histology as the reference standard confirmed that CBCT is more accurate than periapical radiographs at detecting signs of periapical pathosis (Kanagasingam *et al.*, 2017b). A CBCT study looking at the outcome of 354 primary and secondary endodontic treatments found a success rate of 75.5%, 90.6% and 91.1% for molar, premolar and anterior teeth, respectively, thereby suggesting the use of molars for future endodontic outcome studies comparing different instrumentation or infection control protocols (Al-Nuaimi *et al.*, 2018). With this more sensitive method to detect new and residual periapical pathology, failure of the root canal treatment was found to be two times higher than that detected by traditional periapical radiographs (Aminoshariae *et al.*, 2018). Thus, it was recommended to re-evaluate the outcomes of root canal treatments and the prognostic factors using CBCT and long-term

follow-up (Wu *et al.*, 2009, Kanagasingam *et al.*, 2017b). Although an increased use of CBCT in assessing outcome of primary and secondary root canal treatment is notable (Liang *et al.*, 2011, Patel *et al.*, 2012a, Metska *et al.*, 2013, Zhang *et al.*, 2015, Davies *et al.*, 2016, Al-Nuaimi *et al.*, 2017, Al-Nuaimi *et al.*, 2018, Curtis *et al.*, 2018, Zhang *et al.*, 2021), there are still very limited numbers of randomised clinical trials evaluating the effectiveness of different protocols or procedures on treatment outcomes using CBCT.

It is essential to detect variables and factors predicting outcomes of root canal treatment and thus improve best practice. Many studies reported different factors to affect outcomes using periapical radiographs. These factors included pre-operative periapical status, tooth type, obturation length and quality, procedural errors, and quality of coronal restoration (Ng *et al.*, 2008). To date, CBCT outcome studies on primary root canal treatment are limited (Liang *et al.*, 2011, Patel *et al.*, 2012a, Liang *et al.*, 2013, van der Borden *et al.*, 2013, Zhang *et al.*, 2015, Kamburoglu *et al.*, 2017).

4.2. Aims and objectives

Based on the pilot study findings in Chapter 2, as well as previous reports (Williams *et al.*, 2003, Niazi *et al.*, 2016, Saeed *et al.*, 2017), this clinical trial was set to determine the clinical significance of iatrogenic contamination and to minimise intra-operative cross infection. The aim of this randomised clinical trial was to (i) compare the outcome of primary root canal treatments undertaken using a standard protocol (SP) or an enhanced infection control protocol (EP) in molars, and (ii) to develop an evidence-based enhanced infection control protocol for endodontic treatment to improve the best practice.

4.3. Materials and method

4.3.1. Study design and ethical approval

A single-blind, two-armed, randomised controlled clinical trial was conducted, with patients and radiographic examiners blinded to which protocol was used. This trial compared two protocols for root canal treatment: standard (SP) and enhanced infection control protocol (EP). Prior to commencing the study, the study was reviewed and approved by London - Surrey Research Ethics Committee (reference no. 18/LO/1661) (Appendix 4), as well as Health Research Authority (HRA), Health and Care Research Wales (HCRW) approval (Appendix 5). The study was conducted in compliance with the principles of the Declaration of Helsinki and good clinical practice (GCP). A description of the purpose of the study, detailed treatment protocol, follow-up examinations, as well as total radiation dose anticipated when participating, were distributed to all patients as patient information sheets (Appendix 6). Patients were informed clearly about their voluntary participation, and that not taking part in the study or withdrawal at any point would not adversely affect the standard of treatment they were receiving. Patients were also reassured that the treatment outcome would not be adversely affected with the treatment protocol. Moreover, all information collected during the course of the research would be kept strictly confidential and accessed only by authorised people. Informed written consent (Appendix 7) was obtained prior to the implementation of the study. The trial was registered in ClinicalTrials.gov registry (ID# NCT03636087).

4.3.2. Sample size and recruitment

Statistical power calculations were estimated prior to enrolling patients using STATA v15. Sjögren reported a success rate of 94% in cases with absence of culturable bacteria prior to obturation using conventional radiographs (Sjögren *et al.*, 1997). Based on this data, it was estimated that a minimum of 106 independent teeth (53 in each group) would be necessary in a logistic model to reach a power of 80% in detecting favourable outcome rates of 95% and

80% in the two protocols (standard and enhanced) as significantly different, under a confidence interval of 95%. A dropout rate at 25% was expected and, therefore, the initial sample was increased to 132 teeth (or patients).

4.3.3. Recruitment and randomisation

This study was conducted at King's College London, Faculty of Dentistry, Oral & Craniofacial Sciences at Guy's Hospital, London, UK. Patients were recruited from endodontics consultant clinics to which they were referred from general dental practices for primary endodontic treatments. Patients were also enrolled from the Acute Dental Care (ADC) seeking emergency endodontic treatment.

Randomisation was performed by the Biostatistics Unit, King's College London. Randomisation by blocks of four was followed.

4.3.4. Patient selection, inclusion and exclusion criteria

Patients with a non-contributory medical history were included in this study with at least one molar tooth requiring primary root canal treatment. Eligibility criteria required individuals to have restorable, posterior molars with different pulpal and periapical diagnoses and not to have received any endodontic treatment previously. Table 4.1 describes inclusion and exclusion criteria.

Table 4.1: Inclusion and exclusion criteria of patients recruited in clinical trial

Inclusion criteria	Exclusion criteria
1. Age range 18-80 years old. 2. Healthy patients with no significant medical history*. 3. Molar teeth. 4. Diagnosed with symptomatic/ asymptomatic irreversible pulpitis, pulpal necrosis or previously initiated root canal treatment. 5. Radiographic analysis showing mature root apexes with or without periapical lesions. 6. Restorable teeth.	1. Patients younger than 18. 2. Patients unable to give consent. 3. Pregnant women. 4. Patients with compromised medical condition that affects the outcome of root canal therapy. 5. Patients who received antibiotic therapy within the previous three months. 6. Patients with clinical and radiographic diagnosis of previously treated root canal. 7. Evidence of external or internal root resorption. 8. Anterior or premolar teeth. 9. Non-restorable teeth.

* Medical condition that could potentially affect the outcome of root canal therapy and were not included in this study: Diabetes (type I or II), HIV/AIDS, Hepatitis (B or C), cancer and chemotherapy, autoimmune disease, anaemia, patients taking bisphosphonates or immune-suppressive drugs, impaired non-specific immune diseases, and medically compromised patients.

4.3.5. Pre-operative assessment: clinical and radiographical

All included patients were assessed at baseline clinically and radiologically. Routine medical and dental history, pre-operative pain history, assessment of hard and soft tissues were taken as part of the clinical examination.

Assessment of target teeth included pulp sensibility tests with thermal test (Roeko Endo-Frost, Coltène/Whaledent, Germany) and electronic pulp test (Kerr Vitality Scanner 2006; SybronEndo, Orange, CA, USA) using a healthy contralateral tooth as a standard control. Moreover, palpation of surrounding soft tissues and percussion test results were collected together with signs of pulpal/periapical diseases (pain, abscess, sinus tract, deep pockets and abnormal mobility). All clinical data were anonymised and then recorded in electronic datasheets (Microsoft Excel 16.38, Microsoft.).

Periapical radiographs (PA) using paralleling technique were obtained using a digital imaging system Digora Optime (Soredex, Tuusula, Finland) with a beam aiming device (Dentsply Rinn, Elgin, IL, USA) to allow for standardisation of radiographs using a dental x-ray machine (Heliodent, Sirona, Bensheim, Germany). Exposure parameters were set at 65 kV, 7 mA and an exposure time of 0.25-0.32 seconds, with scanning resolution of 400 dpi. Processing of the raw data images was done with Digora default software.

CBCT scans were acquired using a 3D Accuitomo CBCT scanner (J. Morita MFG. CORP, Kyoto, Japan), with a 4 x 4 cm field of view (FOV) and 0.125 mm of voxel size. The exposure parameters were standardised at 90 kVp, 4 mA, the exposure time was set at 17.5 seconds. Following manufacturer's instructions, the degree of beam angulation was set to position the

target tooth in the centre of the FOV. CBCT scans were reformatted (0.125 slice intervals and 1.5mm slice thickness) using the system's proprietary software (i-Dixel.images, J. Morita).

4.3.6. Clinical intervention / root canal treatments procedures

Operative procedures were undertaken by four operators: endodontic specialists and endodontic residents in their final year. The operators were trained and instructed to follow a standard treatment protocol provided to them, which is consistent with the European Society of Endodontology (ESE) guidelines (European Society of Endodontology, 2006). A detailed step by-step form was given to operators to follow in case of tooth allocated to enhanced infection control protocol (EP) group.

Regardless of the treatment protocol, all treatment was performed under local anaesthesia and in a single visit. When the endodontic treatment had to be completed in two visits, teeth were excluded from the study. A dental operating microscope (3 step entree; Global, St Louis, MO, USA) was used throughout the treatment. Before rubber dam application, supragingival biofilms were removed from each tooth by scaling and cleansing with pumice using a slow hand piece. Single tooth isolation was undertaken using rubber dam and gingival barrier; OpalDam Green™ (Ultradent, South Jordon, UT, USA). The surface of the rubber dam and the tooth were disinfected by swabbing for 60 seconds with 2.5% sodium hypochlorite. Where appropriate, the tooth was built up with a direct plastic composite restoration (SDR flow +, Dentsply Sirona, Baillagues, Switzerland) to facilitate rubber dam isolation.

After caries removal and pulp chamber access with sterile bur and saline irrigation, the operative field including pulp chamber was then decontaminated again with 2.5% NaOCl and the NaOCl was then inactivated with 5% sodium thiosulphate. Initial scouting of canals was

achieved with #10 or #15 K-flexofile (Dentsply Sirona). The working length was determined using an electronic apex locator (Root ZX, J. Morita Corp., Kyoto, Japan) and confirmed with a digital periapical radiograph. The working length was always 1mm short of the '0' apex locator reading length, canals were then prepared to at least size 15 Flexofile to the working length, after which canal preparation was accomplished in a crown-down approach using ProTaper® Gold Universal rotary instruments (Dentsply Sirona) at 300 RPM and a torque of 4N to at least a F2 master apical rotary file. Canals were frequently irrigated with 2.5% NaOCl (Adams Healthcare, Leeds, UK) delivered using a side-vented 27-gauge needle inserted 1 mm short of the apex throughout instrumentation.

After completing the chemomechanical preparation and after taking the master point radiographs, additional steps were taken for teeth assigned to the enhanced infection control protocol (EP) group. These steps included replacing the rubber dam, gloves, surface barriers, and all instruments with a new, sterile set (Table 4.2). In the standard group (SP), only the gloves were changed after the master point radiograph was taken and none of the instruments were replaced and treatment was followed conventionally.

In both treatment groups, canals were irrigated with 2.5% NaOCl and activation with Endoactivator system (Dentsply Sirona) using medium activator tips (25/04) measured 1mm short of the apex for 30 seconds. Sterile saline solution was used to flush the NaOCl, the canals were then irrigated with 17% EDTA (Pulpdent, Watertown, Massachusetts, USA). After a final rinse with saline, the root canals were dried with sterile paper points (Dentsply Sirona). In both treatment groups the tried master GP points were disinfected for three minutes in 2.5% NaOCl before obturation. The canals were obturated with gutta-percha (Dentsply Sirona) and AH Plus resin-based root canal sealer (Dentsply Sirona) using a warm compaction technique with

System B endodontic heat source unit (EIE-Analytic Technology, Orange, CA, USA) and B&L Biotech Beta Obturation Gun (B&L BioTech, VA, USA).

Access cavities were restored with flowable hybrid composite; CORECEM^{TM} (RTD, Saint-Egrève, France). A final radiograph was taken after placement of the restoration. Teeth were then referred to the patient's general dentist for cuspal coverage. A summary of all steps undertaken in both treatment groups is shown in Table 4.2.

In the event of pre-existing perforations (three cases), mineral trioxide aggregate (MTA) (ProRoot MTA, Dentsply Sirona) was used to seal the perforation area.

All the pre-operative and intra-operative information such as patency of canals, working lengths achieved, pre-operative cracks presence and the occurrence of procedural complications such as perforation were recorded.

Table 4.2: A summary of the treatment protocols used in the enhanced infection control group (EP), and (SP) of root canal-treated teeth.

Steps followed in both groups

- All treatment carried out in single visit.
- Single tooth isolation using a rubber dam and light-cured gingival barrier OpalDam.
- The surface of the rubber dam and the tooth were disinfected by swabbing for 60 seconds with 2.5% NaOCl.
- Access cavity preparation, canal instrumentation using ProTaper® Gold.
- Canals disinfected with 2.5% NaOCl, delivered using side-vented 27-gauge needles.
- Completion of chemomechanical preparation and master point radiographs taken.

Steps followed in EP group	**Steps followed in SP group**
• The access cavity was filled with Cavit™ material (3M ESPE, St Paul, MN). • All existing instruments were replaced with new sterile sets. This included dental mirror, tweezers, DG-16 endodontic explorer, heat plugger, flat plastic instrument, and endodontic rulers that were provided in a new sterile tray. • The existing rubber dam was replaced with a new rubber dam placed with new sterile clamps and gingival barrier. • Gloves were replaced. • Surface barriers were replaced, including those of the microscope and light surface. • The temporary filling was removed using sterile diamond burs. • Decontamination of the operative field with 2.5% NaOCl for one minute was performed.	• The treatment was not discontinued; chemomechanical preparation was followed immediately with final irrigation. • Instruments were not replaced, and same sets were used throughout the procedure. • Existing rubber dam was not changed at time of obturation. • The gloves were changed after the master point radiograph was taken. • Surface barriers were not replaced.

Steps followed in both groups

- Final irrigation with 2.5% NaOCl and activation with the Endoactivator and 17% EDTA.
- Sterile saline solution was used to flush the NaOCl.
- The root canals were dried with sterile paper points.
- The master GP points were disinfected for three minutes in 2.5% NaOCl before obturation.
- The canals were obturated with GP and resin-based root canal sealant using a warm compaction technique.
- Access cavities were restored with flowable hybrid composite.

4.3.7. Patients recall

After treatment completion, follow-up appointments were scheduled to be 12 months (+/-4 weeks) to assess the outcomes clinically and radiographically. All patients were contacted by phone. A letter was mailed to those patients who did not respond after six additional attempts by phone. The letter explained the reason for the follow-up.

4.3.8. Follow-up: clinical and radiographic examination

All patients were examined at the review appointment by the same investigator for assessment of periapical healing. In the follow-up appointment, changes to the medical and dental history including pain assessments were recorded. For each tooth, the following clinical criteria were evaluated: pain, swelling, tenderness to apical and gingival palpation and percussion, periodontal probing, mobility, and the type and quality of coronal restorations. All post-operative information was recorded.

Radiographic assessment was carried out using PA radiographs and CBCT scans with the same settings used for the pre-operative images. Sagittal, coronal, and axial CBCT slices from each root were selected based on the apical area showing the presence of the lesion and/or the area showing the largest periapical radiolucency using Accuitomo software (One Volume Viewer, J. Morita). Contrast and brightness were adjusted to improve the visualisation. Identical slices were chosen for the follow-up scans. All scan adjustments were made by the same endodontist who did not participate in the radiographic evaluation. Images were then exported to TIFF format and collected in a PowerPoint presentation.

Two presentations were prepared, for PA and CBCT scans. Slides were randomly ordered and patients' identifying information was removed (Figure 4.1 and 4.2). The CBCT raw data were also made available to examiners on the Accuitomo viewer.

The scoring system used is shown in Table 4.3. A periodontal ligament space greater than 2mm was diagnosed as a periapical radiolucency (Low *et al.*, 2008, Bornstein *et al.*, 2011). The radiographic outcome (Table 4.3) was scored using a six-point classification (Patel *et al.*, 2012a). Each case was scored after both examiners of the consensus panel were in agreement.

Assessing molar teeth allowed direct evaluation of like pairs of specific roots (Patel *et al.*, 2012a). The overall outcome of the tooth was considered as the unit of assessment based on the root that had the worst treatment outcome.

The radiographic images were assessed by two experienced endodontists familiar with interpretation of CBCT imaging and were blinded to the treatment protocols used. The examiners were calibrated in a preliminary session which involved assessing 50 pairs of matched PA and CBCT images which were not part of the clinical study. The pre-operative and the one-year follow-up PA (session 1) and CBCT (session 2) of each case were viewed together so the development, complete resolution of PARL, or the change (increase/decrease) in size of an existing periapical radiolucency could be assessed. The images within each session were randomly ordered, and to minimise the likelihood of examiner's fatigue, viewing sessions were divided into at least two separate periods within the day.

The intra-examiner reliability was determined in another session by re-evaluating 98 pairs of randomly selected periapical radiographs and CBCT images. There was at least a two-week interval between sessions.

Based on the post-operative clinical signs/symptoms, alongside periapical and CBCT outcome evaluation, cases were classified into *favourable* and *unfavourable* outcomes. The treatment was considered *unfavourable* when clinical signs and symptoms were present (pain to percussion/palpation, swelling or discomfort), and if there was an unchanged/increased lesion size relative to the pre-existing periapical radiolucency, or if a de novo lesion developed (outcome classes 1, 2, 3). Treatment was considered *favourable* when teeth were asymptomatic (no pain, swelling or discomfort), and there was a reduction in size, or absence of periapical radiolucency (outcome classes 4, 5, 6) (Table 4.3).

The radiographic images were also assessed for apical extension and quality of root canal obturations, missed canals, intra-operative procedural errors, and the quality of the marginal fit of the coronal restoration. Root canal obturation was considered *adequate* if there was a good adaptation to the root canal walls and absence of voids. Apical extent of root canal filling was recorded as *adequate* (0-2mm short of radiographic apex), *short* (2mm short of radiographic apex), and *long* (2mm beyond radiographic apex). Missed canals were detected from axial CBCT reconstructions as shown in Table 4.4.

The quality of the coronal final restoration was recorded as adequate or inadequate based on clinical and radiographic evaluation. A restoration was considered adequate when there was a smooth transition of the probe across the restoration margin, absence of marginal discrepancy, and no clinical or radiographic signs of caries (Table 4.4).

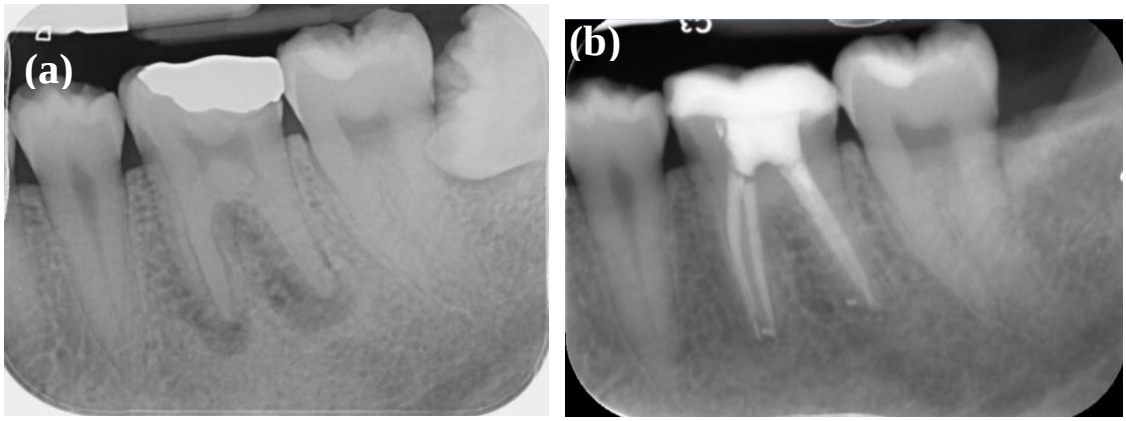


Figure 4.1: An example of periapical radiographs evaluation slides.

Viewing two images; for baseline (a) and one-year follow-up (b) showing complete resolution of the periapical radiolucency on both roots.

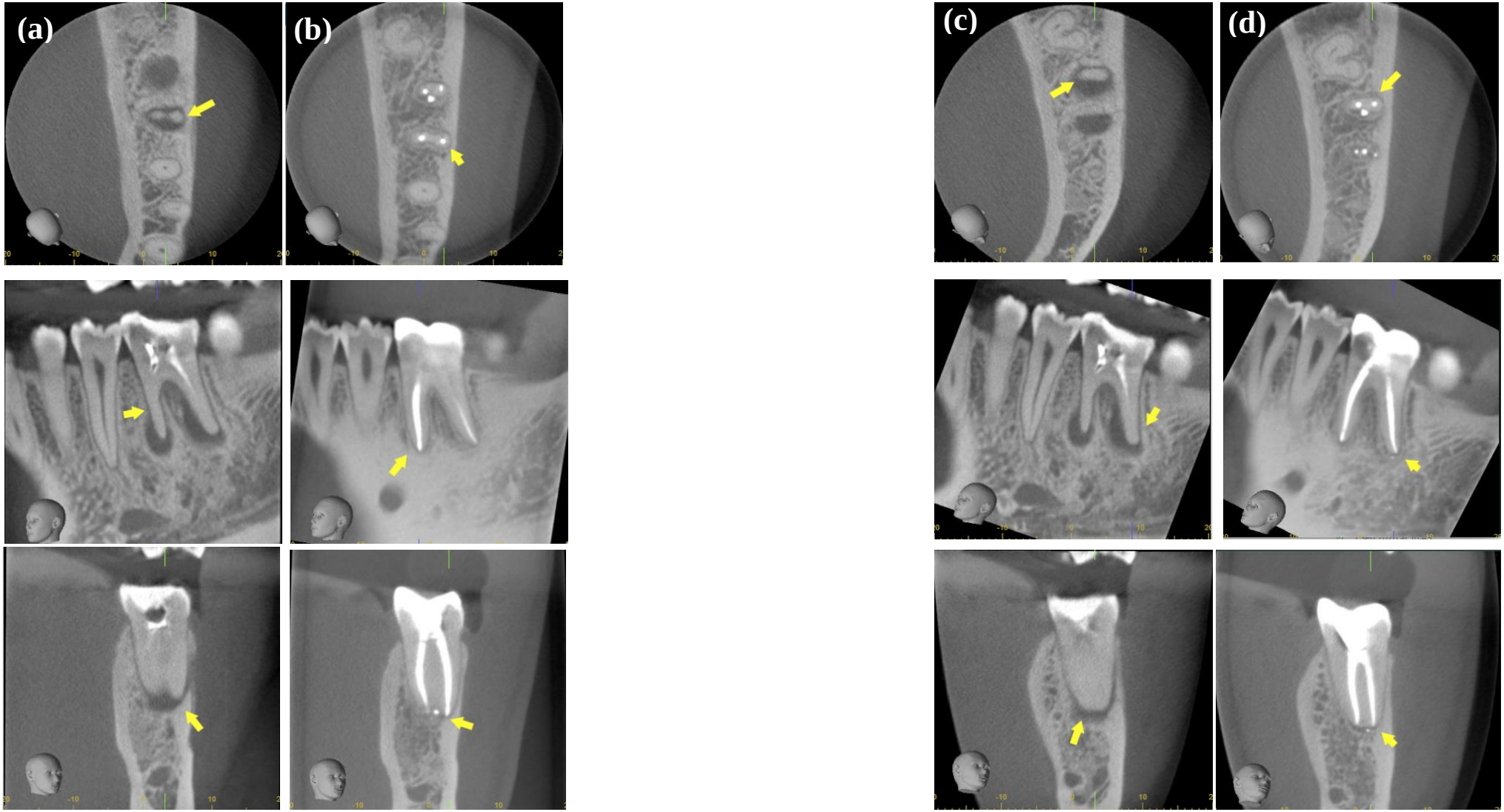


Figure 4.2: An example of CBCT scan evaluation slides.

Images of different spatial planes at baseline (a and c) and one-year follow-up (b and d) of lower left first molar. Mesial root (a and b) and distal root (c and d). Yellow arrows indicate the root to be evaluated. The images show a complete resolution of the periapical radiolucency (score 5) on both roots.

Table 4.3: The outcome categories for root canal retreatment (Patel *et al.*, 2012a)

Score	Description	Outcome
1	New periapical radiolucency	Unfavourable
2	Enlarged periapical radiolucency	Unfavourable
3	Unchanged periapical radiolucency	Unfavourable
4	Reduced periapical radiolucency	Favourable
5	Resolved periapical radiolucency	Favourable
6	Unchanged healthy periapical status	Favourable

Table 4.4: The criteria used to evaluate quality and length of root canal filling as well as quality of coronal restoration

Adequate	Inadequate
Quality of root canal fillings	
• Adequate density. • Proper adaptation to the lateral canal walls. • Absence of voids (homogeneity).	• Voids were present within the root canal filling.
Apical extent of root canal filling	
• 0-2mm short of radiographic apex.	• **Short** 2mm short of radiographic apex. • **Long** 2mm beyond radiographic apex.
Quality of the coronal restoration	
• Smooth transition of exploration probe across restoration margin. • Absence of marginal discrepancy. • No clinical or radiographic signs of caries.	• Loss of marginal adaptation. • Open margins. • Recurrent caries.

4.3.9. Statistical analysis

All the analyses were carried out using IBM SPSS software (SPSS version 23, IBM). The significance level was set at 5% (α=0.05).

Study participants' characteristics and outcome findings were summarised using descriptive statistics (absolute frequencies and percentages) and continuous, like age (mean, standard deviation, range and median). Chi^2, Fisher´s exact (for nominal data) and Mann-Whitney tests (for quantitative data) were used to study the homogeneity of both protocol groups through demographic and clinical variables.

The association between outcome and other variables, such as treatment protocols, diagnosis, gender, age, pre-operative periapical status, type of tooth, quality and length of obturation, and quality of coronal restoration, were assessed using binary logistic regression models. Non-adjusted odds ratio (OR) and 95% confidence intervals (CI) were obtained. This was followed by a multivariate binary logistic regression model selecting the significant variables only ($p<0.1$) obtaining adjusted OR.

Intra-consensus panel agreement and inter-examiner agreement were estimated using linear weighted Kappa´s index for the outcome scoring of PA and CBCT. Regarding assessments of obturation length, quality and restoration quality, conventional unweighted Kappa´s index was computed. In both cases, 95% confidence intervals were estimated.

4.4. Results

4.4.1. Demographic characteristics of teeth in both groups at T0:

From November 2018 to October 2019, a total of 173 patients were initially approached. 154 teeth (148 patients) met the inclusion criteria and agreed to participate. The patients were then randomised into 77 teeth in the enhanced infection control protocol (EP), and 77 teeth in the standard protocol (SP) group. During root canal treatment, 10 teeth (6%) were excluded. Reasons for exclusions are provided in Table 4.5. After exclusions, 144 teeth remained (73 in SP and 71 in EP).

The average age of patients was 36.6 years and the sample included 64 males and 80 females. A total of 144 molars received primary root canal treatment as a part of this study, comprising 96 first molars, 47 second molars and one third molar. Teeth were almost equally distributed within maxillary and mandibular arches (42% in the upper arch and 58% in the lower).

Of the 144 teeth, 85 (59%) were initially accessed in the ADC unit and were diagnosed as previously initiated (PI). The remaining 59 teeth (19.4%) were diagnosed as pulpal necrosis (PN), and 21.5% teeth were symptomatic/asymptomatic irreversible pulpitis (SIP/AIP). The percentage of teeth with pre-operative periapical radiolucency was 60% (86 teeth) when assessed by CBCT.

Baseline characteristics were balanced across the treatment groups. Pre-operative PARL was evident in 56% and 63% of teeth in EP and SP respectively. Almost 60% of teeth in each group were accessed at ADC. All other clinical factors were comparable in the two treatment protocol groups as shown in Table 4.6.

Table 4.5: Reasons for exclusion before or during root canal treatment

Reasons for exclusion	Number of teeth
Not meeting inclusion criteria	9
Declined participation	2
Pre-operative CBT scan was not taken	8
VRF diagnosed	7
Tooth found to be non-restorable	1
Treatment completed in two visits	2
Total	**29**

Table 4.6: Characteristics of the patients at baseline (T0) in total and in standard (SP) and enhanced infection control (EP).
Number of teeth (%) or mean ± standard deviation. Results of Chi^2, Fisher´s exact test or Mann-Whitney test.

	Treatment protocol		Total in both groups	*p-value*
	SP (n=73)	**EP (n=71)**	**(n=144)**	
Age (years)				
Mean	35.5	37.8	36.6	0.25
Range	18-80	20-70	18-80	
Gender				
Male	32 (43.8)	32 (45)	64 (44.4)	1.0
Female	41 (56.2)	39 (55)	80 (55.6)	
Arch				
Upper	32 (43.8)	28 (39.4)	60 (41.7)	0.6
Lower	41 (56.2)	43 (60.6)	84 (58.3)	
Pulpal diagnosis				
Irreversible pulpitis	17 (23.3)	14 (19.2)	31 (21.5)	0.8
Pulpal necrosis	15 (20.5)	13 (18.3)	28 (19.4)	
Previously initiated	41 (56.2)	44 (62)	85 (59)	
Tooth type				
First Molar	46 (63%)	50 (70.4)	96 (66.5)	0.29
Second molar	27 (36%)	20 (28.2)	47 (32.6)	
Third Molar	0	1 (1.4)	1 (0.7)	
Pre-operative PARL (CBCT)				
Present	46 (63)	40 (56.3)	86 (59.7)	0.49
Absent	27 (37)	31 (43.3)	58 (40.3)	

4.4.2. Clinical and radiographic outcomes in total at T12:

Patients' recall was planned to be conducted from January to October 2020. Due to COVID-19 lockdown, all patient recalls were completed in the period from August-October 2020. All follow-up appointments were completed 12 months to 18 months post-endodontic treatment.

At the T12 review, 115 teeth (110 patients, 42.6% male and 57.4% female) of the original 144 teeth were returned for clinical and radiographic evaluation. The overall recall rate was 80% for teeth, and patients (86% in the EP group and 73% in the SP group).

From the outcome analysis, 29 patients did not attend and were excluded. The most frequent reason for discontinuation and not attending the follow-up review was safety concerns due to the COVID-19 pandemic. All reasons for not including these 29 patients were summarised in Table 4.7. A modified PRIRATE flow diagram of the patient's recruitments, exclusions and follow-ups is illustrated in Figure 4.3. At follow-up, both treatment groups were extremely homogeneous and different factors were equally distributed as shown in Table 4.8.

The clinical examination revealed that 90% (104/115) teeth were asymptomatic at T12 recall appointment, while 11 teeth (10%) were tender to percussion. All teeth presenting with clinical sign and symptoms were associated with unfavourable radiographic outcomes on CBCT. On the other hand, only 11/27 teeth classified as unfavourable outcome on CBCT had clinical symptoms at follow-up.

Regardless of the treatment group, the overall percentage of favourable outcomes (outcome 4, 5, 6) was 92% using periapical radiographs, and 76.5% using CBCT (Table 4.9 and Figure 4.4). A significant difference in the favourable and unfavourable outcomes of teeth was noted when assessed by PA and CBCT (Fisher exact test, $p<0.001$).

Table 4.7: Reasons for patients' non-attendance at the review appointments

Reason for not attending follow-up appointment:	Number of patients
Safety concerns due to COVID-19	16
Loss of contact with patient	2
Pregnancy at follow-up	3
Change of residence / relocation to another country	4
Declined invitation to return	4
Total	**29**

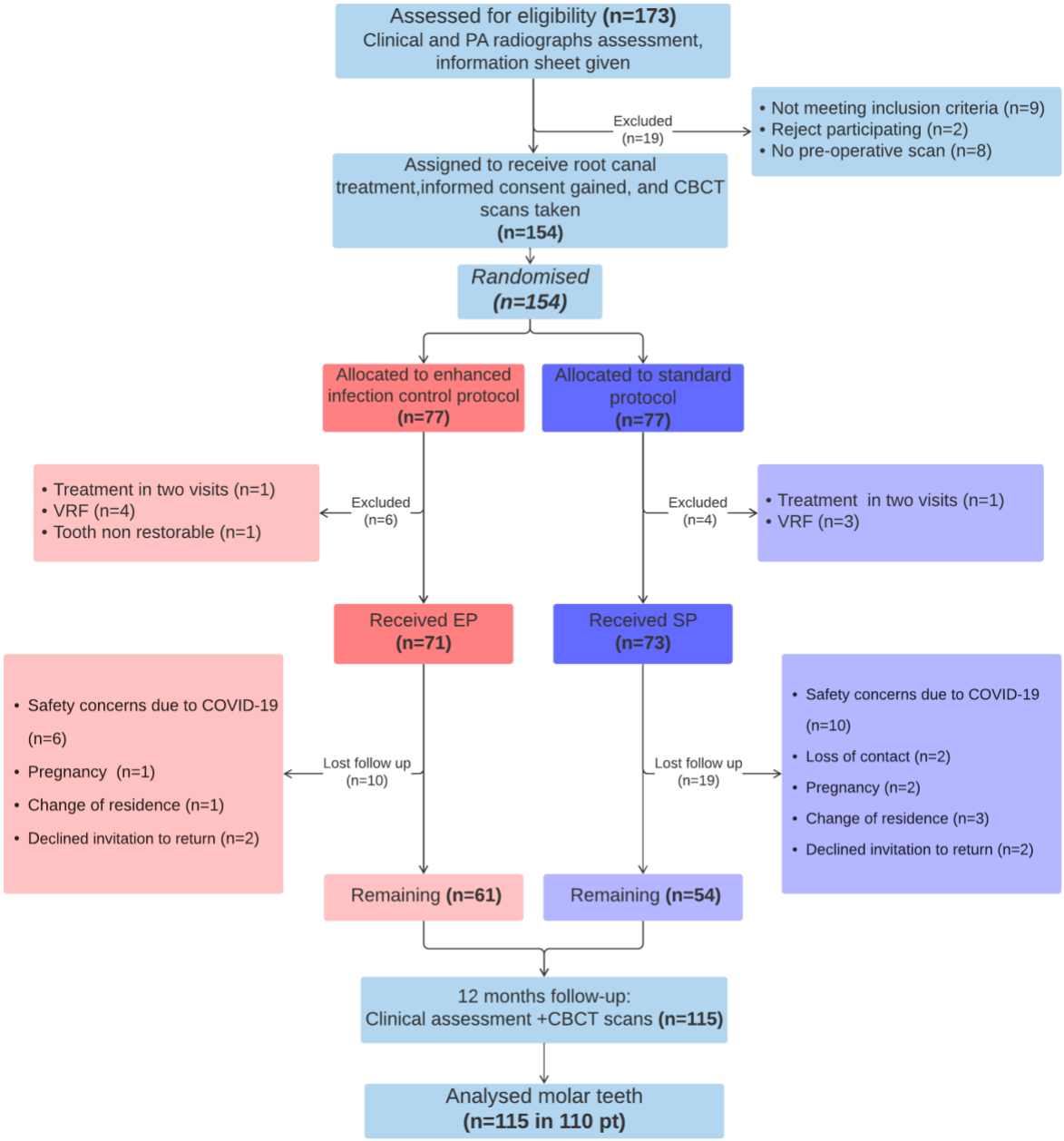


Figure 4.3: Flowchart of the trial showing the process of patient recruitment, exclusion and follow-up. Chart modified from PRIRATE 2020.

Table 4.8: Demographic and clinical characteristics by protocol group at one-year follow-up (T12).
Number of teeth (%) or mean ± standard deviation. Results of Chi^2, Fisher´s exact test or Mann-Whitney test.

Prognostic factor	Treatment protocol		*p-value*
	SP (n=54)	EP (n=61)	
Age (years)			
	37.05 ± 12.9	38.0 ± 12.3	0.601
Gender			
Male	21 (38.9)	28 (45.9)	0.458
Female	33 (61.1)	33 (54.1)	
Tooth type			
1st molar	31 (57.4)	42 (68.9)	0.174
2nd molar	23 (42.6)	18 (29.5)	
3rd molar	0 (0.0)	1 (1.6)	--
Arch			
Upper	23 (42.6)	24 (39.3)	0.84
Lower	31 (57.4)	37 (60.7)	
Pulpal diagnosis			
Irreversible pulpitis	14 (25.9)	12 (19.7)	0.698
Pulpal necrosis	8 (14.8)	11 (18.0)	
Previously initiated	32 (59.3)	38 (62.3)	
Pre-operative PARL (PA)			
No	29 (54.7)	36 (59.0)	0.706
Yes	24 (45.3)	25 (41.0)	
Pre-operative PARL (CBCT)			
No	21 (38.9)	29 (47.5)	0.45
Yes	33 (61.1)	32 (52.5)	
Pre-operative cracks			
No	48 (88.9)	55 (90.2)	0.823
Yes	6 (11.1)	6 (9.8)	
Unfilled canals			
No	50 (92.6)	60 (98.4)	0.146
Yes	4 (7.4)	1 (1.6)	
Perforation			
No	52 (96.3)	60 (98.4)	0.531
Yes	2 (3.7)	1 (1.6)	
Obturation length			
Adequate	50 (92.6)	59 (96.7)	0.185
Short	4 (7.4)	1 (1.6)	
Long	0 (0.0)	1 (1.6)	
Obturation quality			

Inadequate	6 (11.1)	4 (6.6)	0.512
Adequate	48 (88.9)	57 (93.4)	
Restoration type			
Permanent coronal coverage	32 (59.3)	43 (70.5)	0.207
Temporary (CORECEM)	22 (40.7)	18 (29.5)	
Restoration quality			
Inadequate	6 (11.1)	10 (16.4)	0.24
Adequate	48 (88.9)	51 (83.6)	

Table 4.9: Frequency distribution of outcome of treatment for each tooth assessed using periapical radiographs (PA) and cone-beam computed tomography (CBCT).
Number of teeth (%) in enhanced infection control protocol (EP) n=61, standard protocol (SP) n=54 and in total n=115.

	Enhanced protocol		Standard protocol		Total in both groups	
Outcome category	PA	CBCT	PA	CBCT	PA	CBCT
1- New PARL	2 (3.3)	4 (6.6)	3 (5.6)	5 (9.3)	5 (4.3)	9 (7.8)
2- Enlarged PARL	0	3 (4.9)	4 (7.4)	5 (9.3)	4 (3.5)	8 (7)
3- Unchanged PARL	0	2 (3.3)	0	8 (14.8)	0	10 (8.7)
4- Reduced PARL	11 (18)	9 (14.8)	9 (16.7)	11 (20.4)	20 (17.4)	20 (17.4)
5- Resolved PARL	14 (23)	16 (26.2)	12 (22.2)	9 (16.7)	26 (22.6)	25 (21.7)
6- Unchanged healthy PDL	34 (55.7)	27 (44.3)	26 (48.1)	16 (29.6)	60 (52.2)	43 (37.4)
Favourable (4, 5, 6)	59 (96.7)	52 (85.2)	47 (87)	36 (66.7)	106 (92.2)	88 (76.5)
Unfavourable (1, 2, 3)	2 (3.3)	9 (14.8)	7 (13)	18 (33.3)	9 (7.8)	27 (23.5)

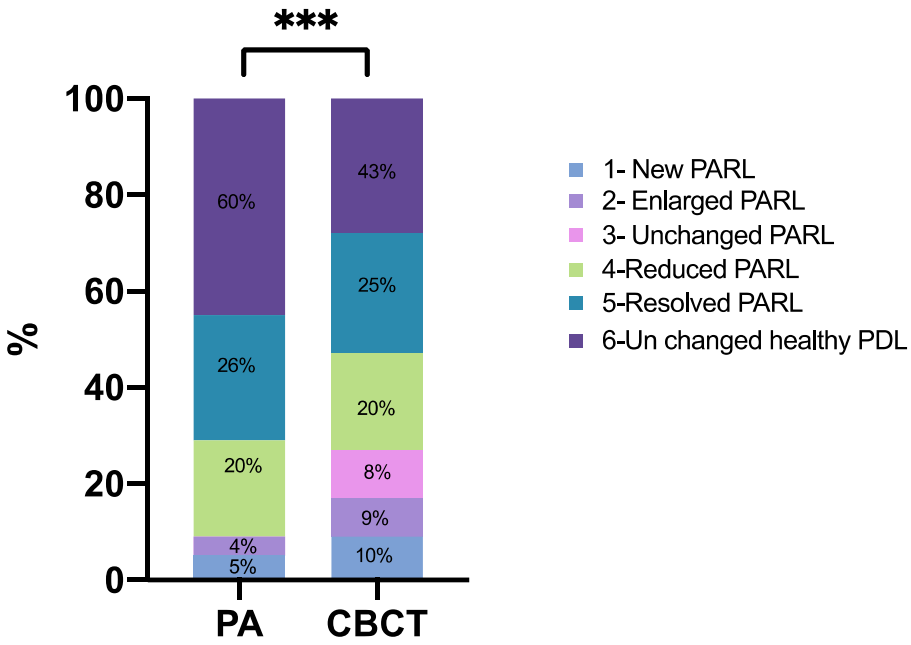


Figure 4.4: Frequency distribution of outcome of treatment for each tooth (n=115) assessed using periapical radiographs PA and CBCT.

4.4.3. Effect of enhanced infection control protocol (EP) on treatment outcomes

Using CBCT, favourable outcomes were observed in 85.2% of molars in the EP group compared to 66.7% of teeth in the SP group. Using PA on the other hand, 96.7% of teeth assigned to EP were favourable, compared to 87% treated following SP as shown in Table 4.10.

Binary logistic regression showed that for CBCT analysis, EP was associated with a significantly higher odds of favourable outcome compared to the SP (OR=2.89; p=0.022). The odds of favourable outcomes increased three times when EP was used (Table 4.10). Periapical radiograph results were close to statistical significance (OR=4.39; p=0.073).

Table 4.10: Favourable root canal treatment outcomes using PA and CBCT in two treatment groups:
Total number of teeth n=115, favourable outcome (%) and results of simple binary logistic regression: OR and 95%CI.

		PA				CBCT			
	Total	Favourable	OR	95%CI	*p-value*	Favourable	OR	95%CI	*p-value*
SP	54	47 (87.0)	1			36 (66.7)	1		
EP	61	59 (96.7)	4.39	0.87 – 22.1	0.073	52 (85.2)	2.89	1.17 – 7.15	**0.022***

*p<0.05

4.4.4. Other factors affecting the outcomes of primary root canal treatment in molars

Besides the treatment protocols, other variables influenced the outcome of root canal treatment based on CBCT. Pre-operative periapical radiolucencies, first molars, presence of cracks, unfilled canals and inadequate obturation quality also affected the outcome negatively. All these factors were included in a multivariate regression model (Table 4.11).

Pre-operative PARL was present in 65 teeth (57%). CBCT-based outcome revealed a favourable outcome of 67.7% and 88% in cases presented with and without pre-operative PARL, respectively. The presence of a pre-operative PARL was revealed as an unfavourable prognostic factor, reducing likelihood of favourable outcome at follow-up (OR=0.29; p=0.014).

Unlike CBCT, when pre-operative PARL were assessed with PA radiographs, presence of PARL pre-operatively was not a significant prognostic factor (OR=0.63;p=0.289).

Second molars showed a significant increment of likelihood of favourable outcome compared to first molars (OR=3.11; p=0.036). This increment was estimated more than three times in which 88% of second molars had favourable outcomes compared to 69.9% of first molars.

Pre-operative cracks were detected clinically in 12 teeth. All cracks were not extending to the pulpal floor and teeth were deemed restorable. Of those, six teeth (50%) had favourable outcomes while the remaining 50% had unfavourable outcome. Presence of pre-operatory crack was also a prognostic factor, reducing favourable outcomes (OR=0.21; p=0.030).

Unfilled canals were present in five teeth, all of which were maxillary first molars. Two out of five teeth had favourable outcome at the one-year follow-up, while the other three teeth had

increased PARL at follow-up. Unfilled canal showed a strong association with the final CBCT outcome (OR=0.19; p=0.074), reducing likelihood of favourable outcomes.

Regarding the obturation length and quality, ten teeth out of the recalled 115 (8.6%) were assessed as having an inadequate root canal filling quality (voids present). Only four of these 10 teeth had favourable outcome at follow-up. Six teeth had inadequate root canal length (four short and two long). The quality of obturation was found to be highly correlated to the odds of favourable outcome increasing the odds of success six times (OR=6; p=0.009). On the other hand, the apical extension of root canal filling did not have an impact on the outcome of treatment in this study (OR=0.6; p=0.562).

Regarding the coronal restoration, 75/115 (65%) of teeth received a full coverage crown/onlay. The remaining 40 teeth (35%) did not receive any full coverage restoration and presented at the follow-up with the build-up material (Corecem) which was placed immediately after completion of root canal treatment. Eighty-six percent (99/115) of restorations were considered adequate while 14% were not adequate. Favourable outcomes were associated with 76% and 75% of teeth with adequate and inadequate coronal restoration.

A multivariate regression model was carried out to identify the prognostic factors that influenced the outcome of root canal treatment including the significant predictors (treatment protocol, pre-operative periapical radiolucency, tooth type, presence of cracks, and quality of obturation). Prognostic factors that remained significant and affected the outcome were the treatment protocol, the presence of a pre-operative radiolucency and the presence of pre-operative cracks (Table 4.12). The effectiveness of the EP remained significant with a threefold increase of the odds of a favourable outcome (OR=3.6; p=0.015).

The presence of PARL pre-operatively reduced likelihood of favourable outcome at follow-up (OR=0.31; p=0.04). Pre-operative cracks also reduced the odds of favourable outcomes (OR=0.18, p=0.019).

When outcome was assessed using PA, none of the tested variable was significantly associated to the likelihood of favourable outcome. Details on PA radiograph-based outcomes are presented in Appendix 8.

Strict criteria for favourable outcomes were tested for possible prognostic factors. Using strict criteria, favourable outcomes included only teeth with completely healed periapical radiolucency and healthy periapical tissue on CBCT (score five and six). Using CBCT, 70.5% of teeth that underwent EP were completely healed, compared to 46.3% treated with the SP. EP involved a significantly higher odds of healed outcome compared to the standard protocol (OR=2.77; p=0.009). Odds of healed status was increased more than 2.7 times. Details about prognostic factors affecting the completely healed outcome are shown in Appendix 9.

Table 4.11: Outcome using CBCT by independent factors: total number of teeth, favourable outcome (%) and results of binary logistic regression: OR and 95%CI.

	Total	Favourable	OR	95%CI	*p-value*
Age (years)	37.6 ± 12.6	38.3 ± 12.6	1.02	0.98 – 1.06	0.317
Gender					
Male	49	40 (81.6)	1		
Female	66	48 (72.7)	0.60	0.24 – 1.48	0.268
Tooth type					
1st molar	73	51 (69.9)	1		
2nd molar	41	36 (87.8)	3.11	1.08 – 8.97	**0.036***
3rd molar	1	1 (100)	--	--	--
Arch					
Upper	47	35 (74.5)	1		
Lower	68	53 (77.9)	1.21	0.51 – 2.89	0.666
Pulpal diagnosis					
Irreversible pulpitis	26	20 (76.9)	1		
Pulpal necrosis	19	14 (73.7)	0.84	0.21 – 3.30	0.803
Previously initiated	70	54 (77.1)	1.01	0.35 – 2.95	0.982
Pre-operative PARL (PA)					
No	65	52 (80.0)	1		
Yes	49	35 (71.4)	0.63	0.26 – 1.49	0.289
Pre-operative PARL (CBCT)					
No	50	44 (88.0)	1		
Yes	65	44 (67.7)	0.29	0.11 – 0.78	**0.014***
Pre-operative cracks					
No	103	82 (79.6)	1		
Yes	12	6 (50.0)	0.25	0.08 – 0.89	**0.030***
Unfilled canals					
No	110	86 (78.2)	1		
Yes	5	2 (40.0)	0.19	0.03 – 1.18	0.074
Perforation					
No	112	87 (77.7)	1		
Yes	3	1 (33.3)	0.14	0.01 – 1.65	0.119
Obturation length					
Adequate	109	84 (77.1)	1		
Short	5	3 (60.0)	0.60	0.10 – 3.44	0.562
Long	1	1 (100)			
Obturation quality					
Inadequate	10	4 (40.0)	1		
Adequate	105	84 (80.0)	6.0	1.55 – 23.2	**0.009****

Restoration type					
Permanent coronal coverage	75	59 (78.7)	1		
Temporary (CORECEM)	40	29 (72.5)	0.72	0.29 – 1.74	0.458
Restoration quality					
Inadequate	16	12 (75.0)	1		
Adequate	99	76 (76.8)	1.10	0.32 – 3.75	0.877

*p<0.05; **p<0.01; ***p<0.001

Table 4.12: Results of multiple binary logistic regression of root canal treatment outcome using CBCT by independent relevant factors from simple binary models: total number of teeth, favourable outcome (%). Adjusted OR and 95% CI

	Total	Favourable	OR	95%CI	*p-value*
Treatment group					
SP	54	36 (66.7)	1		
EP	61	52 (85.2)	3.6	1.3 – 10.6	**0.015***
Tooth type					
1st molar	73	51 (69.9)	1		
2nd molar	41	36 (87.8)	4.6	1.3 – 16.4	0.054
3rd molar	1	1 (100)	--	--	--
Pre-operative PARL (CBCT)					
No	50	44 (88.0)	1		
Yes	65	44 (67.7)	0.31	0.1 – 0.9	**0.040***
Pre-operative cracks					
No	103	82 (79.6)	1		
Yes	12	6 (50.0)	0.18	0.05 – 0.75	**0.019***
Obturation quality					
Inadequate	10	4 (40.0)	1		
Adequate	105	84 (80.0)	4.7	0.98 – 23.1	0.052

*p<0.05; **p<0.01; ***p<0.001

4.4.5. Intra-consensus panel agreement and the inter-examiner agreement

In assessing outcomes, the kappa scores for intra-examiner error were 0.90 and 0.82 for CBCT and PA, respectively. Estimations for CBCT assessments per root were higher than those obtained from PA conventional radiographs. For obturation length and quality, a perfect agreement was reached with kappa scores of 1.00 and 0.85, respectively (Table 4.13). The inter-examiner agreement for treatment outcome was 0.71 and 0.72 for CBCT and PA, respectively.

Table 4.13: Intra-consensus reliability of outcome PA and CBCT as well as obturation length and quality: Kappa´s concordance index and 95% confidence interval.

	Number of teeth	Kappa	95% CI	Assessment
Tooth outcome scoring (CBCT)	49	0.90	0.79 – 1.00	Almost perfect
Tooth outcome scoring (PA)	49	0.82	0.67 – 0.97	Almost perfect
Obturation length	49	1	1.00 – 1.00	Perfect
Obturation quality	49	0.85	0.55 – 1.00	Almost perfect

4.5. Discussion

In this CBCT-based single-blind, two-armed, randomised controlled clinical trial involving patients undergoing primary root canal treatment, the enhanced infection control protocol resulted in a significantly higher percentage of favourable outcomes than did the standard protocol.

Patients were randomly assigned in a 1:1 ratio to receive one of the following regimens: standard root canal treatment protocol (SP) or enhanced infection control protocol (EP). Our pilot study in Chapter 2 showed that around half of the rubber dam surfaces were contaminated with bacteria at the time of obturation. Bacteria were also detected in initial files, gloves, instruments and rulers prior to obturation. The pilot study findings suggested the risk of introducing bacteria into the root canal space after chemomechanical preparations. Thus, this clinical trial aimed to evaluate the effectiveness of implementing an enhanced infection control protocol as a prognostic variable for favourable outcome in non-surgical root canal treatment of molars after one-year follow-up.

The enhanced infection control protocol employed in the present study was set to follow the standardised root canal treatment protocol (European Society of Endodontology, 2006), with changes after the completion of instrumentation and irrigation. This protocol was adopted considering the available data in the literature reports (Ng *et al.*, 2003, Williams *et al.*, 2003, Niazi *et al.*, 2016, Rorslett Hardersen *et al.*, 2019) and the findings of our pilot study. These changes included replacing rubber dam, gloves, instruments and surface barriers to reduce the chances of iatrogenic contaminations at the time of obturation.

Previous recommendations suggested the change of instruments to a sterile set after caries removal (Bergenholtz *et al.*, 2013, Ørstavik, 2020). The infected root canal system is a source

for contaminating instruments and surfaces within the dental surgery (Ng *et al.*, 2003, Williams *et al.*, 2003, Niazi *et al.*, 2016, Rorslett Hardersen *et al.*, 2019). This inadvertent contamination from the work environment may result in reinfection of the disinfected root canal and ultimately result in treatment failure (Hargreaves *et al.*, 2016, Rotstein and Ingle, 2019). Thus, the timing of changing instruments in this study was modified, in which all instruments (tweezer, DG-16 endodontic explorer, plugger and flat plastic instrument), rubber dam and gloves, and surface barriers were changed at the end of the chemomechanical preparation to eliminate these contaminant sources.

A recent survey assessing infection control routine and asepsis maintenance measures such as rubber dam disinfection, gloves, and instruments changing during root canal treatment showed a low compliance of general dentist and endodontists (Shuen *et al.*, in press). Therefore, it does appear that there are opportunities to reduce the contamination of the root canal system/operative field during treatment.

Effect of implementing EP on outcomes

After one-year follow-up, 66% of molars in the standard protocol had favourable outcome whereas the enhanced infection control protocol increased the success rate to 85%. Precisely, the EP increased the odds of favourable outcomes three times. Even after covariables adjustments, the EP remained strong and independently associated with favourable outcomes increasing the odds of success more than three times.

The percentage of favourable outcomes (85%) in the EP compares favourably with previous CBCT outcome-based studies (Patel *et al.*, 2012a, van der Borden *et al.*, 2013, Al-Nuaimi *et al.*, 2018). One study reported 76% of molars had favourable outcomes one year after primary root canal treatment (Al-Nuaimi *et al.*, 2018), while the other reported 77% of molars,

premolars and anterior teeth had favourable outcomes after 10–37 months' follow-up (van der Borden *et al.*, 2013).

Moreover, when success was defined using strict criteria (score five and six only), the difference between the two protocols remained significant. Seventy percent of teeth that underwent EP completely healed compared to 46.3% under SP.

One of the main limitations of clinical studies is the presence of confounding factors that are usually difficult to control and affect the outcomes (Ng *et al.*, 2008). The experimental and the control group in our study were very well balanced in relation to all pre-, intra-, and post-operative factors which have been shown to affect the outcome of root canal treatment. Four well standardised operators carried out all the treatments using the same instrumentation and obturation techniques, all root canal treatments were undertaken in one visit, all molar teeth were enlarged to size S2 ProTaper® Gold Universal with apical size prequal to #25 to allow NaOCl reaching apical third (Saini *et al.*, 2012).

Also, to reduce the clinical variabilities, homogenous distribution of clinical characteristics such as age, gender, pulpal diagnosis, periapical radiolucency, arch and tooth among the two treatment protocols was noted at baseline and, to a slightly lesser extent, at follow-up. Moreover, other intra-operative factors were also homogenously distributed among the two groups at time of follow-up, such as pre-operative cracks, obturation quality, restoration types and quality.

Only molars were included; the number of maxillary and mandibular molars was similar in the two groups. Molar teeth are more difficult to standardise due to challenging anatomical variations; however, Al-Nuaimi *et al*. in a pooled data analysis of 354 teeth included in CBCT

studies showed that the success rate of molars was far lower than that of premolars and anterior teeth (Al-Nuaimi *et al.*, 2018). Thus, with such high success rate, incorporating an enhanced infection control protocol in single-rooted teeth might not be able to show any differences in outcomes; in fact a previous CBCT study comparing the success of single-rooted teeth treated with and without the aid of ultrasonically activated irrigants failed to detect any significant difference between the two groups (Liang *et al.*, 2013).

All cases included in this study were treated in one visit. The reason was to eliminate the effects of $Ca(OH)_2$ intracanal medications, leakage between appointments, and the effect of time lapsing between visits and to maximise the influence of using new sterile instruments, gloves, rubber dams at time of obturation, whilst strictly adhering to best practice. In our study, GP points of both treatment protocols were disinfected with 2.5% NaOCl for at least one minute as suggested previously, to reduce the risk of contaminating the root canal space (Gomes *et al.*, 2005, Royal *et al.*, 2007).

The overall success rate (loose criteria: healed and healing) was 76.5% when CBCT was used as a diagnostic method, in line with CBCT outcome studies on molars primary treatment (Patel *et al.*, 2012a, van der Borden *et al.*, 2013, Al-Nuaimi *et al.*, 2018). Favourable outcomes when assessed with PA radiographs were 92%, which is also in agreement with previous reports (Ng *et al.*, 2007, de Chevigny *et al.*, 2008, Ng *et al.*, 2011, Patel *et al.*, 2012a, Al-Nuaimi *et al.*, 2018). Using PA radiographs, 93% of molars had favourable outcomes in one year (Patel *et al.*, 2012a, Al-Nuaimi *et al.*, 2018) and 86% to 87% after 2-4 years (de Chevigny *et al.*, 2008, Ng *et al.*, 2011), while the pooled meta-analysis using loose criteria was 85.5% (Ng *et al.*, 2007).

When favourable outcome was defined as complete absence of PARL (strict criteria), the success rate of our study dropped to 60% with CBCT and 74% with PA radiographs. These results are consistent with those of meta-analysis and other prospective studies (Ørstavik *et al.*, 2004, Ng *et al.*, 2007, van der Borden *et al.*, 2013). The pooled success rates estimated by meta analyses were 74.4% using strict periapical radiographic criteria (Ng *et al.*, 2007). Complete absence of PARL was reported in one study to be 89% using PAs and 75% using CBCT (Liang *et al.*, 2011), which is higher than our findings. The reason is that all teeth included in the mentioned study were vital and the absence of PARL pre-operatively probably contributed to the increased prevalence of completely healed cases. Another explanation is the longer observation period of two years probably attributed to greater likelihood of complete resolution of PARL (Liang *et al.*, 2011).

The lower success of molars compared to anterior and premolars has been acknowledged previously (de Chevigny *et al.*, 2008, Patel *et al.*, 2012a, Gomes *et al.*, 2015a, Al-Nuaimi *et al.*, 2018, Zhang *et al.*, 2021) and is possibly attributed to the challenges associated to the cleaning and shaping of root canals with a more complex anatomy (Cleghorn *et al.*, 2006, Valencia de Pablo *et al.*, 2010).

The use of CBCT for outcome

It is agreed that assessment of root canal treatment is based on clinical and radiographic findings through evaluating and monitoring the periapical status (European Society of Endodontology, 2006). Using CBCT in detecting periapical radiolucency is a well-established method with an increased accuracy especially in terms of sensitivity, compared to periapical radiographs (de Paula-Silva *et al.*, 2009, Patel *et al.*, 2009).

A limitation of the present study was the potential of false positives with CBCT. A human cadaver study revealed that approximately 20% of roots with canal fillings and histologically 'healthy' periapical tissues may have been judged as diseased on CBCT. However, in that study there were no pre-treatment CBCT scans for comparison, meaning that an objective comparison of radiographic changes was not possible (Kruse *et al.*, 2019). Studies also confirmed that PA radiographs are most likely to overestimate the success rate of treatment due to their lower sensitivity (de Paula-Silva *et al.*, 2009, Patel *et al.*, 2009, Cheung *et al.*, 2013, Liang *et al.*, 2013). Limited values of PA radiographs in diagnosing periapical radiolucencies were asserted in our study, in line with all previous evidence supporting the superiority of CBCT as a diagnostic modality (de Paula-Silva *et al.*, 2009, Patel *et al.*, 2009, Patel *et al.*, 2019).

Many well-known factors contribute to the limited information provided with the two-dimensional PA radiographs resulting in underestimating the number of pre-treatment or post-treatment periapical radiolucencies. Orientation of the x-ray beams, the presence of a lesion in cancellous bone and the superimposition of anatomical structures masking the lesions or changes in the lesion in a buccolingual direction are among the reasons for reduced PA radiograph accuracy (Bender *et al.*, 1982, de Paula-Silva *et al.*, 2009, Patel *et al.*, 2009). Moreover, lesions smaller than 2mm and molar teeth were among the factors which significantly reduced PA radiograph sensitivity (Low *et al.*, 2008, Cheung *et al.*, 2013, Jang *et al.*, 2020). This is particularly relevant for molar teeth such as the ones included in our study, due to the thickness of the cortical plate in the lower molar region and the presence of the zygomatic arch in the maxillary molar area.

In our study, the overall number of favourable outcomes was significantly lower when assessed with CBCT (76%) compared to PA radiographs (92%). More new lesions were detected with CBCT (nine vs five with PA), and this was in agreement with other studies where up to 20% more post-treatment lesions were detected with CBCT (Patel *et al.*, 2012a, Liang *et al.*, 2013, van der Borden *et al.*, 2013, Al-Nuaimi *et al.*, 2018). Additionally, PA did not reveal any prognostic factor affecting the outcome including the treatment protocol used.

In five teeth, pre-operative PARL were evident in PA radiographs but not on CBCT. Similar observation was reported previously (Liang *et al.*, 2011, Fernandez *et al.*, 2013). Because of the lack of histological findings, it is unknown whether these lesions were false positive in PAs or false negative in CBCT. Given the greater accuracy of CBCT (de Paula-Silva *et al.*, 2009, Patel *et al.*, 2009, Kanagasingam *et al.*, 2017b), the overall outcome of this study was based on CBCT and clinical outcomes rather than PA radiographs.

The apical extent and quality of root canal filling in this study was based on periapical radiograph observations. It has been reported that CBCT is less sensitive than periapical radiographs in detecting voids and length evaluation (Soğur *et al.*, 2007, Huybrechts *et al.*, 2009). This is perhaps due to images noise and artefacts generated due to the presence of radiopaque filling material (Soğur *et al.*, 2007). CBCT images did not offer an advantage of detecting voids in clinical studies and an agreement was reported between the PA radiographs and CBCTs in detecting voids and length (Restrepo-Restrepo *et al.*, 2019). On the other hand, higher sensitivity of CBCT was reported in detecting voids and evaluating the apical extent of root canal filling in some clinical studies (Liang *et al.*, 2011, Liang *et al.*, 2012).

One-year recall and outcome findings

The six-point outcome classification was used as it took into account how the periapical status changed at the one-year follow-up (Patel *et al.*, 2012a). A one-year follow-up of patients followed the European Society of Endodontology Quality Guidelines (European Society of Endodontology, 2006). Ørstavik concluded that the peak incidence of healing and emerging of apical periodontitis as detected on periapical radiographs is at one year (Ørstavik, 1996). Dropouts were frequently reported in clinical and prospective endodontic studies (Ross *et al.*, 2009). The recall rate in our study was 80%, which compares favourably to similar studies (Patel *et al.*, 2012a, Davies *et al.*, 2016), and is higher than the median recall rate of prospective clinical studies (Ng *et al.*, 2007, Zhang *et al.*, 2021). The major reason for dropout was the COVID-19 pandemic, during which patients were unwilling to travel. All reached patients were asked about the tooth condition and confirmed that the tooth was functional.

There is no agreement on defining success in endodontic treatment; in the present study, the outcome was based on the comparison of the post-operative with pre-operative radiographs and CBCTs. This method was used in the majority of outcome studies (de Chevigny *et al.*, 2008, Ng *et al.*, 2011, Patel *et al.*, 2012a, Zhang *et al.*, 2015, Azim *et al.*, 2016, Al-Nuaimi *et al.*, 2018), unlike some CBCT-based studies, in which pre-operative CBCT scans were not available and outcome was based only on post-operative CBCT scans (Fernandez *et al.*, 2013, Restrepo-Restrepo *et al.*, 2019). This is methodologically wrong, as to radiographically establish the response to a treatment comparing baseline images of low sensitivity (PAs) with recall images of high sensitivity (CBCT) may clearly lead to an overestimation of the number of failures. Additionally, changes and resolution of PARL were recorded for each root allowing matching root comparisons, then the worst score given to a root was assigned to the tooth, thus greater accuracy was assumed (Patel *et al.*, 2012a).

Other variables, which were strongly and independently associated with unfavourable outcomes of the root canal treatment based on CBCT analysis after covariables adjustment, include the presence of pre-operative cracks and presence of pre-operative PARL.

Presence of pre-operative periapical radiolucencies

The presence of a pre-operative periapical radiolucency was found to have a negative impact on outcome in the present study, similarly to many other periapical radiographs and CBCT-based outcome studies (Hoskinson *et al.*, 2002, Friedman *et al.*, 2003, de Chevigny *et al.*, 2008, Ng *et al.*, 2008, Ng *et al.*, 2011, Liang *et al.*, 2012, Patel *et al.*, 2012a, Liang *et al.*, 2013, Azim *et al.*, 2016). The presence of periapical radiolucencies in CBCT was shown corresponding to histological inflammation (de Paula-Silva *et al.*, 2009). Unlike teeth with intact periradicular tissue, teeth presenting with pre-operative periapical radiolucency are always associated with radicular infection, thus their outcome is less favourable (Sundqvist, 1976).

Presence of pre-operative cracks

Pre-operative cracks were detected clinically in 12 teeth. Half of these had unfavourable outcomes. Presence of pre-operative crack was among the factors impacting outcome negatively in previous studies (Ng *et al.*, 2011). A recent retrospective cohort study showed a success rate of 75% in cracked teeth. The presence of PARL and lack of permenant coronal restoration were among the prognostic factors (Chen *et al.*, 2021), while a recent meta-analysis revealed that the survival and success rate of posterior teeth with cracks were 88% and 82%, respectively. The presence of deep periodontal pocket was associated with failure (Olivieri *et al.*, 2020).

Patients were advised to receive a full coverage restoration as soon as possible to prevent cracks propagation. Most teeth with pre-operative cracks had a full coverage restoration at follow-up (9/12); still, four teeth with the full coverage restoration had unfavourable outcomes. A possible explanation of this failure is the timing of the coronal restoration received. This information was not available in our data. Although all cracks clinically were not extending to the pulpal floor and were not associated with deep pockets pre-operatively, the diagnosis of vertical root fractures cannot be absolutely excluded. Interestingly, almost half of the failed cases in the EP group (5/9) had cracks diagnosed pre-operatively.

Tooth type, and location

Although second molars showed significantly greater success (87.8%) than first (69.9%) in the simple regression, this was not confirmed in the multi-regression model. All unfilled canals were the mesio-lingual canals of first molar teeth, and this perhaps justifies the difference. The same explanation also applies to the greater success of lower teeth in our study.

Unfilled canals

Unfilled canals were present in 5/115 recalled teeth. The prevalence of missed canals was 4.3% of all treated teeth, which is lower than what was reported in retrospective and cross-sectional studies (Karabucak *et al.*, 2016, Costa *et al.*, 2019). Clinical notes reported the inability to negotiate mineralised (three cases) and transported (one case) canals. Three out of five teeth with unfilled canals in the present study had unfavourable outcomes. When pre-operative periapical radiolucencies were present, all cases with unfilled canals failed (two cases). These areas of untreated root canal spaces can contribute to failure by harbouring microbial components (Ricucci and Siqueira, 2010). Although studies associated unfilled canals with failure (Karabucak *et al.*, 2016, Costa *et al.*, 2019, Leprince and Van Nieuwenhuysen, 2020),

the presence of unfilled canals did not affect the outcome in our multivariate analysis, probably due to the small number of teeth involved.

Quality of root canal filling

In our study, the quality of obturation was found to be correlated to odds of favourable outcome. As in previous studies (Sjögren *et al.*, 1990, Liang *et al.*, 2012), this association did not remain strong in the multivariate analysis. On the contrary, one CBCT-based outcome study found that the density of the root canal obturation was a significant factor in the context of a multivariate analysis (Liang *et al.*, 2011). The reason for such different findings may be the rate of cases with inadequate root canal filling. In that study, 16% of the roots were considered inadequate when assessed with PA (Liang *et al.*, 2011), while only 8.6% had inadequate filling quality in our study and thus did not reach a significant level.

Apical extent of root canal filling

Regarding the apical extent of root canal filling, a short filling was detected in 5/115 cases. A lower success rate was noted in cases with short filling compared to those with adequate filling, but the difference was not significant. Cross-sectional studies based on CBCT found that the length of root canal filling was significantly associated with periapical status (Gomes *et al.*, 2015a, de Sousa Gomide Guimaraes *et al.*, 2019, Meirinhos *et al.*, 2020). However, prospective CBCT studies failed to show such significant association (Liang *et al.*, 2011, Zhang *et al.*, 2021). One retrospective study found an association between root canal filling length and absence of PARL at follow-up (Liang *et al.*, 2012). In their study, 42% of examined roots had either short or long root canal fillings when assessed with PA radiographs, compared to 5% of teeth in our study. Thus, the lower number of cases with inadequate obturation length in our study is probably attributed to the absence of a significant difference.

Patient-related factors

Age and gender were not among the predictable variables affecting the outcome in our study. This is in agreement with previous studies (Peters and Wesselink, 2002, Friedman *et al.*, 2003, Farzaneh *et al.*, 2004, de Chevigny *et al.*, 2008, Liang *et al.*, 2011, Patel *et al.*, 2012a, Al-Nuaimi *et al.*, 2018, Zhang *et al.*, 2021).

Coronal restoration quality

Inadequate coronal restoration was reported to affect outcomes negatively (Hoskinson *et al.*, 2002, Gillen *et al.*, 2011, Liang *et al.*, 2011, Liang *et al.*, 2012), but did not impact the retreatment outcomes in a four-year CBCT prospective study (Zhang *et al.*, 2021). In our study, restoration quality was not among the factors predicting the outcomes. This is probably due to the lower number of cases with inadequate restoration quality in this study (16 /115). Moreover, in a short period of one year, the influence of leakage associated with inadequate coronal restoration quality might not be readily detected.

Pulpal diagnosis

Pulpal diagnosis did not significantly impact the outcome of our study although favourable outcome of irreversible pulpitis teeth was higher than necrotic (76.9% and 73.7% respectively). The success rate of vital teeth in our study (76.9%) is within the range of CBCT-based outcome study including only vital teeth (Liang *et al.*, 2011). When periapical radiographs are considered as a diagnostic measure, the success rate of vital teeth in our study was 88.5%, which is comparable to the pooled success rate for vital teeth of 89%, although not only molars were included in the pooled analysis (Ng *et al.*, 2008).

Panel agreement

Both examiners who evaluated the CBCT and PAs were experienced endodontists and were long-term CBCT users. Consensus panel agreement on CBCT outcome scoring was 0.90. The intra-examiner agreement with CBCT was higher than for periapical radiographs (0.82) which was pointed out previously (Liang *et al.*, 2011, Patel *et al.*, 2012a, Zhang *et al.*, 2021). These intra-consensus panel agreement scores are considered as very good (Landis and Koch, 1977).

Radiation in CBCT

A concern might be raised due to the radiation dose associated with the use of CBCT in this study. This study used 3D Accuitomo with a small 4 x 4cm field of view that adhered to the ALARA (as low as reasonably achievable) principle (ICRP 2007) and a thyroid collar protection was used for all patients. The protocol used in this study was 90kV and 4mA leading to an effective dose of 54.8 µSv. The maximum effective dose was 66µSv accounting for 20µSv more exposure of the salivary glands, patient and technique variation. This makes a CBCT scan radiation equivalent to 11 days of background radiation (annual natural background radiation dose is 2.2mSv in the UK).

Additionally, all patients were given a detailed information sheet about the total radiation exposure upon participating in this study and an informed consent was signed before commencing the treatment.

4.6. Conclusion

This clinical randomised trial showed the effectiveness of implementing an enhanced infection control protocol on primary root canal treatment of molar teeth. The enhanced infection control protocol was associated with a threefold increase in the odds of favourable outcome of root canal treatments. Findings are suggesting that incorporating simple steps such as changing instruments, gloves and rubber dams during root canal treatment are important in improving the asepsis and have a great impact on treatment outcomes.

Chapter Five: The Efficacy of Implementing an Enhanced Infection Control Protocol on Microbial Reduction During Root Canal Treatment of Molars: A Quantitative, *In Vivo* Molecular Study

5.1. Introduction

The preceding chapter demonstrated that the implementation of the enhanced infection control protocol was associated with a higher percentage of favourable clinical outcomes. In this chapter, we aimed to explore the effect of the amended protocol on total microbial numbers in the root canal space after chemomechanical preparation.

In Chapter 2 we examined the qualitative and quantitative contribution of bacteria to iatrogenic introduction of microbes during treatment. However, to date, there is no consensus on whether the quantity of bacteria and their products or specific bacterial types elicit a pathogenic phenotype. In this and the following chapter, we will establish whether total microbial load or specific pathogens are affected by the implementation of the enhanced infection control protocol.

The evaluation of the bacterial population within the root canal space has been traditionally undertaken using culture-based studies (Byström and Sundqvist, 1985, Sjögren *et al.*, 1997, Siqueira *et al.*, 2007a). Culture studies have been extensively used to measure the effectiveness of irrigation protocols or to generally measure the bacterial reduction after treatment (Byström and Sundqvist, 1985, Sjögren *et al.*, 1997, Siqueira *et al.*, 2007a).

Molecular microbiology was shown to be far more sensitive in evaluating and identifying microbial communities. Methods such as qPCR with a broad-ranged primer pair directed against the bacterial 16S ribosomal RNA (rDNA genes) can be more accurate, especially when evaluating disinfection protocol influencing microbial load (Siqueira and Rôças, 2005b). Shortcomings with these methods still exist and include the presence of PCR inhibitors within saliva or blood (Ochert *et al.*, 1994), incomplete coverage of universal primers (Nadkarni *et al.*, 2002), contamination, and false-positive amplifications (Corless *et al.*, 2000).

Endodontic diseases are polymicrobial in origin, and culture studies found that no specific predefined pathogens determine the disease development and progression (Kakehashi *et al.*, 1965, Möller, 1966, Möller *et al.*, 1981, Fabricius *et al.*, 1982). To better represent the gene copy numbers measured with qPCR, where different bacteria with different gene copies per cell are expected, we used the mixed microbial community (ZymoBIOMICS^{TM} microbial community) as our positive control in qPCR assay. Moreover, universal primers that were previously validated (Nadkarni *et al.*, 2002) were used to quantify all type of bacteria existing within the samples rather than targeted taxa, including cultivable and as-yet-uncultivated bacteria.

This part of the randomised clinical trial was designed to evaluate the effectiveness of the enhanced infection control protocol (EP) on the intracanal bacterial number after chemomechanical preparations by comparing the bacterial load in two groups that were randomly assigned to standard protocol (SP) and enhanced infection control protocol (EP).
The key research question of this study was whether or not the new protocol would reduce the risk of contamination during treatment.

Multiple factors affect the outcome of root canal treatments as shown in Chapter 1 (Table 1.8, 1.9 and 1.11). Pre-operative periapical status, tooth type, obturation quality and length and restoration quality were frequently reported to impact the outcomes (Ng *et al.*, 2008). The effect of bacterial presence or absence at time of obturation on outcome is debatable (Sathorn *et al.*, 2007). The secondary aim of this chapter was to examine whether presence of bacteria / bacterial load before obturation is associated with an increased percentage of failures.

5.2. Materials and method

This chapter was conducted as a part of the clinical trial described in Chapter 4. Patient recruitment, randomisation, inclusion and exclusion criteria were described in Section 4.3.1 to Section 4.3.4.

5.2.1. Sampling procedures

The pre-operative assessment, root canal treatment procedures, and steps implemented for the enhanced infection control protocol group, were all detailed in the preceding chapter, Sections 4.3.5 and 4.3.6.

Sampling protocols were performed under aseptic conditions, as described in the pilot study, Section 2.3.2. In this study, collected samples were contamination control (CC), initial intracanal samples (S1), initial file (F) and pre-obturation samples (S2), in addition to negative control samples (NC) collected from sterile paper points and files. All samples were transferred into sterile Eppendorf microtubes containing 200µl of phosphate-buffered saline and immediately snap-frozen at -20°C. Table 5.1 shows all samples taken in this trial during root canal treatment.

Table 5.1: Clinical samples collected for microbiological analysis

Sample	Abbreviation	Description and time of collection
Negative control	NC	Samples from sterile paper points, and files.
Contamination control	CC	Samples from teeth surface after rubber dam placement, OpalDam and decontamination with 2.5% NaOCl.
Initial intracanal sample	S1	Sampling the intracanal space once the chamber was accessed and before the use of any irrigant. Paper point inserted 1mm short of canal's working length and held in position for 30 seconds.
Initial file	F	The first #10 K file introduced to the canal before irrigant use.
Final intracanal sample (pre-obturation)	S2	After completion of chemomechanical preparation, after rinsing with saline and drying the canals, with paper points held in place for 30 seconds.

5.2.2. DNA extraction, quantification and total bacterial enumeration by quantitative polymerase chain reaction

DNA extractions and quantification of all collected samples were undertaken as described in Section 2.3.3. Bacterial quantification before and after treatment procedures using 16S rRNA gene-targeted qPCR was performed on collected samples as described previously in Section 2.3.4. In this study, instead of using *Enterococcus faecalis* DNA extracts as positive control, quantified ZymoBIOMICS^{TM} microbial community DNA standard was used (Zymo Research, Irvine, CA, USA). The diverse community used included several Gram-positive and negative bacteria with known gene copy number, namely *Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella enterica*, *Lactobacillus fermentum*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Bacillus subtilis.*

Validations of the assay performance and standard curves were carried out using ZymoBIOMICSTM DNA standard of known gene copy number and bacterial copy number based on a series of 10-fold dilutions. All reactions were run in triplicate. Sensitivity of the qPCR assays was set at 10^2 bacterial cell equivalents. Bacterial load and gene copy numbers were inferred from the standard curves and quantified as Ct values, absolute read counts and log10-transformed reads.

A flow diagram of the patient's recruitments and exclusions is illustrated in Figure 5.1.

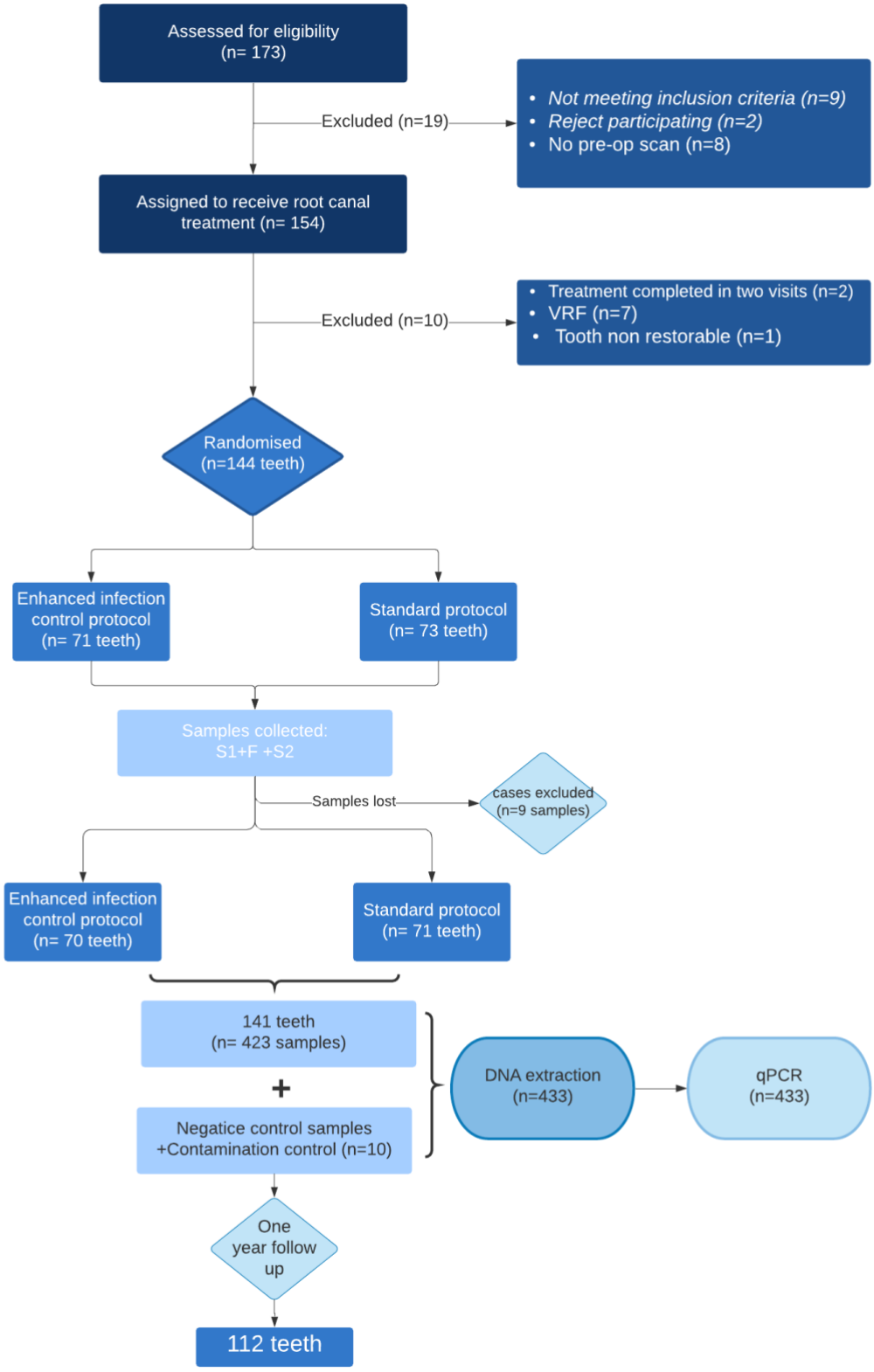


Figure 5.1: Flow diagram of included cases subjected to qPCR.

After exclusions, 144 teeth were randomised into standard or enhanced infection control protocol group. During laboratory DNA extraction, three samples were lost, and the associated cases were excluded (n=3). Remaining teeth were 141 teeth in which initial, pre-obturation and files samples were collected from each tooth. A total of 423 samples were subjected to qPCR after DNA extraction. At one-year follow-up, clinical and radiographic examination (CBCT and PA) were completed for 112 teeth.

5.2.3. Statistical analysis

All the analyses were carried out using SPSS version 23.0, and the level of significance was assumed at or below 5% ($p \leq 0.05$).

Descriptive statistics were used to summarise various study characteristics including all factors described in Chapter 4.

The homogeneity of both protocol groups was tested through demographic and clinical variables with Fisher's exact test for categorical data (gender, arch, pulpal diagnosis, tooth type, periapical condition) and Mann-Whitney test for continuous data (age, and bacterial number).

Data of bacterial load at S1 and S2 were tested for normality and found to be non-parametric. Wilcoxon signed-rank test was used to compare the reduction in bacterial counts before and after chemomechanical preparation (from S1 to S2).

The delta Ct (δCt*)* value was calculated, indicating the relative change in bacterial load between the initial (S1) and pre-obturation (S2) samples ($\delta Ct = Ct_{obturation(S2)} - Ct_{initial(S1)}$).

Mann-Whitney U test (Wilcoxon rank-sum test) was used to compare bacterial numbers in pre-obturation samples and δCt among the two treatment protocols.

Binary logistic regression models were performed to study probability of bacterial absence in pre-obturation samples according to treatment protocol. Binary logistic regression models were also performed to study the occurrence of cases with increased/decreased microbial load and treatment protocol. Non-adjusted odds ratio (OR) and 95% confidence intervals were obtained.

The association between bacteria in pre-obturation samples and one-year CBCT outcomes were tested with Mann-Whitney U test. Two-tailed Fisher exact test was used for the

association between outcomes and number of cases with/without genomic DNA (gDNA) amplification.

Kruskal Wallis test was used to compare initial bacterial load in teeth with different pulpal diagnoses, while Mann-Whitney U test was used to compare initial bacterial load in cases with or without pre-operative periapical radiolucencies.

Clinical variables affecting the microbial change were evaluated using a multiple logistic regression model including all clinical characteristics: age, gender, pulpal diagnosis, arch, tooth type, initial bacterial load at S1, and presence of PARL (Appendix 10).

5.3. Results

Amplification was successful in all clinical samples (n=423). The negative control samples (paper points and files) were considered negative as those samples yielded a signal less than the detection level of 10^2 copy numbers per reaction. The exception was with one sample (NC) paper point with a median gene copy number of 4.6×10^3.

Correlation coefficient (r^2) and amplification efficiency (E) values of the standard curves for the qPCR assay using universal primers were >0.98 and 95-103% respectively.

5.3.1. Demographic characteristics of teeth in both groups

All patients included as a base line in Chapter 4 were included in this study (n=144); samples belonging to three teeth were lost during the DNA extraction process and were excluded from the analysis, leaving 141 for further analysis. The enhanced protocol (EP) group included 70 teeth, while 71 teeth were included in the standard protocol (SP) group. The distribution of demographic characteristics including age, gender, pulpal diagnosis and tooth type from 141 teeth is shown in Table 5.2.

The age of all participants ranged between 18 and 80 years; mean age 36.5 years. More than half of the recruited teeth, 84/141 (60%), were previously accessed as an emergency treatment in the Acute Dental Care department or by the referring GDP, i.e., diagnosed as "previously initiated". Forty percent of the recruited teeth were presented with symptoms of pulpal necrosis or symptomatic irreversible pulpitis (around 20% in each group). The pre-operative periapical radiolucency was present in 86/141 teeth (61%) based on CBCT analysis.

One-year follow-up was carried out as described in Chapter 4. The CBCT-based outcome data were available for 112 teeth out of the 141 teeth sampled and processed for qPCR analysis.

Table 5.2: Distribution of demographic and clinical characteristics of randomised patients in each group.

SP: standard protocol, EP: enhanced protocol, number of teeth (%) or mean ± standard deviation for age, or median for bacterial gene copy number. Results of Chi2, Fisher's exact test for categorical data (gender, arch, pulpal diagnosis, tooth type, periapical condition) or Mann-Whitney test for continuous data (age, and bacterial number) showing homogenous distribution of cases among the two treatment protocols.

	Treatment protocol		**Total in both groups**	
	SP (n=71)	**EP (n=70)**	**(n=141)**	***p-values***
Gender				
Male	30 (41.7)	31 (44.9)	61 (43.3)	0.86
Female	41 (57.7)	39 (55.7)	80 (56.7)	
Age (years)				
Mean	35 ±12.2	37.7 ±12.1	36.5 ±12.2	0.14
Range	18-80	20-70	18-80	
Arch				
Upper	32 (45.1)	27 (38.6)	59 (41.8)	0.49
Lower	39 (54.9)	43 (61.4)	82 (58.2)	
Pulpal Dx				
Irreversible pulpitis	16 (22)	14 (20)	30 (21)	0.93
Pulpal necrosis	14 (19.4)	13 (18.8)	27 (19.1)	
Previously initiated	41 (56.9)	43 (62.3)	84 (59.6)	
Tooth type				
First Molar	50 (70.4)	49 (70)	99 (70.2)	1.0
Second molar	21 (29.6)	20 (28.6)	41 (29.1)	
Third Molar	0	1 (1.4)	1 (0.7)	
Pre-operative PARL (CBCT)				
Present	46 (64.8)	40 (57)	86 (61)	0.22
Absent	25 (35.2)	30 (42.9)	55 (39)	
Bacterial number in S1				
Median	8.1×10^3	8.6×10^3		0.76
Mean	5.7×10^4	2.8×10^4	2.29×10^3	

5.3.2. Total bacterial load before and after chemomechanical preparation and cleaning efficiency across all samples

Overall, the median bacterial number in initial samples (S1) was 8.6×10^3, after instrumentation (S2), the median bacterial number decreased significantly to 2.1×10^3 (Wilcoxon signed-rank test, $p<0.001$).

Intra-group quantitative analysis demonstrated that in both treatment groups, a highly significant bacterial reduction was achieved as shown in Table 5.3. In the SP group, a median bacterial number of 8.1×10^3 was found in S1 samples, and decreased significantly in S2 to 3.5×10^3 (Wilcoxon signed-rank test, $p=0.005$). Similarly, in the EP, the median number of bacteria reduced significantly from 8.6×10^3 in S1 to 1.3×10^3 in S2 (Wilcoxon signed-rank test, $p<0.001$).

The δCt value was calculated, indicating the relative change in bacterial load between the initial (S1) and pre-obturation (S2) samples ($\delta Ct = Ct_{obturation(S2)} - Ct_{initial(S1)}$). Positive values represent the log2-transformed *reduction* in bacterial numbers while negative values indicated an *increase* in bacterial numbers after chemomechanical debridement.

When δCt was examined, the range was -5.6 to 9 (with an average of 1.5). This highlighted that most samples had significant reductions in bacterial numbers after chemomechanical preparation shown by the positive values of the δ*Ct*. On the other hand, some cases had increased bacterial load after instrumentation represented by the negative values of δ*Ct* (Figure 5.2).

Table 5.3: Quantitative qPCR findings before and after chemomechanical preparation in both treatment groups.

Treatment group	Initial samples (S1)	Pre-obturation(S2)	*p-value*
Both groups			
Mean (SD)	4.3×10^4 ($\pm1.3\times10^5$)	9.1×10^3 ($\pm1.9\times10^4$)	**<0.001*** **
Median (IQR)	8.6×10^3 (1.2×10^3, 2.9×10^4)	2.1×10^3 (5.2×10^2, 1×10^4)	
Enhanced infection control protocol			
Mean (SD)	2.8×10^4 ($\pm4.7\times10^4$)	4.9×10^3 ($\pm7.8\times10^3$)	**<0.001*** **
Median (IQR)	8.6×10^3 (1.3×10^3, 3.1×10^4)	1.3×10^3 (4.3×10^2, 6×10^3)	
Standard protocol			
Mean (SD)	5.7×10^4 ($\pm1.7\times10^5$)	1.3×10^4 ($\pm2.5\times10^4$)	**0.005****
Median (IQR)	8.1×10^3 (1.1×10^3, 2.8×10^4)	3.5×10^3 (8.7×10^2, 1.4×10^4)	

**p<0.01; ***p<0.001

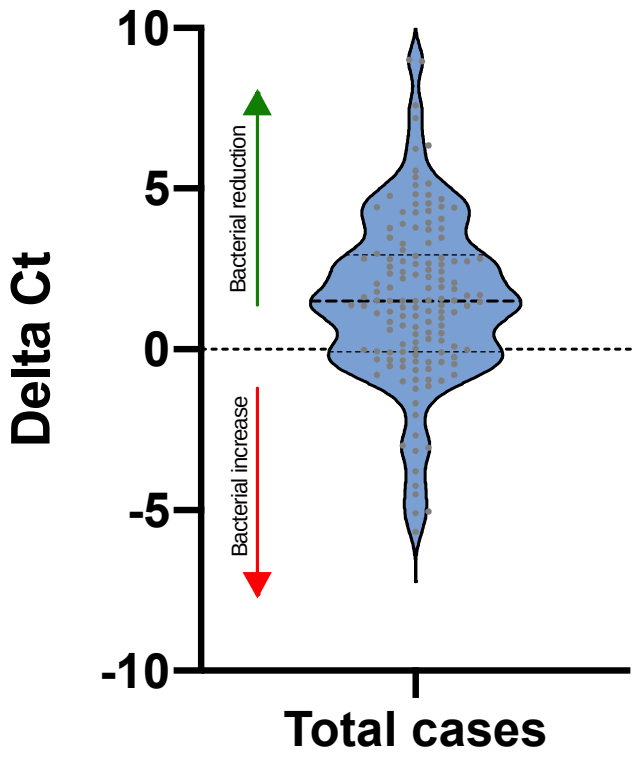


Figure 5.2: Violin plot highlighting the range of δCt.

δCt ($Ct_{obturation}$ - $Ct_{initial}$) in all samples, highlighting that most samples had significant reductions in bacterial numbers (green arrow) as positive values of δCt indicate reduction of bacterial number after chemomechanical preparation: the higher the positive values, the greater the reduction. Red arrow represents the cases where increase in bacterial number was evident after chemomechanical preparation (negative values of δ*Ct*). Dashed line indicates the mean, while top and bottom dotted lines indicate the 25th and 75th quartiles.

5.3.3. The effect of enhanced infection control protocol (EP) on microbial load

A) Delta Ct

When δCt was examined as a measure of bacterial load change, the average δCt values were 0.9 and 2.1 in the SP and EP, respectively. The difference in δCt was significant (Mann-Whitney U test, p=0.010) between the two treatment protocols and highlighted a greater reduction in bacterial number noted in the EP. This was reflected with the higher δCt values within this group (Table 5.4, Figures 5.3 and 5.4).

Table 5.4: Delta Ct values reflecting microbial reduction in the two treatment protocols.
Mean and median of the δCt values in the treatment groups and showing results of Mann-Whitney U test.

δCt values	Standard protocol (SP)	Enhanced infection control protocol (EP)
Mean (SD)	0.9 (±2.7)	2.1 (±2.3)
Median (IQR)	1.04 (-0.5,2.8)	1.7 (0.7,3.7)
Range	-5 to 9	-5 to 8.9
p-value		**0.01***

*p<0.05

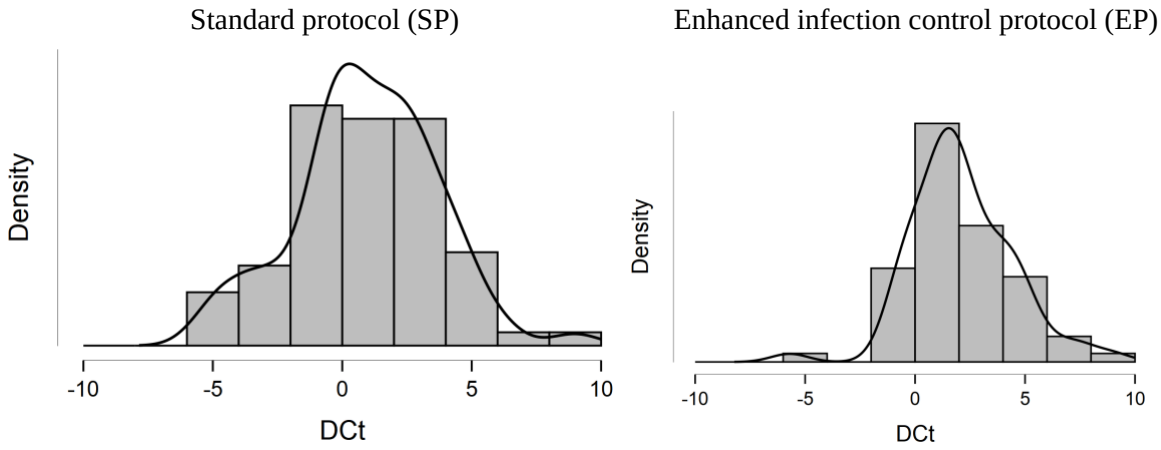


Figure 5.3: Density plot of Ct values (δCt) differences between experimental groups (SP and EP).

More negative δCt values are present in the SP group, which indicates increase in bacterial load after chemomechanical debridement, while the EP was associated with greater reduction in bacterial number (positive δCt values).

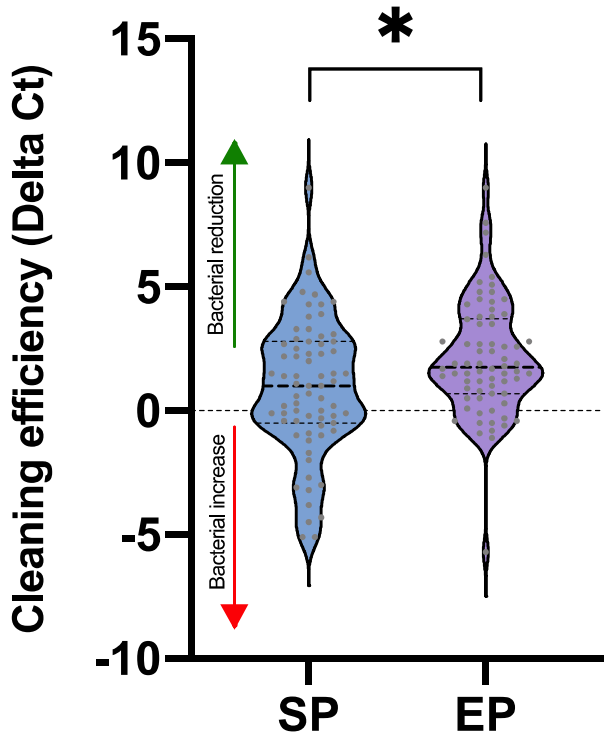


Figure 5.4: Violin plots highlighting the range of δCt within the two treatment protocols.

A significantly higher mean of δCt is observed in cases treated with EP, indicating a greater reduction in bacterial number noted within this group.

Green arrow indicates positive values in which a reduction in bacterial number after chemomechanical preparation is noted. Red arrow represents the cases where increase in bacterial number was evident after chemomechanical preparation (negative values of δ*Ct*). Dashed line indicates the mean, while top and bottom dotted lines indicate the 25th and 75th quartiles.

B) Total estimated bacterial number in pre-obturation samples

The median bacterial number of pre-obturation samples in the EP was 1.3×10^3 compared to 3.5×10^3 bacteria in the SP pre-obturation samples. As shown in Figure 5.5, the enhanced protocol significantly reduced the estimated bacterial count in pre-obturation samples when compared to the standard protocol (Mann-Whitney U test, p=0.009).

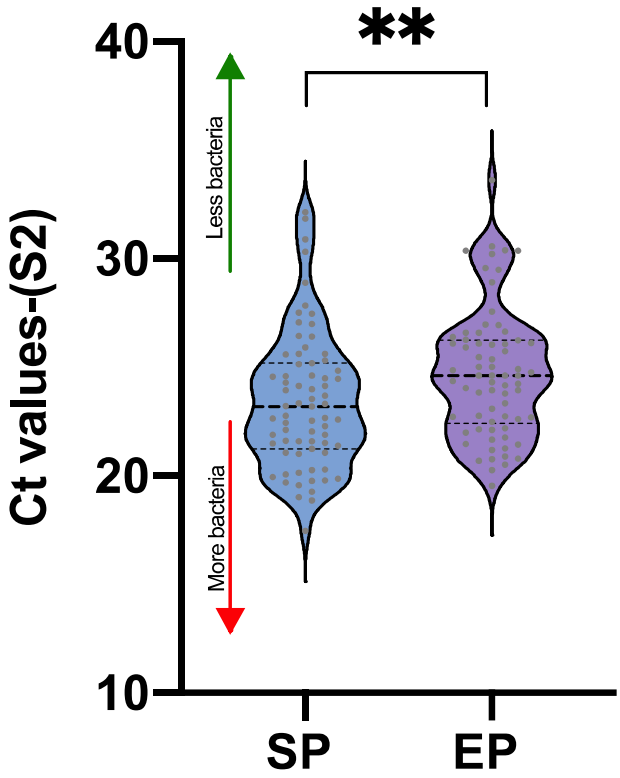


Figure 5.5: Ct values of pre-obturation samples in standard (SP) and enhanced protocol (EP).
Significantly less bacteria detected in pre-obturation samples in the EP compared to the SP (shown by higher Ct values in the SP group samples).
Dashed line indicates the mean while top and bottom dotted lines indicate the 25th and 75th quartiles.

C) Cases with negative genomic DNA amplification in pre-obturation samples of experimental groups

The 16S rRNA gene copies in S2 was categorised into two categories: *negative* and *positive*. *Negative* (no detectable gDNA amplification) in S2 was considered when bacteria were below the detection level of 10^2 copy number per reaction, while the S2 sample was categorised as *positive* when gDNA bacteria amplification was greater than that detection threshold level.

In the EP, after chemomechanical preparations, 15 of the 70 pre-obturation samples (21.4%) did not show detectable gDNA amplification above detection threshold and considered negative. On the other hand, only five of the 71 teeth (7%) in the SP showed absence of gDNA amplification in S2 samples using qPCR (Figure 5.6).

The binary logistic regression model demonstrated, using absence of gDNA amplification as an outcome measure, that cases in the EP were involved with a significantly higher odds of undetectable gDNA amplification in pre-obturation samples when compared to SP (OR=3.6, p=0.019). The odds increased more than threefold (Table 5.5).

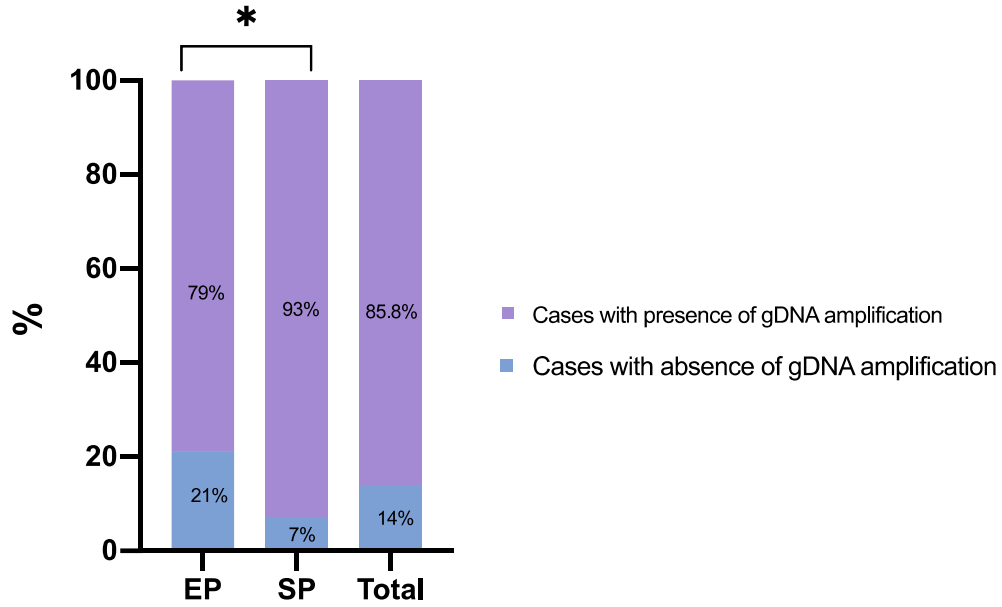


Figure 5.6: Cases with absence/presence of gDNA amplification in S2 samples of both groups.
SP (n=71) and EP (n=70) and in total (n=141).
Higher proportion of cases with no gDNA amplification (negative qPCR results) in the EP group compared to SP. Negative gDNA amplification was considered if at or below background amplification detection level of 10^2 copies per reaction.

Table 5.5: Number and percentage of S2 samples with no gDNA amplification in the two treatment protocols.
The total number of teeth, the number of cases with reduced microbial load (%) and results of simple binary logistic regression: OR and 95% CI.

		Negative gDNA amplification is S2 samples			
	Total	Negative gDNA	OR	95%CI	*p-value*
SP	71	5 (7)	1		
EP	70	15 (21.4)	3.6	1.2 – 10.5	**0.019***

*p<0.05

D) Cases with bacterial reduction/increase before obturation (S2)

Based on the δCt values, the change of bacterial number was categorised into two categories; *increased* and *decreased* after chemomechanical preparation. Positive δCt values represent a reduction in bacterial numbers from S1 to S2, while negative δCt values indicated an increase in bacterial numbers after chemomechanical debridement.

In the EP group, 59 of 70 teeth (84.3%) had bacterial reduction after chemomechanical preparation compared to 43 of 71 teeth (60%) in the SP group. In the SP, 39% of the cases had increased bacterial number after chemomechanical debridement, and 15.7% in the EP cases (Figure 5.7).

Binary logistic regression was carried out to compare the difference between the two treatment protocols. Cases in the EP group involved a significantly higher odds of cases with reduced bacterial load after chemomechanical preparation than standard protocol (OR=3.4, p=0.002). The odds increased threefold (Table 5.6).

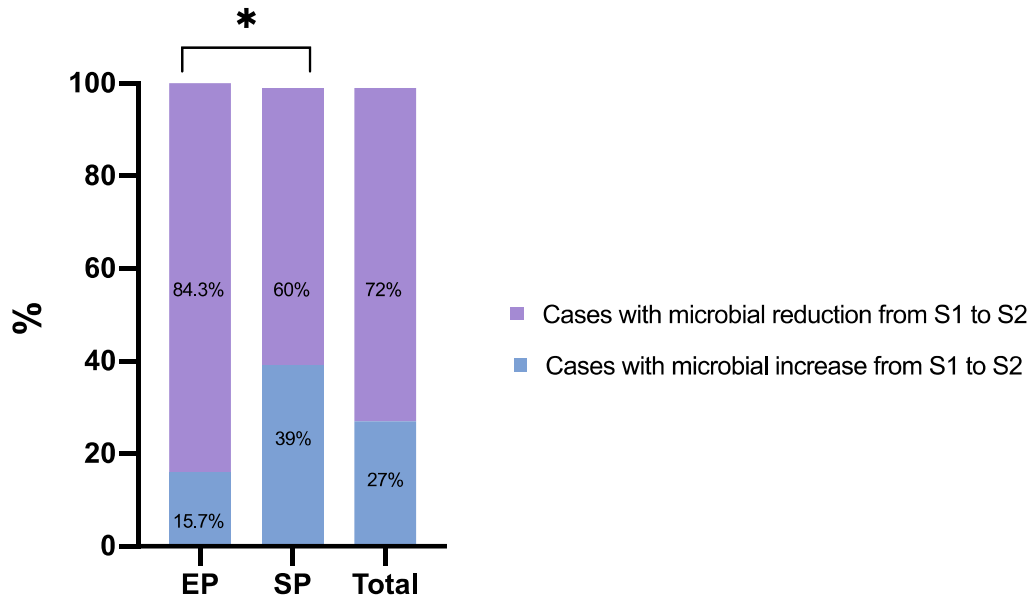


Figure 5.7: Cases with increased/decreased microbial load from S1 to S2 in both treatment groups.
SP (n=71) and EP (n=70) and in total (n=141).
After chemomechanical preparation, cases with reduced bacterial load were significantly more in the EP (84.3%) compared to SP (60%).

Table 5.6: The change in microbial load after chemomechanical preparation in the two treatment groups.
SP: standard protocol, EP: enhanced infection control protocol. The total number of teeth, the number of cases with reduced microbial load (%) and the results of simple binary logistic regression: OR and 95%CI.

	Change of microbial load from S1 to S2				
	Total	Decreased microbial load	OR	95%CI	*p-value*
SP	71	43 (60)	1		
EP	70	59 (84.3)	3.4	1.5 – 7.7	**0.002***

*p<0.05

5.3.4. Association between bacterial numbers prior to obturation and outcomes of root canal treatment

A) Quantitative association

At one-year follow-up, out of the 141 teeth sampled and processed for qPCR analysis, the clinical and CBCT-based outcome data were available for 112 teeth. Cases with favourable outcomes at one-year follow-up were associated with a median number of bacteria at S2 samples of 1.6×10^3 (average of 24.6 Ct values). On the other hand, significantly higher median bacterial number of 5.7×10^3 (average of 22.9 Ct values) in S2 samples of cases with unfavourable outcomes (Mann-Whitney U test, p=0.019) as shown in Table 5.7 and Figure 5.8.

Table 5.7: Quantitative findings of bacterial number in S2 samples in cases with CBCT-based favourable and unfavourable outcomes.

Bacteria number at S2	Favourable outcome	Unfavourable outcome	*p-value*
Number of bacteria			
Mean (SD)	8.6×10^3 ($\pm2.2\times10^4$)	1.3×10^4 ($\pm1.8\times10^4$)	
Median (IQR)	1.6×10^3 (3.6×10^2,7.8×10^3)	5.7×10^3 (1.3×10^3,1.6×10^4)	**0.019***
Ct values			
Mean (SD)	24.6 (±3.3)	22.9 (±2.9)	

*p<0.05

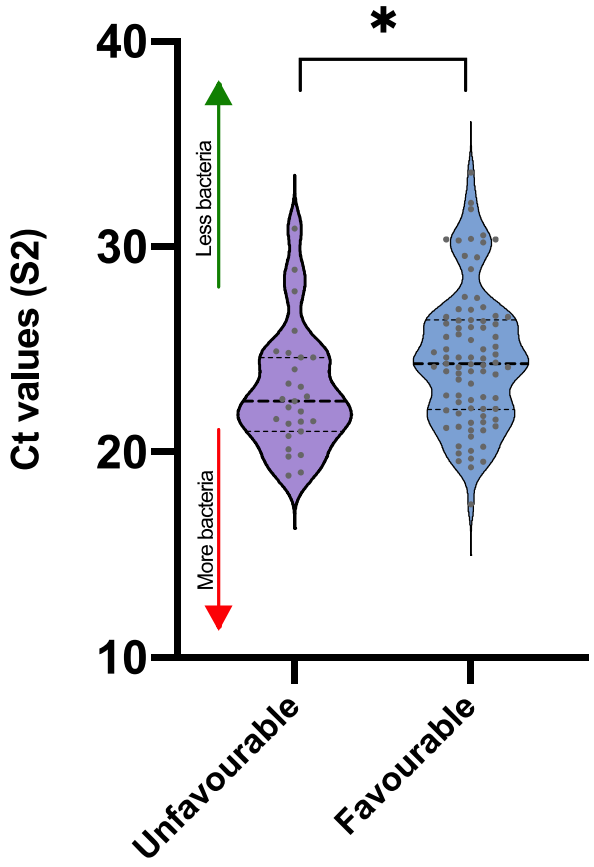


Figure 5.8: Ct values of pre-obturation samples (S2) and outcomes.

qPCR-based amplification (Ct) values of pre-obturation samples (S2) and outcomes, highlighting the correlation between lower bacterial numbers and favourable outcomes. A reduced bacterial count prior to obturation (higher Ct values) was significantly associated with improved odds of favourable outcomes. Treatment was considered favourable when teeth were asymptomatic and there was a reduction in size or no periapical radiolucency present on CBCT scans. Dashed line indicates the mean, while top and bottom dotted lines indicate the 25th and 75th quartiles.

B) *Association between outcomes and absence of genomic DNA amplification prior to obturation*

Prior to obturation, only 19 out of 112 teeth (17%) did not have gDNA amplification (lower than the detection level of 10^2 copy numbers per reaction) when detected with qPCR. All except one of the 19 cases with no bacterial amplification above threshold (94.7%) had a favourable outcome at follow-up. Twenty percent of the cases with favourable outcome did not show detectable gDNA amplification prior to obturation, compared to only 3% of cases with unfavourable outcome (Table 5.8, Figure 5.9). The Fisher exact test showed a significant difference in healing with the presence or absence of gDNA amplification prior to obturation (OR=6.9, p=0.04, CI=0.8-55).

Table 5.8: The effect of pre-obturation microbial status on treatment outcomes in primary root canal treatment of molars.

The number of teeth (%), positive: Microbial load ≥ 10^2 prior to obturation, negative: microbial load below than detection level of 10^2 gene copy number.

Microbial status at S2	Favourable outcome	Unfavourable outcome	Total
Presence of gDNA	67 (72)	26 (28)	93
Absence of gDNA	18 (94.7)	1 (5.2)	19
Total	85	27	112

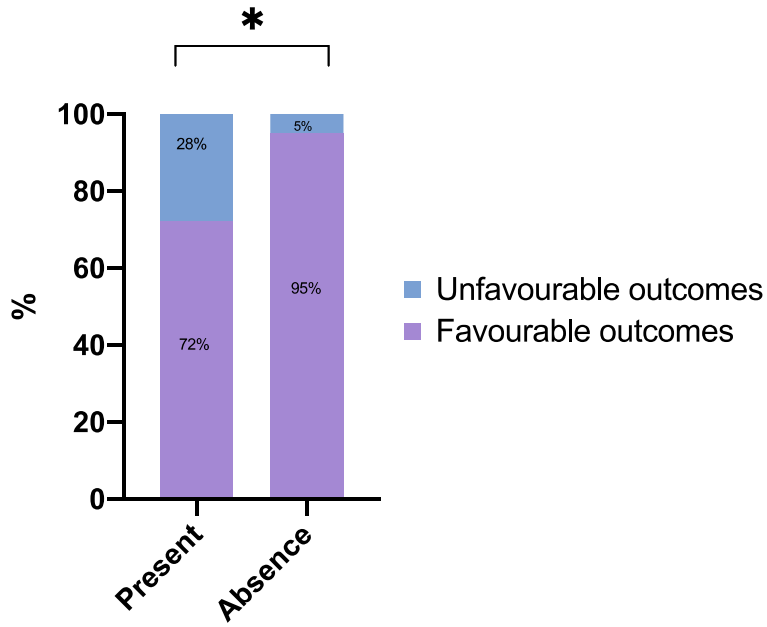


Figure 5.9: Presence and absence of gDNA amplification in cases with different treatment outcomes.

Favourable (n=85) and unfavourable (n=27). Absence of gDNA amplification was considered if below the detection level of 10^2 copy numbers per reaction. Treatment was considered favourable when teeth were asymptomatic and there was a reduction in size or no periapical radiolucency present on CBCT scans.

5.3.5. Differences in initial bacterial load (S1) and pulpal diagnoses / pre-operative periapical status

Regarding pre-operative periapical status, teeth with intact PDL pre-operatively in CBCT sampled a median of 3.1×10^3 bacteria initially, those with PARL pre-operatively had significantly higher microbial load initially with a median of 1.1×10^4 bacteria (Mann-Whitney U test, p=0.001) as shown in Table 5.9, Figure 5.10.

Regarding pulpal diagnosis, teeth presenting with irreversible pulpitis had a median bacterial count of 8×10^3 which was lower than necrotic teeth with a median of 8.4×10^3. The difference in bacterial number between different pulpal diagnosis was not significant (Kruskal Wallis test, p=0.6), as shown in Table 5.9, Figure 5.11.

Table 5.9: Bacterial load in initial samples (S1) in cases with different pulpal diagnoses and in different radiographic periapical conditions.

Median bacterial number and IQR, results of Kruskal Wallis test and Mann-Whitney U test.

	Median (IQR)	*p-value*
Pulp diagnosis		
Irreversible pulpitis	8×10^3 (1.2×10^3, 1.6×10^4)	0.6
Pulpal necrosis	8.4×10^3 (2.1×10^3, 6.7×10^4)	
Previously initiated	8.6×10^3 (1×10^3, 3×10^4)	
Pre-operative periapical radiolucency		
Present	1.1×10^4 (3.2×10^3, 3.5×10^4)	**0.001****
Absent	3.1×10^3 (4.3×10^2, 1.4×10^4)	

**p<0.01

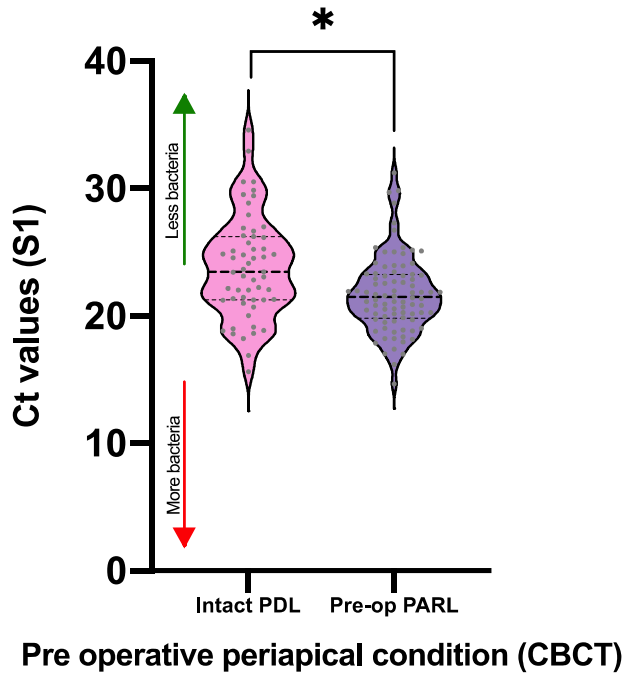


Figure 5.10: Violin plots showing different microbial load initially (S1) among cases with or without periapical radiolucency (PARL) pre-operatively.

Highlighting the significantly increased microbial load (lower Ct values) **i**n cases with pre-op PARL detected with CBCT compared to cases with intact PDL. Dashed line indicates the mean, while top and bottom dotted lines indicate the 25th and 75th quartiles.

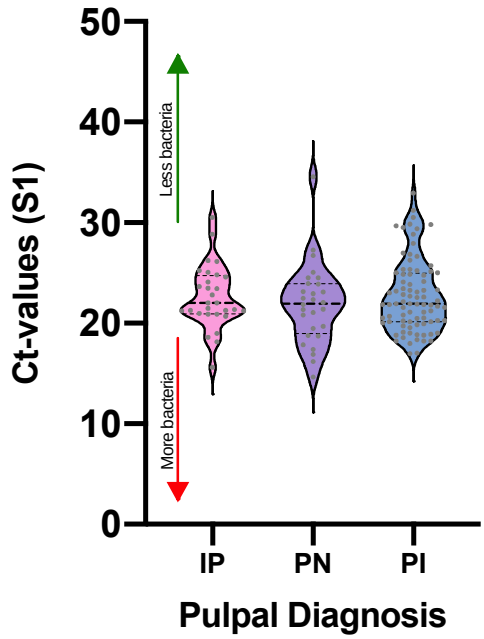


Figure 5.11: Violin plots showing different microbial load initially (S1) among cases with different pulpal diagnoses.

Highlighting the reduced microbial load in irreversible pulpitis (IP) cases (higher Ct values) compared to necrotic (PN) and previously initiated (PI) but significant differences were not noted among groups. Dashed line indicates the mean, while top and bottom dotted lines indicate the 25th and 75th quartiles.

5.4. Discussion

Several studies in the literature reported increased bacterial levels on gloves, rubber dams, and operative surfaces during treatment (Ng *et al.*, 2003, Williams *et al.*, 2003, Niazi *et al.*, 2016, Rorslett Hardersen *et al.*, 2019). This chapter explored the effect of introducing an enhanced infection control protocol on the root canal space microbiological load after chemomechanical preparations. Our null hypothesis was that there is no significant difference in intracanal bacterial load prior to obturation between the enhanced infection control protocol or standard protocol. Since the risk of iatrogenic bacterial introduction was reduced in the EP group, our null hypothesis was rejected, and our findings further validated those of our pilot study.

Effectiveness of enhanced infection control protocol on bacterial reduction

We evaluated the effectiveness of the implemented protocol on microbial load by four different measures: the change of bacterial load from S1 to S2 (δCt), total bacterial number in pre-obturation samples, the number of cases with no gDNA amplification, and the number of cases with increased microbial load after chemomechanical preparation. Besides showing better clinical outcome associated with EP as shown in Chapter 4, using quantitative microbial analysis, all four measures highlighted the significant influence of EP on microbial load during molar root canal treatment and ascertaining that implementing an enhanced infection control protocol have a greater potential of reducing bacteria prior to obturation.

Although both treatment groups showed significant reduction of microbial load after chemomechanical preparation, the δCt which is indicative of the microbial reduction was significantly higher in the enhanced protocol.

Moreover, a similar distribution of cases in regard to initial microbial load before treatment was achieved in the two treatment groups. This was shown by the intergroup comparison in which there were no differences in the bacterial load in S1 samples between the two groups.

This allowed for the comparison of bacterial load in the pre-obturation samples of both groups. The bacterial number prior to obturation was significantly less in the enhanced protocol samples; thus, suggesting the reduced risk of contamination and introducing bacteria prior to obturation in the EP group when compared to SP.

To promote tissue healing after root canal treatment, the etiological factor should be removed. Since it is virtually impossible to completely eradicate bacteria from the root canal space, reducing the bacterial count to a specific threshold is a more realistic goal of endodontic treatment (Siqueira and Rôças, 2008). Besides the quantitative difference in bacterial number in the two groups, we also qualitatively compared the number of cases with no detectable gDNA amplification, which has been shown to be one of the methods to determine such effectiveness (Paiva *et al.*, 2012). The odds of yielding negative gDNA amplification prior to obturation was increased almost fourfold when cases were treated with EP compared to SP.

Finally, there was less chance of iatrogenically introducing bacteria during treatment in EP group cases. This was shown by the threefold increase in the odds of reducing bacteria in the EP compared to SP. The chances of increasing bacteria during treatment were significantly reduced in the EP group. This finding further supports the impact of introducing an enhanced infection control protocol.

It is worth highlighting that in cases previously initiated by a general dentist, the EP increased the odds of having bacteria-free canals prior to obturation up to sixfold (OR=6.7, p=0.018). This finding indicates the validity of implementing this protocol in cases where initial treatment was provided in the context of a general dental practice, in which infection control processes might be less strict than those applied by a specialist in endodontics.

Effect of endodontic treatment on bacterial load

Intra-group comparison between S1 and S2 samples revealed that, regardless of the treatment protocol, there was a substantial bacterial reduction after chemomechanical preparation. This finding is in line with earlier studies highlighting the effectiveness of NaOCl in reducing endodontic microflora in infected root canals (Byström and Sundqvist, 1983, Byström and Sundqvist, 1985, Siqueira *et al.*, 2000a, Vianna *et al.*, 2006b, Sakamoto *et al.*, 2007). However, the degree to which bacterial reduction was attained is lower than that stated in other studies, which were largely conducted on single-rooted teeth (Siqueira *et al.*, 2007a, Rôças *et al.*, 2013). The magnitude of bacterial reduction and effectiveness of sampling from curved and narrower canals might again have had an impact on this. Moreover, the amount of microbial reduction is probably underrepresented in the present study since the sampling surface areas of S1 and S2 are very different. S1 samples were taken from narrow unprepared canals while S2 samples were taken from properly shaped and enlarged canals; thus, the initial microbial load is possibly underrepresented. Moreover, other factors might contribute to such differences and are difficult to control in clinical studies such as canal anatomy and diameter, initial bacterial load, instrumentation and irrigation protocols and sampling protocols, in addition to the DNA extraction methods, qPCR protocols and primers used, as well as positive and negative standards.

The initial microbial load was one of the factors which affected the microbial reduction significantly. Our data showed that there is a greater chance of detecting bacteria in the pre-obturation samples when there is a greater microbial load initially (Appendix 10). Moreover, cases with lower microbial load initially were at a higher risk of introducing bacteria during treatment which indicates possible contamination (Appendix 10).

Association between microbial status prior to obturation and clinical/radiographic outcomes

Regardless of the treatment group, 85.8% of cases had positive bacterial amplification at S2, highlighting the challenges involved in root canal disinfection (Siqueira *et al.*, 2007a, Rôças *et al.*, 2013). Detecting positive bacteria has been shown in the literature. When using culture method, cases with positive cultures were reported in 25% to 85% of the cases after chemomechanical preparation (Byström and Sundqvist, 1983, Byström and Sundqvist, 1985, Sjögren *et al.*, 1997, Peters and Wesselink, 2002, Vianna *et al.*, 2006b, Siqueira *et al.*, 2007d, Nardello *et al.*, 2020b). While molecular methods detected more positive bacteria at time of obturation than culture (Sakamoto *et al.*, 2007, Rôças and Siqueira, 2010, Rôças and Siqueira, 2011b, Rôças and Siqueira, 2011a, Paiva *et al.*, 2012, Paiva *et al.*, 2013), the greater incidence of positive bacteria at S2 is not surprising and probably attributed to the higher sensitivity of such molecular methods, besides its ability in detecting as-yet-uncultivated bacteria. Furthermore, false-positive results from detecting DNA from recently killed bacteria cannot be completely disregarded (Siqueira and Rôças, 2005b).

Absence of gDNA amplification in S2 samples was significantly associated with favourable outcomes compared to cases with positive amplification. The debate about bacterial presence prior to obturation impacting the outcome is well documented (Sathorn *et al.*, 2007), and the literature lacks studies examining this association using highly sensitive molecular methods. Negative cultures prior to obturation were regarded as a prognostic factor predicting a favourable outcome (Sjögren *et al.*, 1997, Waltimo *et al.*, 2005). In Waltimo's study (2005), samples were taken at the beginning of the obturation appointment rather than after completion of chemomechanical preparation and it was concluded that microbiological status at the root canal filling appointment has a remarkable impact on healing (Waltimo *et al.*, 2005). One study measured the risk difference and odds ratio of Sjogren's study and concluded that the

confidence interval reflected a low precision of the study (Sjögren *et al.*, 1997, Sathorn *et al.*, 2007). Two other studies did not find microbial status to be a significant factor in predicting outcome (Peters and Wesselink, 2002, Molander *et al.*, 2007). Because of the host-immune response and the multifactorial nature of endodontic diseases, as well as the internal limitations of paper points sampling, absence at time of obturation cannot solely be a perfect predictor for outcomes (Sathorn *et al.*, 2007). Rather than examining the absence of bacteria as an outcome predictor with such a sensitive molecular method, quantitative data were used to determine such an association. Our findings showed that cases with favourable outcomes at one-year follow-up had significantly lower bacterial number in S2 samples compared to unfavourable outcomes.

Findings of microbial load in initial samples (S1)

The median bacterial count in initial samples was similar to earlier qPCR studies where the initial bacterial load of infected root canals ranged from 3×10^5 to 4.5×10^7 (Sakamoto *et al.*, 2007, Siqueira *et al.*, 2007a, Siqueira *et al.*, 2007d, Blome *et al.*, 2008, Rôças *et al.*, 2013, Neves *et al.*, 2014). An inevitable variability among qPCR studies can be associated with different microbial targets, different qPCR cycling conditions, different diagnosis of included cases, and sampling methods. Moreover, all other studies included single-rooted teeth in which sampling is more controllable (Sakamoto *et al.*, 2007, Siqueira *et al.*, 2007a, Blome *et al.*, 2008, Paiva *et al.*, 2013, Rôças *et al.*, 2013, Neves *et al.*, 2014). Another possible contributor to such variabilities is the inclusion of teeth with different pulpal diagnoses and periapical area statuses. Cases with a clinical diagnosis of pulpal necrosis were 19% of the total, and 60% of teeth had pre-operative PARL. The reason for this inclusion was to detect the effect of an external source of iatrogenic contamination which might be easily detected in canals with fewer bacteria initially. Finally, the number of cases included in the present study is significantly greater (141)

compared to previous qPCR-based studies mentioned (11-50 cases) (Vianna *et al.*, 2006b, Sakamoto *et al.*, 2007, Siqueira *et al.*, 2007a, Blome *et al.*, 2008, Paiva *et al.*, 2013, Rôças *et al.*, 2013, Neves *et al.*, 2014).

Cases with periapical radiolucencies detected in CBCT had significantly greater bacteria number than cases with healthy PDL, in line with previous studies where the bacterial load was shown to be greater in cases when periapical radiolucencies were present, varying from 10^3 to 10^8 (Vianna *et al.*, 2006b, Sakamoto *et al.*, 2007, Siqueira *et al.*, 2007b, Blome *et al.*, 2008, Siqueira and Rôças, 2009b). The detection of radiolucencies in the present study was based on CBCT, which detects smaller radiolucencies than those detected with PA radiographs (Patel *et al.*, 2009, Patel *et al.*, 2012a), thus possibly contributing to the lower bacterial load compared to what has been reported earlier (Vianna *et al.*, 2006b, Sakamoto *et al.*, 2007, Siqueira *et al.*, 2007a, Blome *et al.*, 2008, Paiva *et al.*, 2013, Rôças *et al.*, 2013, Neves *et al.*, 2014).

Teeth presenting with irreversible pulpitis had a median bacterial count of 8×10^3, which agrees with our findings of Chapter 3 indicating bacteria presence in irreversible pulpitis root canal spaces, albeit lower numbers than those typically reported in infected root canals of 10^3-10^8 cells (Siqueira and Rôças, 2009c).

Cases that were previously accessed also harboured bacterial DNA. The median bacterial number was 8.6×10^3. Despite the use of Ledermix® intracanal medicament, this group of teeth harboured slightly greater bacterial numbers than those observed in cases sampled immediately after access (necrotic and irreversible pulpitis). This increased microbial load might be attributed to the amplification of non-viable DNA. Moreover, unlike $Ca(OH)_2$, degrading DNA is probably not achieved with Ledermix® use due to the lower alkalinity measured (pH=8)

compared to $Ca(OH)_2$ (pH=12). Also, incomplete canal instrumentation was evident in all cases previously initiated in our study, resulting in limited coronal placement of the medicament used and eventually not achieving its effectiveness in the root canal system. Furthermore, potential contamination might have taken place between the visits before our initial intracanal sampling.

Limitations of the present study

Finally, a number of limitations of this study were recognised. Only bacteria sampled by paper points were examined. The shortcomings of paper points sampling include inconsistently or under-sampling bacteria, due to bacteria in difficult-to-reach lateral canals, ramifications or deep within the dentinal tubules, the depth of penetration, absorption power and the size of the root canal space. Besides paper points sampling, this study also utilised files for initial intracanal samples. An agreement between files and paper points sampling was shown but a lower bacterial load was noted in files samples (Appendix 11).

The current investigation included teeth with different clinical diagnoses, which might account for the variability of the results in comparison with previous reports. A special concern might arise from including cases that were previously accessed. Lastly, the concern of molecular methods' inability to differentiate between viable and non-viable bacterial cells has been discussed previously (Siqueira, 2008); however, remaining dead cells might trigger host inflammatory reactions, leading to persistence of apical periodontitis. Moreover, one study evaluated the effectiveness of NaOCl detecting nucleic acids, which is commonly used to assess cells viability, and compared it to DNA findings. Only three out of 45 samples showed disagreement between rRNA and DNA bacterial PCR (Rôças and Siqueira, 2010). This figure suggests that the high copy number of 16S rDNA detected with such sensitive methods is unlikely to be originating from dead cells (Zandi *et al.*, 2016). A recent study was conducted to analyse the microbial cells activity in ribosomal RNA and compared it to the rDNA (rRNA

genes) levels after chemomechanical preparations. They found that although rDNA-based qPCR was less sensitive than real-time qPCR (targeting rRNA), the rDNA qPCR assay had high PPV suggesting a low risk of false-positive detection. They also found that all samples that were positive in rDNA-based qPCR assay after chemomechanical preparation were also rRNA-positive for qPCR assays, indicating bacterial activities after chemomechanical instrumentation (Nardello *et al.*, 2020b).

When S2 samples were collected in both groups, sterile gloves and tweezers were used for sampling to avoid false-positive contamination and assuring that bacteria were not introduced at the sampling step. Although all our negative control samples were considered negative, with the exception of one sample, still, the risk of contamination originating from paper points cannot be radically excluded (van der Horst *et al.*, 2013).
Although qPCR assay and sampling have their limitations, still it is considered the best available valid method to evaluate such an effect (Siqueira and Rôças, 2005b).

This study highlighted the possible impact of implementing an enhanced infection control protocol. Still, many other reasons besides iatrogenic contaminations might explain bacteria persistence after chemomechanical preparation. These reasons possibly include: (i) the presence of bacteria in inaccessible areas to irrigation or their intrinsic resistance to irrigant; (ii) the presence of bacteria in biofilms shielded them from the effect of antimicrobial agents; (iii) the presence of dentine, necrotic tissue, bacterial by-products that inactivate or reduce the efficiency of irrigant.

5.5. Key findings and conclusion

This part of our clinical study showed that both treatment protocols were effective in reducing the microbial load after chemomechanical preparation of molars. Moreover, one-year favourable outcomes were associated with significantly fewer bacteria in the pre-obturation samples. Implementing an enhanced infection control protocol was suggestive of minimising iatrogenic contamination. This was shown by: (i) a significantly greater bacterial reduction in the EP group, (ii) a threefold increase in the odds of having bacteria-free canals prior to obturation in the EP group, (iii) a threefold reduction of the odds of introducing bacteria during treatment in the EP group.

Chapter Six: Microbial Profile Changes after Endodontic Treatment Procedures Based on Treatment Protocols: An *In Vivo* NGS Molecular Study

6.1. Introduction

The preceding chapters demonstrated the clinical benefit of the enhanced infection control protocol, with improved clinical outcomes in this group, as well as the effectiveness of this protocol microbiologically. Although complete disinfection of root canals was not achieved in any group, the implemented protocol significantly reduced bacterial number in pre-obturation samples and increased the odds of having pre-obturation canals free from bacteria. Furthermore, the EP investigated reduced the odds of introducing bacteria to the root canal system during instrumentation.

In this chapter we sought to explore the microbial community shifts during endodontic treatment and the resident microbiome of the subgroups (enhanced and standard protocol groups), as well as the potential contribution of external contaminants.

Origins of contaminants

Previous works have demonstrated the presence of bacteria typically associated with whole saliva or skin to be present in gloves, rubber dam and operatory surfaces (Williams *et al.*, 2006, Niazi *et al.*, 2016, Saeed *et al.*, 2017, Rorslett Hardersen *et al.*, 2019), rather than the intrinsic endodontic microbiome. In Chapter 2, our trial study revealed that surfaces of the rubber dam, gloves, rulers and instruments carried bacteria such as members of *Streptococci, Rothia, Granulicatella, Cutibacterium* (formerly *Propionibacterium*), *Lactobacilli, Sphingomonas, Veillonella,* and *Fusobacterium,* suggesting contamination from both oral and non-oral sources. We aimed to examine whether the enhanced infection control protocol would reduce the risk of such iatrogenic bacterial contamination to the root canal space during treatment. The source of contaminating microorganisms is anticipated to arise from either patient's saliva, skin microbiota, gloves, operatory surfaces, or endodontic microorganisms reintroduced to the root canal space during treatment. Examples of common salivary contaminants are members of the

genera *Actinomyces (A. oris, A. odontolyticus); Aggregatibacter (A. segnis, A. aphrophilus); Fusobacterium (F. nucleatum, F. periodonticum); Gemella haemolysans; Haemophilus (H. influenzae, H. parainfluenzae); Oribacterium (O. parvum, O. sinus); Peptostreptococcus stomatis; Porphyromonas (P. endodontalis, P. pasteri); Prevotella (P. histicola, P. melaninogenica, P. nigrescens, P. oris); Propionibacterium acnes; Rothia (R.aeria, R. mucilaginousa, R. dentocariosa); Streptococcus (S. pneumoniae, S. salivarius, S. sanguinis);* and *Veillonella (V. dispar, V. parvula, V. rogosae)* (Lim *et al.*, 2017). While taxa of skin contaminants include *Propionibacterium acnes; Corynebacterium (C. tuberculostearicum, C. simulans, C. capitis, C. amycolatum); Streptococcus (S. mitis, S. oralis, S. pseudopneumoniae, S. epidermidis, S. capitis, S. warneri); Micrococcus luteus,* and *Veillonella parvula* (Byrd *et al.*, 2018).

Databases identifying the microbial composition

The high sensitivity of NGS in bacterial detection, including bacteria thus far uncharacterised, has found widespread use in describing microbiomes in a wide range of applications. High-throughput DNA sequencing technologies known as next-generation sequencing (NGS) have been developed and used to investigate the human microbiome, including the oral cavity, thoroughly, thereby assisting in delineating typical microbial residents within the endodontic niche and their potential role in pathology. Hence, detecting of lower abundance bacterial reads in endodontic infections is crucial. NGS has comprehensively explored the endodontic microbial communities and revealed significantly higher microbial diversity in the root canal system than other molecular methods (Manoil *et al.*, 2020).

Molecular analysis pipeline clusters the bacterial variable regions based on specific sequence into an operational taxonomic unit (OTU) (Schmidt *et al.*, 2014) and these OTUs could be assigned to specific taxa when compared to 16S rRNA gene databases. In this clinical study, the identification of microbial taxa was initially achieved by comparing each OTU sequence against the Human Oral Microbiome Database (HOMD) (Chen *et al.*, 2010) (http://www.homd.org/) version 15.1 (release date January 2018). The HOMD database comprises a total of 998 full length 16S rDNA sequences and 769 representatives of the oral taxa and is frequently updated. In this database, each OTU is assigned to a specific Taxon HOMD ID, which is a unique ID known as Human Microbial Taxa (HMT) and all OTUs are classified to species level. If a genus name has not been formally defined, the taxon is given the upper-level taxonomic name "family name" with a numeric designation (e.g., G-1). Whereas if a species is not yet defined, the taxon will be identified as "sp. oral taxon XXX" where XXX is the specific HOMD ID.

Given the specific adaptation of bacteria to the oral environment, the HOMD database was used to identify OTU in the first instance, with manual searches or use of alternative databases for common, non-oral reads. Since we are investigating endodontic infections and the source of iatrogenic contamination arises from either skin, saliva or endodontic infection, the use of HOMD database was assumed to be more relevant and easier to analyse than other databases.

Measures of microbial communities present within endodontic cases

As stated previously, with the advent of next-generation sequencing, the identification of most bacteria present within endodontic cases is now feasible. In common with most oral, and indeed human microbiome studies, large subject-to-subject variability has been observed (Flores *et al.*, 2014), as well as the many confounders that have resulted in a given endodontic

microbiome. The present use of a longitudinal study design is important to better understand the contribution of endodontic bacteria, therapeutic intervention and potential iatrogenic contamination.

In addition to describing the identity of bacteria present, describing the community makeup or structure, such as frequent predominance of a single taxon or small numbers of taxa within an ecological niche, is essential. A number of measures have been proposed and implemented within microbial ecology (Willis, 2019).

The structure of the microbial community is measured with alpha diversity, which is commonly the first approach to assess differences between microbial communities. Alpha diversity takes into account the microbial richness and evenness. Richness is the number of different species present within the community, while evenness is the uniformity of population size of each species.

Operational taxonomic units (OTUs) richness, ACE (Abundance-based Coverage Estimator) and Chao1 are used to estimate the total richness of the community (number of observed OTUs in all given samples). OTU richness is the count of different species/OTUs. Both Chao1 and ACE are non-parametric measures using the abundance or incidence of rare species in the samples to estimate the total number of species, using a previously formulated non-parametric model (calculate expected OTUs based on observed OTUs) (Kim *et al.*, 2017). Chao1 richness estimator gives more weight to the low abundance species. On the other hand, ACE considers both rare and abundant species (those represented by more than 10 individuals by default) (Kim *et al.*, 2017).

For microbial diversity, different bioinformatic tools are used to compare species diversity in a community. Shannon-Weaver and Simpson are among the methodologies currently used

(Kim *et al.*, 2017). Both measures give deeper insight into community composition by taking into account richness and evenness. The Shannon index places a greater weight on species richness, whereas the Simpson index considers species evenness more in its measurement (Kim *et al.*, 2017).

Contamination during endodontic sampling and processing

As this study is looking into the potential risk of iatrogenic contamination, it was imperative to identify and appropriately address contaminants intrinsically present in the ultralow DNA input approach used in this study. One of the main concerns with sequencing analysis is the microbial contamination during different experimental steps, including NGS "procedural contamination". Besides contamination arising from laboratory solutions used for DNA extraction procedure and/or PCR steps, studies have shown contamination to arise at any stage during sequencing from environmental surfaces and reagents (Strong *et al.*, 2014). When bacterial-free identical cell lines were processed separately in the same lab, or in different labs, different microbial contaminations were notable (Strong *et al.*, 2014). Different taxa were reported associating with contamination during the sequencing process. Examples are *Bradyrhizobium* in ultra-pure water, *Leucobacter* sp., *Acinetobacter* sp., *Pseudomonas* sp., and *P. denitrificans* (Percudani, 2013, Laurence *et al.*, 2014, Strong *et al.*, 2014). Suggestions to overcome contamination issues in open-ended sequencing methods and to minimise data misinterpretations were given: (i) To include negative contamination control/self-control samples as a mock sequence library preparation to detect contamination. (ii) To minimise signal to noise ratio in cases with lower DNA in clinical samples. When insufficient DNA is present in clinical samples, it has been shown that there will be an inevitable overamplification of environmental contaminants in a concentration-dependent manner (van der Horst *et al.*, 2013, Strong *et al.*, 2014). (iii) To remove known common contaminants during downstream

analysis, which might result in misrepresenting bacteria also present in the root canal space (Strong *et al.*, 2014, de Goffau *et al.*, 2018). (iv) The use of highly purified metabolic enzymes and other reagents during sequence library preparation (Strong *et al.*, 2014).

In addition to concerns regarding the introduction of microbial contaminants from other oral sites into the pulp chamber, contaminants from otherwise sterile devices were noted. One such concern is contamination arising from paper points used to collect microbes from the root canal space. Paper points could be considered as a substantial source of "foreign" bacterial DNA (van der Horst *et al.*, 2013). One study evaluated the sequencing profiles of sterile paper points and found *Enterococcus* (25% of reads) and *Exiguobacterium* (21%) predominating (van der Horst *et al.*, 2013), which have also been described as true endodontic microbial contaminants (Siqueira and Rôças, 2005a). Other taxa, *Escherichia-Shigella*, *Anaerosporobacter*, and *Methylobacterium*, were also detected. Their recommendation was given to avoid the use of paper points when open-ended techniques such as sequencing or DGGE were used and replacing it with sterile curettes for peri implants sampling (van der Horst *et al.*, 2013).

Adaptations for low copy number amplification and pitfalls from the pilot study

As the quantities of bacterial DNA isolated from root canal spaces were below standard input amounts for NGS, and due to the risk of substantial contamination from the many steps required to sequence the libraries, multiple displacement amplification (MDA) was used. This method has been reported to have the lowest amplification bias (Hosono *et al.*, 2003). Our pilot study generated a proof of concept using this adjunctive molecular technique for endodontic samples, with very low DNA quantities obtainable within the *pico to femtogram*-range. However, this technique did mandate controlling for contaminants present before or during this further

process. This was addressed by including samples processed through both methods (with and without MDA).

Moreover, the useful insight from the pilot study allowed us to adjust the target region within the 16S ribosomal RNA gene. Since high-quality merging of forward and reverse sequences spanning was not achieved when targeting the V3-V5 region in the pilot study, in this clinical study, we targeted the V3-V4 hypervariable region of the bacterial 16S rRNA to determine the microbial composition.

The first section of this chapter will describe the overall microbial changes in endodontic root canal after chemomechanical preparation. Next, we will describe the main microbial change in pre-obturation samples using a protocol which will further validate the enhanced protocol used. We wanted to investigate whether residual bacteria in pre-obturation samples were arising from incomplete disinfection or from iatrogenic recontamination. The null hypothesis was that the enhanced protocol would not reduce the iatrogenic contamination of the root canal system.

6.2. Materials and method

6.2.1. V3/V4 16S rDNA gene NGS sequencing

This study was conducted to further characterise the samples obtained as part of the randomised clinical trial previously described in Chapter 4 and to correlate to bacterial rDNA copy numbers in Chapter 5. Clinical interventions, symptoms distribution and one-year outcomes were described in detail in Chapter 4. Intracanal sampling and DNA extraction was carried out as described previously from Section 2.3.2 to 2.3.4. Briefly, intracanal samples were taken under aseptic conditions, and bacterial genomic DNA was extracted from clinical samples using the GenElute Bacterial Genomic DNA Kit. Multiple displacement amplification and clean-up of the DNA was carried out using the REPLI-g MDA synthesis kit (Qiagen) and positive DNA amplification by MDA was confirmed by 1% horizontal agarose gel electrophoresis. Where positive amplification was observed, DNA extracts of 179 samples were selected. These samples included 156 intracanal samples from both treatment groups and 23 control samples. Three different types of control samples were included in this part of the study. Contamination control (CC) from the target tooth surface after decontamination (n=5), negative control (NC) samples of sterile paper points and files (n=13), and negative extraction control (NEC) samples from ultra-pure water extraction (n=5). Moreover, matching non-MDA amplified control samples were included for four clinical samples. These intracanal samples were subdivided into identical samples after DNA extraction. MDA was performed on half of the samples while the other half did not have MDA performed. All control samples were processed in the same way as the test samples. Samples were subjected to 2 x 300 bp paired-end, high-throughput sequencing of the V3-V4 hypervariable region of the bacterial 16S rRNA gene by the Illumina MiSeq platform and the v3 chemistry 2x300 bp paired-end reads (Integrated Microbiome Resource, Halifax, Canada) as described previously in Section 2.3.6. Details about the analysed samples are shown in Figure 6.1.

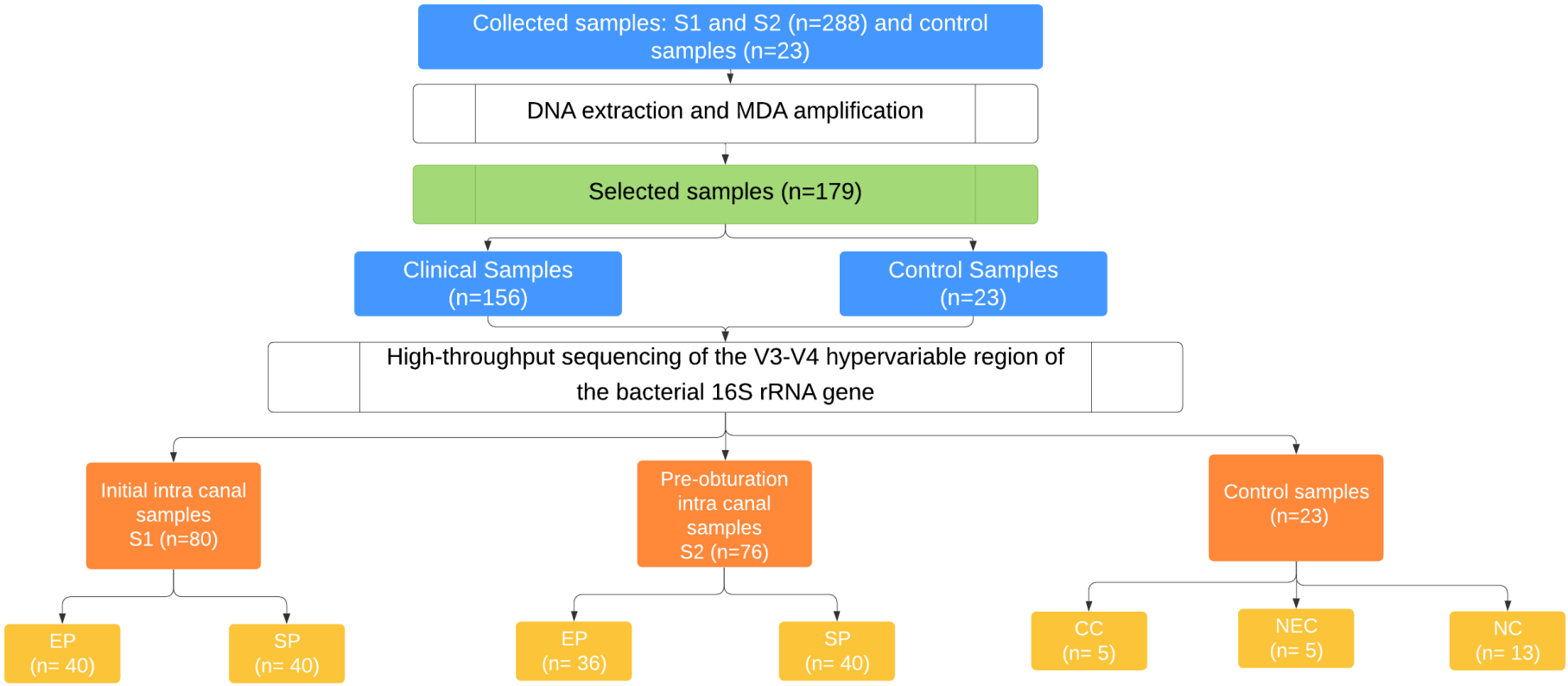


Figure 6.1: Flow diagram of included samples subjected to NGS.

Initial (S1) and pre-obturation samples (S2) collected from intracanal space of molars undergoing primary root canal treatment (n=288) were subjected to DNA extraction. MDA was confirmed by a positive signal on agarose gel electrophoresis, leaving 156 clinical samples. Different control samples were included (n=23): Contamination control (CC), negative extraction control (NEC) and negative control (NC) of sterile paper points and files. All NTC, CC, and files had very low read counts (<200 with an average of 27 reads per sample). Paper points yielded higher reads (164 – 11,388).

6.2.2. Bioinformatic analysis, data processing and statistical analysis

The 16S amplicon processing and bioinformatics analysis was carried out as described in Section 2.3.7 with minor adjustments. DNA sequences quality scores "Phred quality" was set at 16 to allow proper merging of forward and reverse sequences and thus enabled deeper taxonomic classification. All 16S-rDNA sequences were analysed using the Human Oral Microbiome Database (HOMD) (Chen *et al.*, 2010). After low quality reads removal, alpha diversity estimates were calculated on rarefied OTU tables where rarefaction depth was set to 500.

Wilcoxon signed-rank test was used to compare the richness and diversity of bacteria in S1 and S2 samples and in S2 samples of the two protocols. The Mann-Whitney U test and Benjamini-Hochberg corrections and/or Fisher exact test were performed to evaluate whether there were significant differences between any bacterial taxa in the pre-obturation samples of the two treatment groups as well as in S1 and S2. Significance level set in the analysis was 5% (α=0.05).

6.3. Results

A total number of 4,075,560 16S rRNA gene sequence reads were obtained from 179 samples subjected to paired-end sequencing on Illumina MiSeq. After quality filtering, and to correct for unequal sequencing depth, data was normalised by subsampling the 16S rDNA data at 500 reads per sample. 93 samples with 4,064,880 sequence reads remained. After the quality control, a total of 514 distinct OTUs were observed across samples.

6.3.1. Analysis of extraction, amplification, contamination and MDA control samples

All control samples (contamination control, negative control and negative extraction control) yielded weak amplification with the exception of one CC and one paper point of a NC. NTC samples had an average read number of nine reads (one - eight reads per sample); an average of two reads per sample was noted in the CC samples. Whereas sterile file samples had an average read number of 30 (5-74) with the exception of one sample which had higher read numbers of 2260. Paper point samples yielded the highest read numbers among the control samples. One sample had 11,388 reads while remaining samples had an average of 328 reads per sample.

The most abundant taxa in the CC samples were: *Enterococcus italicus*, *Lachnospiraceae bacterium HMT 86*, *Peptostreptococcus stomatis*, *Enterococcus casseliflavus*, *Dialister pneumosintes*, and different species of *Streptococci* as shown in Appendix 12.

Regarding NEC samples, the most abundant taxa in paper points were: *Enterococcus casseliflavus*, *Paracoccus yeei*, *Pseudomonas fluorescens*, *Lactobacillus paracasei*, *Filifactor alocis*, and *Enterococcus italicus*. Sterile files were predominated with *Moraxella osloensis*, *Pseudomonas fluorescens*, *Streptococcus oralis*, *Atopobium rimae*, *Cutibacterium acnes*, and *Lawsonella clevelandensis* (Appendix 12).

The NEC samples were predominantly abundant with *Pseudomonas fluorescens*, *Haemophilus parainfluenzae*, *Peptostreptococcus stomatis*, *Moraxella osloensis*, and *Fusobacterium nucleatum* (Appendix 12).

Additionally, non-MDA samples corresponding to samples subjected to MDA were included to assess if any bias had been introduced in this amplification step.

The Shannon diversity, richness estimates were calculated and compared for each corresponding sample as shown in Table 6.1. Figure 6.2 presents the most abundant genera in the two pairs of samples. Comparison of the microbial profiles showed that the abundance and proportion of microbiome had not changed dramatically.

Table 6.1: Diversity and richness calculations in MDA and non-MDA corresponding samples.
Shows a comparable finding between the matching samples and thus confirms the reliability of introducing such a method with endodontic samples.

Sample ID	MDA	Observed	Chao1	ACE	Shannon	Simpson
090.IC1	Yes	110	121.33	121.96	2.61	0.89
090.IC1.2	No	114	129.48	137.68	2.76	0.90
092.IC1	Yes	39	44.14	48.98	0.43	0.14
092.IC1.2	No	26	35.00	38.89	0.72	0.23
104.IC1	Yes	30	51.86	51.74	2.35	0.75
104.IC1.2	No	20	53.00	52.00	0.58	0.20
107.IC1	Yes	103	142.55	134.66	1.96	0.70
107.IC1.2	No	112	131.09	132.50	2.21	0.81

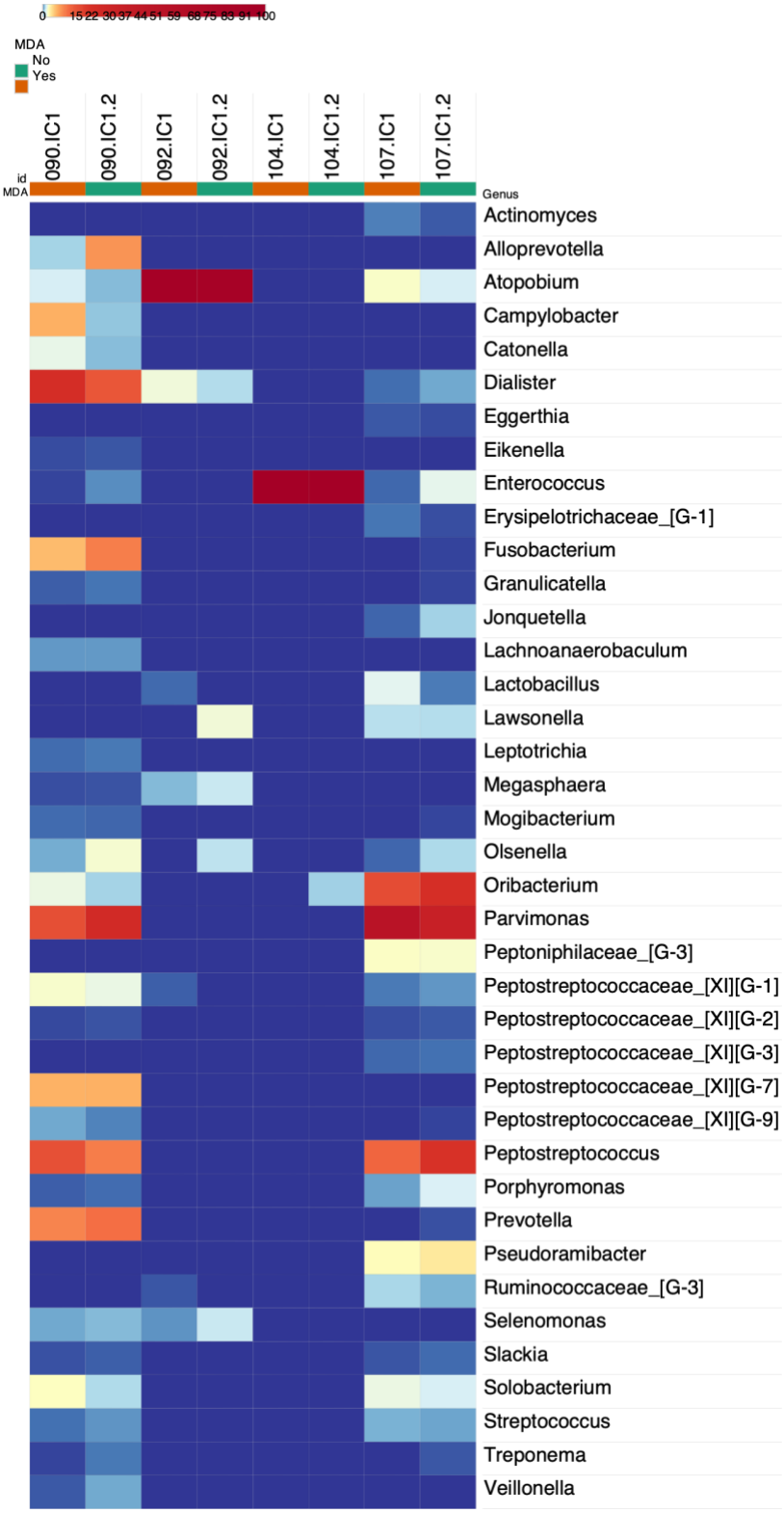


Figure 6.2: Heat map of clinical samples with MDA and matching control samples not subjected to MDA.
Samples not subjected to MDA amplification are denoted X.2 "green bars". A comparable microbial profile is shown between each two corresponding samples.

6.3.2. Initial intracanal samples (S1)

Initial intracanal samples (n=80) were represented by ten known bacterial phyla and 147 genera. The phyla were: Firmicutes, Bacteroidetes Actinobacteria, Fusobacteria Proteobacteria, Synergistetes, Spirochaetes, Saccharibacteria (TM7), and Chloroflexi. Regardless of the pulpal diagnosis or periapical condition, the most abundant phyla were: Firmicutes (relative abundance 70%), Bacteroidetes (11%), Actinobacteria (9%), Proteobacteria (4%), and Fusobacteria (4%).

At the genus level, *Streptococcus* (relative abundance of 19%, present in 98% of samples), *Peptostreptococcus* (8% relative abundance and 72% of cases), *Parvimonas* (5.5% relative abundance and 75% of cases) and *Enterococcus* (4.6% relative abundance and 75% of cases) were the most abundant in primary infected root canal spaces samples (Table 6.2). The most prevalent species in initial samples were: *Peptostreptococcus stomatis* (relative abundance of 7.2%), *Streptococcus oralis* (4.9%), *Rothia dentocariosa* (3.5%), *Bacteroidaceae [G-1] bacterium HMT 272* (3.2%), *Streptococcus anginosus* (3%), and *Streptococcus vestibularis* (3%) as shown in Appendix 13.

Initial intracanal samples (S1) microbial composition and diversity measures did not differ between the two treatment groups as shown in Appendix 13.

Table 6.2: All phyla and most abundant genera (>0.01) after read normalisation in initial intracanal samples (S1) of all samples (n=76).

Phyla	**Relative abundance (%)**	**Frequency in samples n (%)**	**Genera**	**Relative abundance (%)**	**Frequency in samples n (%)**
Firmicutes	70.2	76 (100)	*Streptococcus*	19.1	75 (98.6)
Bacteroidetes	11.5	73 (96)	*Peptostreptococcus*	8.19	55 (72.3)
Actinobacteria	8.9	74 (98.6)	*Parvimonas*	5.5	57 (75)
Fusobacteria	4.2	13 (17.1)	*Enterococcus*	4.59	57 (75)
Proteobacteria	4.08	61 (91)	*Dialister*	4.41	51 (67)
Synergistetes	0.74	32 (42.1)	*Oribacterium*	4.12	52 (68.4)
Spirochaetes	0.13	27 (35.5)	*Peptostreptococcaceae*	4.1	58 (76.3)
Saccharibacteria(TM7)	0.08	22 (28.95)	*Bacteroidaceae*	4.05	43 (56.5)
Chloroflexi	0.001	2 (2.6)	*Prevotella*	4.03	70 (92)
			Fusobacterium	4.03	56 (73.6)
			Actinomyces	3.6	55 (72.3)
			Atopobium	3.5	62 (81.5)
			Shuttleworthia	2.25	36 (47.4)
			Mycoplasma	2.07	20 (26.3)
			Lachnospiraceae	2.01	5 (6.5)
			Pseudoramibacter	1.75	28 (36.8)
			Filifactor	1.51	25 (32.9)
			Catonella	1.32	16 (21)
			Lactobacillus	1.32	34 (44.7)
			Peptoniphilus	1.23	34 (44.7)
			Corynebacterium	1.11	18 (23.6)
			Enterobacter	1.06	9 (11.8)
			Porphyromonas	0.93	32 (42)
			Veillonella	0.92	60 (78.9)

6.3.3. Overall transition of microbial composition after chemomechanical preparation

Regardless of the treatment group, when compared to initial samples, lower richness and diversity metrics were observed after debridement in the pre-obturation (S2) intracanal samples. The mean number of OTUs in the S2 samples were significantly lower than S1 samples (Wilcoxon signed-rank test, p=0.0013). An average of 62 OTUs per sample observed in initial samples reduced to an average of 32 OTUs in pre-obturation samples. Table 6.3 and Figure 6.3 illustrate the number of OTUs as well as richness and diversity measures.

In response to chemomechanical preparations, most taxa decreased significantly after chemomechanical preparation such as *Streptococcus*, *Peptostreptococcus*, *Parvimonas*, *Fusobacterium*, and *Atopobium*. Some other taxa increased significantly in abundance, such as *Actinomyces*, *Enterococcus*, *Corynebacterium*, *Prevotella*, and *Porphyromonas*. Details about different endodontic taxa significantly changed in their abundance and frequency after chemomechanical preparation are presented in Table 6.4. A summarised heat map of the genera before and after chemomechanical preparation is shown in Figure 6.4, while a detailed heat map of all taxa is shown in Appendix 14.

Table 6.3: Richness and diversity indexes of bacterial community in S1 and S2 samples. Results of Wilcoxon signed-rank test. Values of the mean and range.

Indicator	S1 (n=49)		S2 (n=23)		*p-value*
Number of OTUs per canal	62.84	(16-127)	32.35	(7-115)	<0.001***
Chao1 estimator of richness	79.80	(19.75-166.6)	56.78	(13-187.1)	0.006**
ACE estimator of richness	81.82	(20.31-153.7)	59.47	(14.35-195.2)	0.005**
Shannon index of diversity	1.57	(0.004-2.99)	1.18	(0.012-3.15)	0.05*
Simpson index of diversity	0.60	(0.0008-0.91)	0.44	(0.002-0.93)	0.02*

*p<0.05; **p<0.01; ***p<0.001

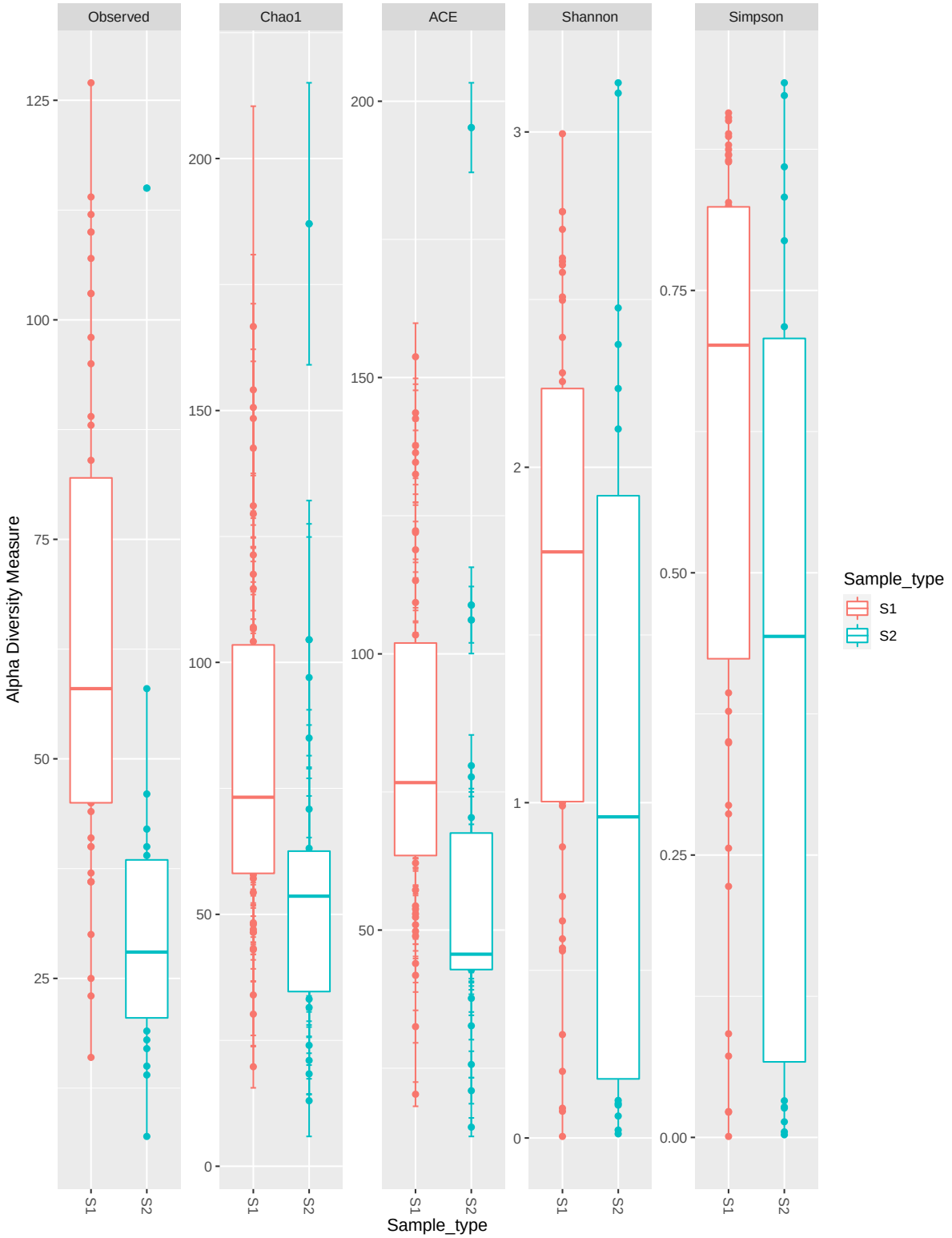


Figure 6.3: Richness and diversity indices of the bacterial community in primary infected root canal system (S1).

A significant reduction is noted in all indexes after chemomechanical preparations (S2). Species richness measures: observed species richness, Chao1, and ACE. Diversity measured with Shannon and Simpson diversity indexes. Outliers were noted within the S2 samples, possibly attributed to poorer quality reads intrinsic to the group.

Table 6.4: Overall list of taxa presented significantly *reduced* in the pre-obturation samples after chemomechanical preparations regardless of the treatment protocol.

Results of Mann-Whitney U test and Benjamini-Hochberg corrections or Fisher exact test presenting the relative frequency in the initial (S1) and pre-obturation (S2) samples. After quality filtering, and read normalisation, 80 samples remained in the S1 group and 75 in the S2. Showing only taxa present in at least two samples.

Genera	Species	Initial samples (S1)		Pre-obturation samples (S2)		Change in abundance	*p-value*
		Relative abundance (%)	Frequency in samples n (%)	Relative abundance (%)	Frequency in samples n (%)		
Actinomyces	*A. sp. HMT 172*	0.158	20 (25)	0.270	12 (16)	Increased	0.0000
Actinomyces	*A. naeslundii*	0.001	8 (10)	0.000	0 (0)	Decreased	0.0367
Actinomyces	*A. sp. HMT 180*	0.048	5 (6.25)	0.014	5 (6.7)	Decreased	0.0006
Bifidobacterium	*B. dentium*	0.028	10 (12.5)	0.000	0 (0)	Decreased	0.0085
Bifidobacterium	*B. animalis*	0.056	4 (5)	0.000	0 (0)	Decreased	0.0089
Campylobacter	*C. gracilis*	0.446	13 (16.25)	0.000	0 (0)	Decreased	0.0080
Corynebacterium	*C. durum*	0.003	3 (3.75)	0.088	1 (1.3)	Increased	0.0000
Cryptobacterium	*C. curtum*	0.004	5 (6.25)	0.000	0 (0)	Decreased	0.0150
Cutibacterium	*C. acnes*	0.283	11 (13.75)	0.060	4 (5.3)	Decreased	0.0486
Eikenella	*E. corrodens*	0.003	3 (3.75)	0.000	0 (0)	Decreased	0.0426
Enterococcus	*E. saccharolyticus*	0.102	13 (16.25)	0.182	14 (18.7)	Increased	0.0000
Enterococcus	*E. durans*	0.205	10 (12.5)	0.088	12 (16)	Decreased	0.0000
Fusobacterium	*F. sp. HMT 203*	0.010	3 (3.75)	0.000	0 (0)	Decreased	0.0078
Fusobacterium	*F. naviforme*	0.014	13 (16.25)	0.000	0 (0)	Decreased	0.0075
Fusobacterium	*F. hwasookii*	0.015	7 (8.75)	0.000	0 (0)	Decreased	0.0098
Gemella	*G. haemolysans*	0.023	11 (13.75)	0.105	14 (18.7)	Increased	0.0000
Gemella	*G. sanguinis*	0.008	6 (7.5)	0.158	8 (10.7)	Increased	0.0001
Granulicatella	*G. elegans*	0.007	2 (2.5)	0.075	7 (9.3)	Increased	0.0000
Haematobacter	*H. missouriensis*	0.026	4 (5)	0.078	5 (6.7)	Increased	0.0000
Lactobacillus	*L. vaginalis*	0.013	4 (5)	0.000	0 (0)	Decreased	0.0092
Lactobacillus	*L. kisonensis*	0.031	4 (5)	0.000	0 (0)	Decreased	0.0073

Lactobacillus	*L. fermentum*	0.000	2 (2.5)	0.018	6 (8)	Increased	0.0000
Lactobacillus	*L. gasseri*	0.784	15 (18.75)	0.010	3 (4)	Decreased	0.0328
Lactobacillus	*L. rhamnosus*	0.001	4 (5)	0.000	0 (0)	Decreased	0.0103
Lactobacillus	*L. crispatus*	0.017	2 (2.5)	0.140	3 (4)	Increased	0.0000
Lactobacillus	*L. reuteri clade 938*	0.061	5 (6.25)	0.000	0 (0)	Decreased	0.0380
Microbacterium	*M. flavescens*	0.008	6 (7.5)	0.038	7 (9.3)	Increased	0.0000
Micrococcus	*M. luteus*	0.174	7 (8.75)	0.099	8 (10.7)	Decreased	0.0000
Neisseria	*N. perflava*	0.033	8 (10)	0.035	9 (12)	Increased	0.0000
Neisseria	*N. subflava*	0.002	3 (3.75)	0.000	0 (0)	Decreased	0.0292
Olsenella	*O. sp. HMT 807*	0.001	3 (3.75)	0.009	1 (1.3)	Increased	0.0371
Parvimonas	*P. sp. HMT 110*	1.995	43 (53.75)	0.267	16 (21.3)	Decreased	0.0376
Parvimonas	*P. sp. HMT 110*	0.005	6 (7.5)	0.000	0 (0)	Decreased	0.0244
Parvimonas	*P. micra*	1.970	47 (58.75)	0.437	20 (26.7)	Decreased	0.0194
Peptoniphilus	*P. sp. HMT 187*	0.496	8 (10)	0.000	0 (0)	Decreased	0.0094
Peptostreptococcaceae	*P. bacterium HMT 495*	0.022	12 (15)	0.000	0 (0)	Decreased	0.0081
Peptostreptococcaceae	*P. saphenum*	0.045	5 (6.25)	0.000	0 (0)	Decreased	0.0196
Peptostreptococcaceae	*P. bacterium HMT 81*	0.009	3 (3.75)	0.000	0 (0)	Decreased	0.0083
Peptostreptococcus	*P. stomatis*	6.828	57 (71.25)	1.791	35 (46.7)	Decreased	0.0138
Porphyromonas	*P. pasteri*	0.002	3 (3.75)	0.018	4 (5.3)	Increased	0.0000
Porphyromonas	*P. pasteri*	0.001	4 (5)	0.041	4 (5.3)	Increased	0.0000
Porphyromonas	*P. uenonis*	0.014	3 (3.75)	0.000	0 (0)	Decreased	0.0084
Porphyromonas	*P. uenonis*	0.002	4 (5)	0.000	0 (0)	Decreased	0.0072
Prevotella	*P. denticola*	1.219	25 (31.25)	0.063	5 (6.7)	Decreased	0.0372
Prevotella	*P. sp. HMT 306*	0.083	14 (17.5)	0.057	7 (9.3)	Decreased	0.0028
Prevotella	*P. sp. HMT 313*	0.000	1 (1.25)	0.025	3 (4)	Increased	0.0303
Prevotella	*P. dentalis*	0.058	6 (7.5)	0.000	0 (0)	Decreased	0.0088
Prevotella	*P. pallens*	0.034	11 (13.75)	0.021	6 (8)	Decreased	0.0031

Ruminococcaceae	*R. bacterium HMT 85*	0.000	2 (2.5)	0.010	3 (4)	Increased	0.0306
Selenomonas	*S. sp. HMT 136*	0.030	5 (6.25)	0.014	4 (5.3)	Decreased	0.0274
Selenomonas	*S. sputigena*	0.121	22 (27.5)	0.004	3 (4)	Decreased	0.0087
Solobacterium	*S. moorei*	0.523	38 (47.5)	0.128	15 (20)	Decreased	0.0136
Stomatobaculum	*S. sp. HMT 910*	0.060	7 (8.75)	0.000	0 (0)	Decreased	0.0247
Streptococcus	*S. thermophilus*	0.000	3 (3.75)	0.054	6 (8)	Increased	0.0000
Streptococcus	*S. intermedius*	0.024	16 (20)	0.000	0 (0)	Decreased	0.0101
Treponema	*T. vincentii*	0.020	5 (6.25)	0.000	0 (0)	Decreased	0.0295
Veillonella	*V. rogosae*	0.038	6 (7.5)	0.231	7 (9.3)	Increased	0.0033
Veillonellaceae	*V. bacterium HMT 129*	0.011	3 (3.75)	0.000	0 (0)	Decreased	0.0420
Veillonellaceae	*V. bacterium HMT 132*	0.067	10 (12.5)	0.002	2 (2.7)	Decreased	0.0250
Veillonellaceae	*V. bacterium HMT 155*	0.018	7 (8.75)	0.000	0 (0)	Decreased	0.0332

Figure 6.4: Heat map presenting the most common genera in S1 and S2 samples and the change in each relative genus abundance.

A reduction of *Streptococcus*, *Peptostreptococcus*, *Fusobacterium* and *Parvimonas* is noted. While the relative abundance of *Actinomyces*, *Lactococcus*, *Enterococcus*, and *Prevotella* increased after treatment.

6.3.4. The effect of EP on the microbial composition of pre-obturation samples

The microbial composition and communities' identities of the two treatment groups initial samples (S1) were found to be not statistically different. This was shown by comparative richness and diversity indices and similar microbial composition (Appendix 13). For this reason, a direct comparison of the pre-obturation samples (S2) of the two treatment protocols was carried out.

In the initial samples, the mean number of OTUs per sample was 62 and 63 in the SP and EP, respectively (Appendix 13). After instrumentation and irrigation, the mean number of OTUs per sample was reduced to 38 and 26 in the pre-obturation samples of SP and EP, respectively. The EP pre-obturation samples harboured significantly less OTUs than their corresponding SP counterparts. Although there was no difference in the diversity and richness measures between the two groups in their initial intracanal samples (Appendix 13), Chao1 and ACE estimators of richness were significantly lower in the S2 samples of EP compared to SP ($p=0.02$ and 0.03, respectively), thus indicating a lower number of observed OTUs in the EP. Additionally, the Shannon and Simpson diversity indexes at 3% distinction were lower in the EP pre-obturation samples, indicating greater microbial diversity in the SP pre-obturation samples. Diversity estimates of the two groups after sequence-size-normalisations are shown in Table 6.5 and Figure 6.5.

A total of 88 taxa were significantly different between the two treatment protocols' pre-obturation samples. Some taxa were only detected in the SP group and completely absent in the EP pre-obturation samples, such as: *Bacteroidetes, Fusobacterium, Peptostreptococcaceae*, *Porphyromonas,* and *Veillonella*. Other taxa were detected with a significantly lower relative abundance in the EP S2 samples, such as: *Actinomyces,*

Haemophilus, *Porphyromonas*, *Cutibacterium*, and *Prevotella* (Table 6.6, Figure 6.6 and Figure 6.7).

Table 6.5: Data from diversity and richness estimate calculations of the pre-obturation samples (S2) in the two treatment protocols: SP and EP.
Results of Wilcoxon signed-rank test.

Indicator	S2-SP (n=11)		S2-EP (n=12)		*p-value*
Number of OTUs per canal	38.36	(14-115)	26.83	(7-58)	0.04*
Chao1 estimator of richness	71.02	(21-187.1)	43.72	(13-104.5)	0.02*
ACE estimator of richness	71.89	(42.93-195.2)	48.08	(14.35-106.12)	0.03*
Shannon index of diversity	1.41	(0.024-3.15)	0.96	(0.012-2.47)	0.18
Simpson index of diversity	0.49	(0.005-0.93)	0.39	(0.002-0.86)	0.32

*p<0.05

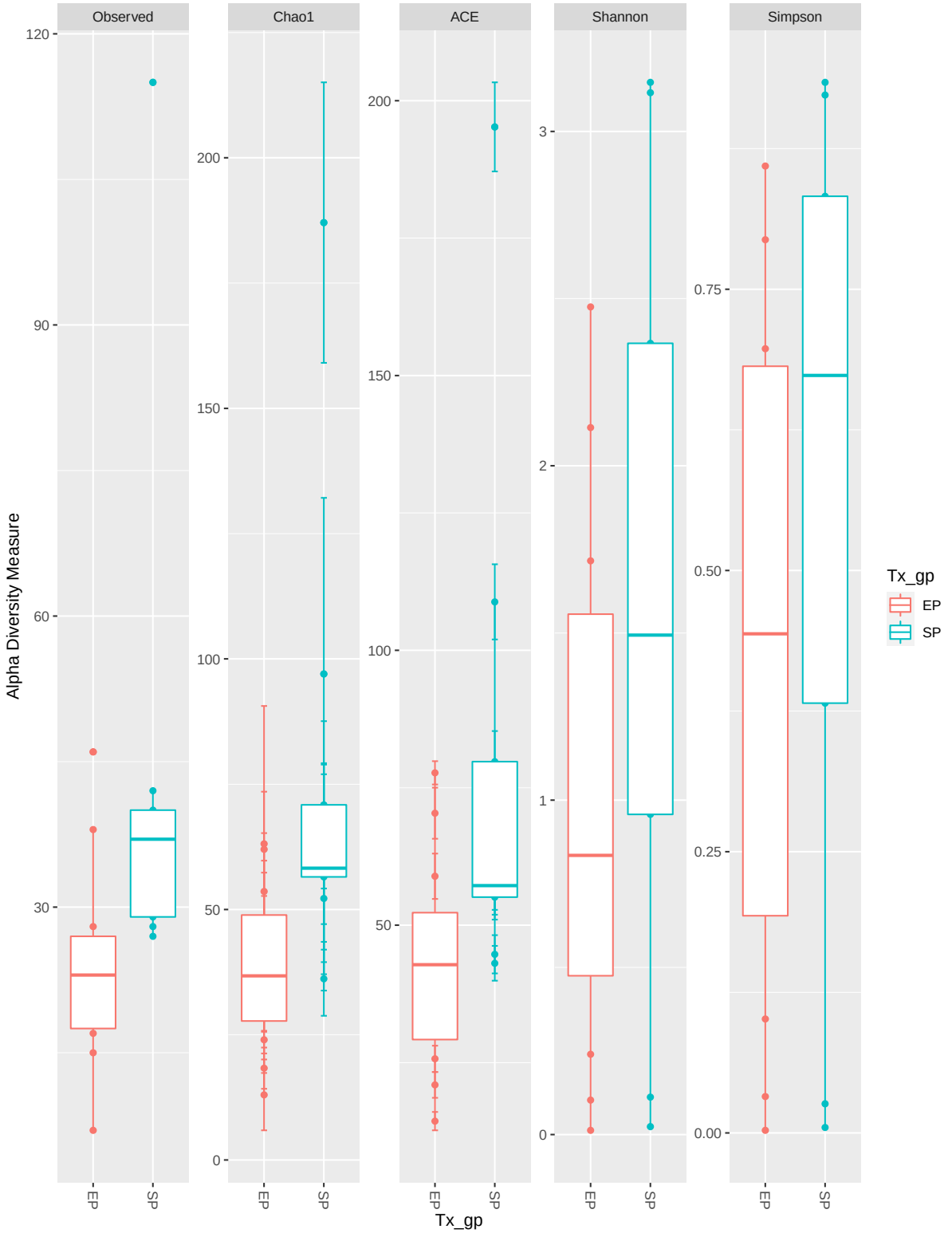


Figure 6.5: Diversity analyses of the microbiomes of the pre-obturation samples (S2) in the two treatment protocols: SP and EP.

Pre-obturation samples of EP had significantly less OTUs, lower values of Chao1 and ACE estimators of richness and lower Shannon diversity index at 3% distinction compared to SP. A greater diversity of all measures was seen on the SP samples, which was attributed to a sporadic but significant iatrogenic introduction of bacteria during the procedure.

Table 6.6: List of taxa presented significantly different in the pre-obturation samples of the two treatment protocols SP and EP.

Results of Mann-Whitney U test and Benjamini-Hochberg corrections or Fisher exact test presenting the relative frequency in the two groups. After quality filtering, and read normalisation, 39 samples remained in the SP group and 36 in the EP pre-obturation samples. All listed taxa had significantly lower relative abundance and were less frequently present in the EP compared to SP samples.

Genera	Species	Standard protocol		Enhanced protocol		*p-values*
		Relative abundance (%)	Frequency in samples n (%)	Relative abundance (%)	Frequency in samples n (%)	
Actinomyces[*‡]	A. *sp. HMT 172*	0.126	8 (20.5)	0.411	4 (11.1)	0.0015
Actinomyces	A. *naeslundii*	0.000	5 (12.8)	0.028	0 (0)	0.0000
Actinomyces	A. *sp. HMT 180*	0.196	17 (43.6)	0.471	12 (33.3)	0.0000
Atopobium[*]	A. *sp. HMT 199*	0.000	3 (7.7)	0.050	1 (2.8)	0.0000
Bacteroidales	B. *bacterium HMT 274*	0.000	1 (2.6)	0.006	0 (0)	0.0087
Bacteroidetes	B. *bacterium HMT 365*	0.000	3 (7.7)	0.078	0 (0)	0.0000
Cutibacterium	C. *acnes*[†]	0.018	3 (7.7)	0.100	1 (2.8)	0.0000
Enterococcus[‡]	E. *durans*	0.071	7 (17.9)	0.105	5 (13.9)	0.0000
Fusobacterium[*‡]	F. *periodonticum*[*]	0.016	6 (15.4)	0.069	3 (8.3)	0.0127
Fusobacterium	F. *nucleatum subsp. Animalis*[*‡]	0.000	4 (10.3)	0.123	0 (0)	0.0005
Haematobacter	H. *missouriensis*	0.028	3 (7.7)	0.125	2 (5.6)	0.0000
Haemophilus[*]	H. *parainfluenzae*[*]	0.010	5 (12.8)	0.111	1 (2.8)	0.0000
Microbacterium	M. *flavescens*	0.037	5 (12.8)	0.039	2 (5.6)	0.0000
Micrococcus	M. *luteus*[†]	0.119	5 (12.8)	0.083	3 (8.3)	0.0021
Neisseria[*]	N. *perflava*	0.007	7 (17.9)	0.062	2 (5.6)	0.0000
Neisseria	N. *mucosa*	0.001	3 (7.7)	0.012	1 (2.8)	0.0088
Olsenella	O. *uli*	0.053	2 (5.1)	0.085	4 (11.1)	0.0000
Oribacterium[*]	O. *sinus*[*]	0.000	4 (10.3)	0.071	1 (2.8)	0.0087
Peptostreptococcaceae[*‡]	P. *yurii subspp. yurii & margaretiae*	0.000	3 (7.7)	0.143	0 (0)	0.0022

Porphyromonas[*]	P. *pasteri*[*]	0.000	4 (10.3)	0.080	0 (0)	0.0000
Prevotella[*‡]	P. *pallens*[*]	0.012	3 (7.7)	0.029	3 (8.3)	0.0128
Shuttleworthia	S. *satelles*	0.296	12 (30.8)	0.411	8 (22.2)	0.0000
Solobacterium	S. *moorei*	0.113	9 (23.1)	0.028	6 (16.7)	0.0000
Streptococcus[*‡]	S. *sp. HMT 56*	0.030	4 (10.3)	0.471	2 (5.6)	0.0088
Streptococcus	S. *sp. HMT 66*	0.133	12 (30.8)	0.006	7 (19.4)	0.0000
Streptococcus	S. *thermophilus*	0.035	3 (7.7)	0.008	3 (8.3)	0.0021
Streptococcus	S. *constellatus*	0.228	3 (7.7)	0.050	4 (11.1)	0.0022
Veillonella[*]	V. *rogosae*[*]	0.000	7 (17.9)	0.003	0 (0)	0.0000
Veillonella	V. *parvula*[*†]	0.000	3 (7.7)	0.171	0 (0)	0.0022

[*]common saliva microbiota, [†]common skin microbiota, [‡] common endodontic microbiota.

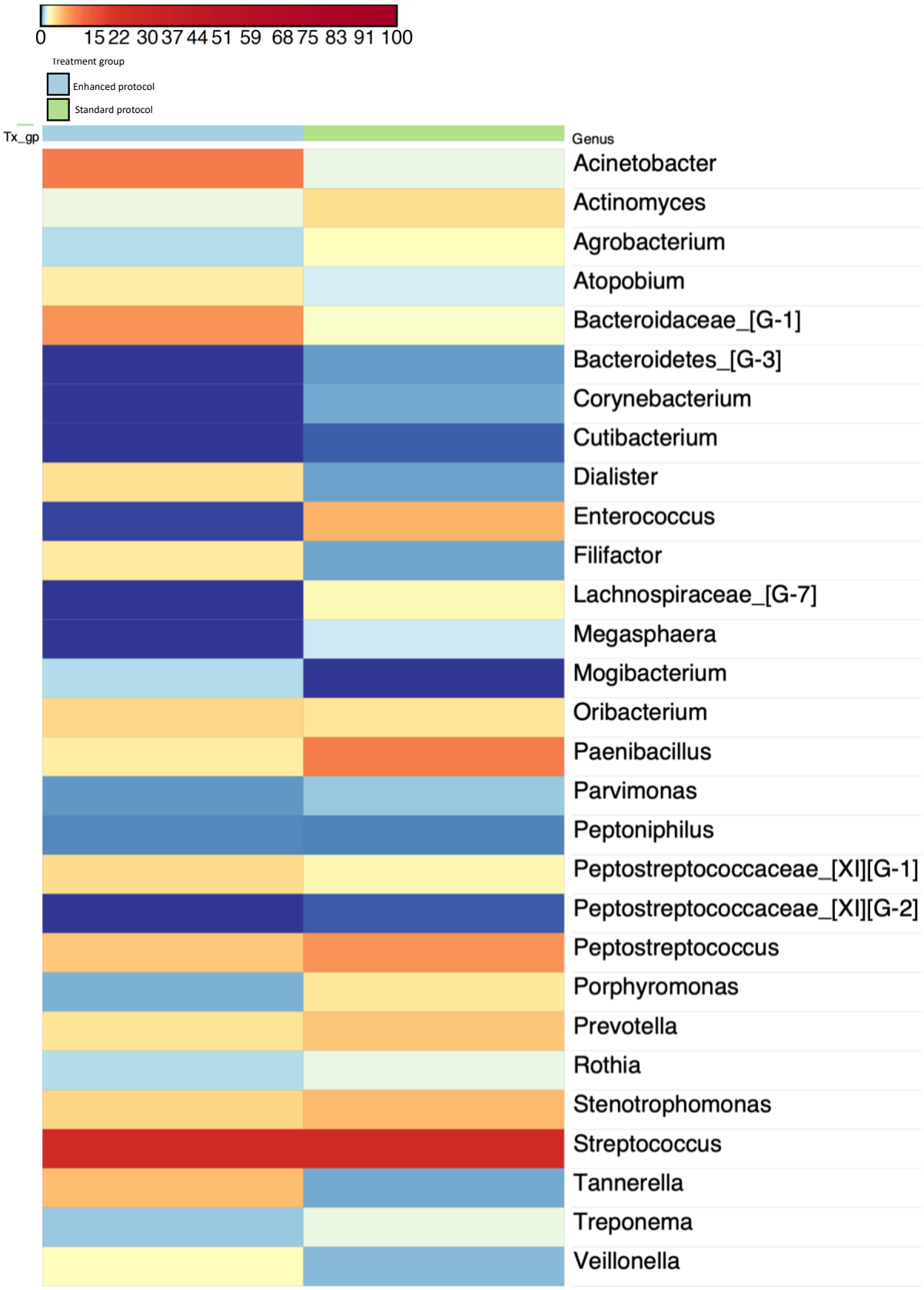


Figure 6.6: Heat map presenting the most common genera in pre-obturation samples of SP and EP.

An enrichment was noted in the relative abundance of *Cutibacterium*, *Enterococcus*, *Bacteroidetes*, *Peptostreptococcaceae*, *Treponema*, and *Rothia* in the SP pre-obturation samples compared to EP.

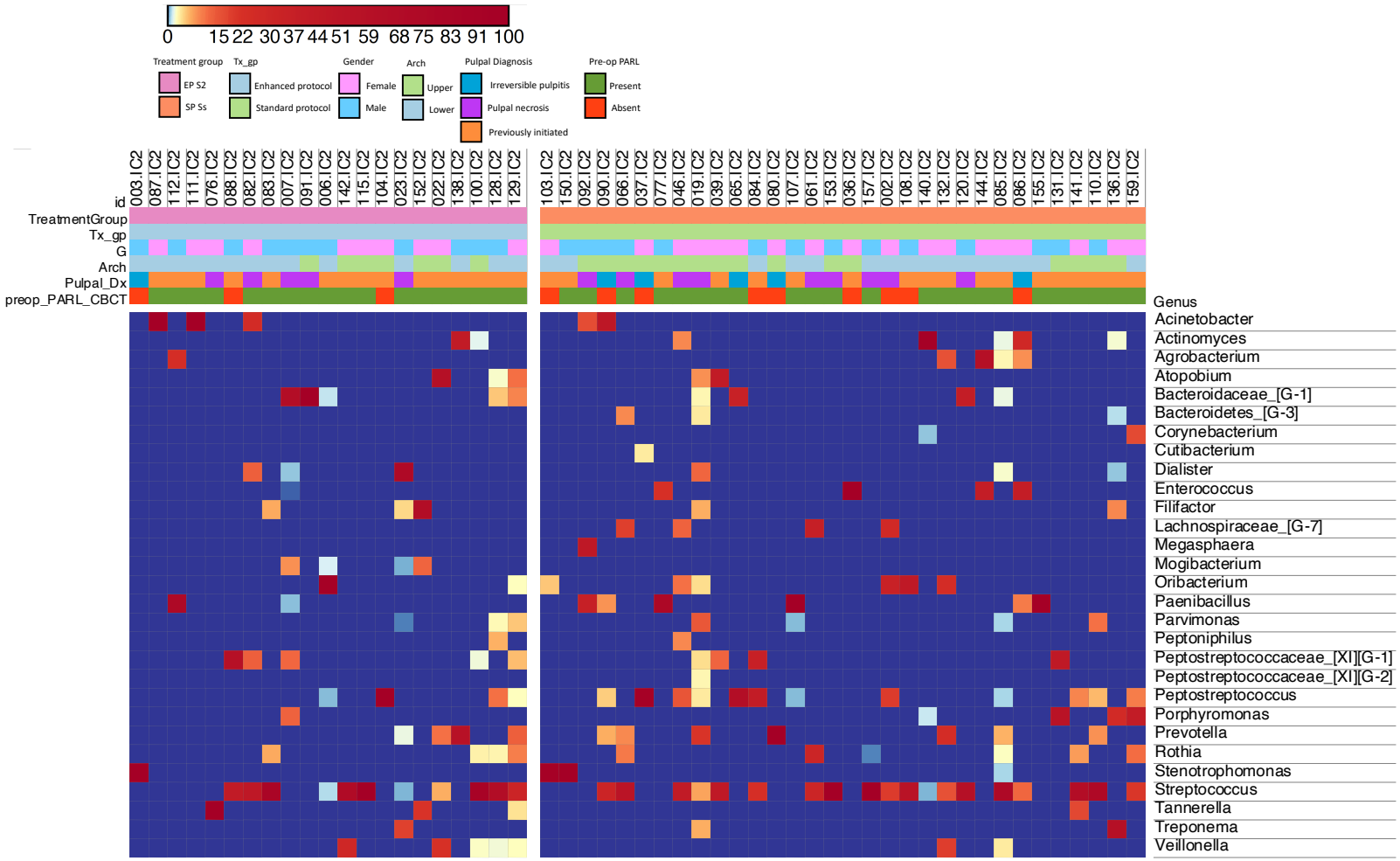


Figure 6.7: Heat map of the pre-obturation samples of two treatment groups after read normalisation.
Showing common endodontic microbiota present in each sample. Note the overall increase in frequency of taxa in the SP compared to EP. A full data set is shown in Appendix 15.

6.4. Discussion

The present chapter aimed to examine the selective changes of the bacterial composition of intracanal samples before and after treatment, using next-generation sequencing (NGS), with the aim to determine if the enhanced infection control protocol would reduce the risk of root canal bacterial contamination by affecting the microbiological profile of pre-obturation intracanal samples. This was supported by the previous datasets examining extraneous contaminants not typically encountered within the endodontic infection space, but frequently present on clinical instruments and surfaces.

While previous NGS studies included 8-24 intracanal samples (Santos *et al.*, 2011, Siqueira *et al.*, 2011, Özok *et al.*, 2012, Hong *et al.*, 2013, Tzanetakis *et al.*, 2015, Keskin *et al.*, 2017, Persoon *et al.*, 2017, Bouillaguet *et al.*, 2018, İriboz *et al.*, 2018), this is the first study to investigate large number of endodontic samples (156 intracanal samples). It thus offers some important insights into endodontic microbiology and enables proximation of the validity of the introduced protocol using open-ended molecular methods.

The sequencing analysis conducted in this chapter showed the benefits of incorporating an enhanced infection control protocol in reducing the risk of microbial contamination during treatment. The present chapter addressed: (i) the microbial profile of the initial intracanal samples, (ii) the overall microbial transition after chemomechanical instrumentation, (iii) the enhanced protocol effect on microbial communities in pre-obturation samples, and (iv) some relevant endodontic microbiota which were notably different within the treatment protocols.

Phylogenetic profile of microbiome in root canal space initial samples

The average number of detected OTUs in the initial samples in our study was 62 OTUs per sample, which is lower than previous studies which reported an average of 79-97 OTUs per

canal or apical segment of infected root canal spaces using similar molecular approaches in primary endodontic infections (Siqueira *et al.*, 2011, Özok *et al.*, 2012, Hong *et al.*, 2013, Persoon *et al.*, 2017). Moreover, Chao1 and ACE richness indexes, as well as Shannon diversity index, were all lower than previous reports. This can be attributed to the fact that some studies only investigated apical segment microflora of anterior teeth (Siqueira *et al.*, 2011, Persoon *et al.*, 2017, Bouillaguet *et al.*, 2018) in which greater bacterial numbers could be expected. Additionally, the inclusion of teeth with different diagnoses in our study compared to exclusively including infected root canals with periapical lesion might explain such differences. Finally, bioinformatic processing methodologies, differences in similarity cut-off for OTU clustering and referenced libraries might also contribute to these differences. Supporting the qPCR findings, greater numbers of OTUs per canal were identified in this study compared to those identified in our investigations on vital teeth (Chapter 3).

In regard to overall microbial composition and communities, a general agreement with previous reports was noted. Using 16S rDNA gene amplicon sequencing investigations on primary endodontic infections, our initial samples were comparable in the composition to previous reports (Özok *et al.*, 2012, Hong *et al.*, 2013, Tzanetakis *et al.*, 2015, Keskin *et al.*, 2017, Persoon *et al.*, 2017, İriboz *et al.*, 2018) in which Firmicutes, Bacteroidetes, Actinobacteria, and Fusobacteria were the most predominant phyla with the most species richness (Siqueira and Rôças, 2009c).

In keeping with the findings of the present study, the common taxa observed agreed with both culture- and non-culture-based studies. Predominant genera described for both were *Streptococcus*, *Peptostreptococcus*, *Fusobacterium*, *Parvimonas*, *Prevotella*, and *Dialister* (Özok *et al.*, 2012, Keskin *et al.*, 2017, Persoon *et al.*, 2017, Bouillaguet *et al.*, 2018). A greater

similarity can be observed when targeting the same V3-V4 16S rRNA hypervariable regions; some studies identified high abundances of similar genera from primary infected root canal spaces of 20 to 23 patients (Özok *et al.*, 2012, Keskin *et al.*, 2017, Persoon *et al.*, 2017, Bouillaguet *et al.*, 2018).

The 16S rRNA V3-V4 hypervariable region is widely targeted in many microbiological studies and became a mainstream amplicon target in microbiota studies (Raju *et al.*, 2018). In endodontics, it is also among the most adopted region to investigate endodontic microbiota (Manoil *et al.*, 2020). The reproducibility of the saliva microbiota shown to be precisely targeting this region compared to other hypervariable regions (Raju *et al.*, 2018, Teng *et al.*, 2018). For accuracy and reproducibility, this region was chosen in our investigation.

In our samples, the most abundant genera were *Streptococcus* and *Peptostreptococcus*, together accounting for 27% of all sequence reads. *Streptococci* and *Peptostreptococci* are among the common Gram-positive cocci, associated with primary endodontic infections and frequently present (Siqueira and Rôças, 2009c, Santos *et al.*, 2011, Özok *et al.*, 2012).

The role of *Streptococcus* in caries has been shown as a synergistic relationship between *Lactobacillus* and *Streptococcus mutans* during caries progression (Willcox *et al.*, 1993), which allows further *Lactobacillus* colonisation, predominance and caries progression (Willcox *et al.*, 1993). *Streptococcus* was recovered from deep carious lesions associated with irreversible pulpitis, especially in the absence of Lactobacillus (Hahn *et al.*, 1991, Zheng *et al.*, 2019). However, *Lactobacillus* sp. is not typically encountered within endodontic infection.

The genus *Parvimonas* was among the most commonly abundant genera in our clinical study, accounting for 5.5% of the reads in the initial samples. Members of *Parvimonas* such as *P. micra* (formerly *Peptostreptococcus micros* or *Micromonas*), another Gram-positive cocci, commonly isolated from primary infections and associated to symptoms in some studies (Gomes *et al.*, 1996, Siqueira *et al.*, 2003) were commonly detected in our samples.

Another genus frequently found among our initial samples is *Dialister*, with a relative abundance of 4.4% and encountered in more than half of the cases. Members of this genus are saccharolytic, obligately anaerobic Gram-negative coccobacilli associated with asymptomatic and symptomatic primary infections, and failed cases (Rolph *et al.*, 2001, Siqueira and Rôças, 2004). This genus was only detected after the invention of molecular methods (Siqueira and Rôças, 2009c), and sequencing methods refined the knowledge about it showing its predominance with greater abundance in primary infected root canals compared to secondary (Bouillaguet *et al.*, 2018, İriboz *et al.*, 2018).

Members of *Fusobacterium* are another Gram-negative species commonly encountered in primary endodontic infections (Siqueira and Rôças, 2009c), and shown to be associated with the growth phase of endodontic pathogenesis (Sundqvist and Figdor, 2003). They were also reported in some failed cases (Rolph *et al.*, 2001, Siqueira and Rôças, 2004). In our study, *Fusobacterium* was detected in 73% of the initial samples.

The effect of chemomechanical preparation on microbial profile

During endodontic treatment, complete sterilisation of the root canal space, although desirable, cannot be attained. To promote tissue healing after endodontic intervention, bacterial reduction should be achieved (Siqueira and Rôças, 2008). This was addressed in the previous chapter,

highlighting the "dose-dependent" effect of the protocols on the outcome (Sections 5.3.3 and 5.3.4).

Preferential bacterial removal during chemomechanical preparation was noted in which selected members of the endodontic microbial community were removed, whilst other members persisted. This can be attributed to either accessibility of taxa within dentine tubules or the presence of some bacteria shown to resist chemomechanical preparation. The effectiveness of chemomechanical debridement in this part of the study was reflected on the significant reduction of bacterial OTUs, richness and diversity measures in S2 samples compared to S1. The significant reduction of bacterial number in qPCR was reflected in this chapter on the significant reduction in relative abundance and reads of most bacteria in sequencing analysis and thus further validated our quantitative analysis using qPCR.

Various molecular techniques have been used to investigate the effect of instrumentation or irrigation protocols on intracanal microbial composition (Siqueira and Rôças, 2005b). Although results vary significantly, there is a general agreement of Gram-negative bacteria to be relatively easily eliminated after treatment with some exceptions such as *F. nucleatum* and *Prevotella* (Chávez de Paz, 2005, Siqueira *et al.*, 2007d). Gram-positive bacteria, on the other hand, were more frequently reported to persist and were repeatedly found in post-instrumentation samples (Chávez de Paz, 2004). Agreeing with the present study, the phylogenetic data showed that, after chemomechanical debridement, a greater reduction of Gram-positive bacteria such as *Streptococcus*, *Peptostreptococcus*, and *Parvimonas*, as well as some Gram-negative as *Fusobacterium* was noted. These same Gram-positive bacteria in our study were previously shown to be significantly reduced after chemomechanical preparation

using molecular methods, or completely eradicated when detected by culture approaches diseases (Siqueira and Rôças, 2009c).

Unlike those taxa readily removed from the endodontic space, as evidenced by the absence (or high-level reduction) of bacteria in S2 samples, an increase of *Enterococcus*, *Actinomyces*, *Veillonella* and *Prevotella* was notable in overall samples. Our findings agree with previous speculations of some bacteria resistant to chemical disinfection (Chávez de Paz, 2004, Siqueira and Rôças, 2009c), such as members of anaerobic bacteria, *F. nucleatum*, and members of *Prevotella*, or some Gram-positive bacteria such as *Streptococci*, *Enterococcus*, *Actinomyces* species, and *Cutibacterium* species (Chávez De Paz *et al.*, 2003, Zandi *et al.*, 2018). The increase in the prevalence of *Enterococcus* and *Tannerella* was also commonly identified as persisting in teeth harbouring periodontal lesions of endodontic origin (Gomes *et al.*, 2015b). One of the drawbacks in molecular methods is that a dramatic change cannot be observed when analysing similar samples and a complete eradication cannot be expected. Thus, both the frequency of detected taxa alongside with their relative abundances were investigated.

The impact of enhanced infection control protocol on microbial community composition

The main purpose of this analysis was to investigate the effectiveness of our enhanced protocol on specific microbial taxa. This was accomplished by comparing the pre-obturation samples of the two groups. The effectiveness of chemomechanical preparation was reflected in the reduction of the relative abundance of most bacterial taxa. Yet, some taxa were detected with a significantly higher relative abundance in the SP compared to EP. Examples are members of *Actinomyces*, *Haemophilus*, *Peptostreptococcaceae*, *Porphyromonas*, *Cutibacterium*, *Bacteroidetes*, *Prevotella*, and *Veillonella*. Looking at the bacterial community, the HOMD

classification of our sequence data detected substantially more OTU, richness and diversity in the pre-obturation samples of the standard group compared to the enhanced protocol. The lower diversity indexes in the EP are suggestive of the effectiveness of the introduced protocol supporting the clinical outcomes and qPCR findings discussed in previous chapters.

Many studies showed the carry-over of iatrogenic contamination in gloves, rubber dam, endodontic materials, and operatory surfaces (Williams *et al.*, 2006, Niazi *et al.*, 2016, Saeed *et al.*, 2017, Rorslett Hardersen *et al.*, 2019). In our study, we are showing the effect of microbial carry-over in surfaces and instruments into the root canal space. The common taxa found in previous reports and in our pilot study were investigated here to study their iatrogenic introduction during treatment.

Enterococcus

Using culture-dependent methods, *Enterococcus* sp. gained a special interest being frequently recovered from post-treatment endodontic failed cases with a proportion ranging from 29% to 77% (Möller, 1966, Molander *et al.*, 1998, Sundqvist *et al.*, 1998, Chávez De Paz *et al.*, 2003, Siqueira and Rôças, 2004). To a notably lesser extent, *Enterococcus* was recovered from primary infected root canal spaces (Byström and Sundqvist, 1985, Siqueira *et al.*, 2002).

Several mechanisms are attributed to the pathogenicity of this microorganisms to overcome treatment and its survival in the root canal environment. First, as it enters a viable non-cultivable state, it can survive starvation and environmental conditions lacking nutritional supplies up to four months (Distel *et al.*, 2002, Evans *et al.*, 2002, Figdor *et al.*, 2003). Next, it has ability to adhere to dentine and is arranged in biofilms (Siqueira and de Uzeda, 1996, Hubble *et al.*, 2003). Moreover, *Enterococcus* has the capability to survive different pH ranges

due to the proton pump inhibition mechanism making it possible to persist calcium hydroxide in a high alkaline environment (Byström *et al.*, 1985, Sjögren *et al.*, 1991, Evans *et al.*, 2002).

Using NGS, the abundance of *Enterococcus* was reported in secondary cases with 2-3% relative abundance (Rolph *et al.*, 2001, Anderson *et al.*, 2013, Siqueira *et al.*, 2016). Some sequencing studies reported less secondary infected cases with *Enterococcus* (2/10 or 7/22), but higher relative abundance, ranging from 13% to 99% (Bouillaguet *et al.*, 2018, Zandi *et al.*, 2018). On the contrary, one sequencing study did not detect any *Enterococcus* in secondary endodontically infected cases (Bouillaguet *et al.*, 2018). In periodontal lesions of endodontic origin, it was recovered from all cases, and the abundance increased after chemomechanical preparations (Gomes *et al.*, 2015b).

Compared to primary infected root canal spaces, *Enterococcus* were significantly more abundant in secondary infection in some reports (Vengerfeldt *et al.*, 2014, Bouillaguet *et al.*, 2018) while the difference was not noted in another study (Keskin *et al.*, 2017). The reduced abundance of *Enterococcus* in open-end molecular studies suggested probably a previous overestimation of its role in treatment failure (Sakamoto *et al.*, 2008, Anderson *et al.*, 2013, Hong *et al.*, 2013, Tzanetakis *et al.*, 2015). Sequencing studies also refuted the speculations of *Enterococcus* as a Koch's pathogen in refractory cases, because cultural methods allowed the solo recovery of *Enterococcus* despite the presence of unculturable bacteria (Peciuliene *et al.*, 2000).

In our study, overall, there was an increase in the relative abundance of *Enterococcus* from S1 to S2 samples. Members of *Enterococcus* were not among the taxa that were significantly present differently in the pre-obturation samples of the two protocols. Since in our pilot study,

Enterococcus was only recovered from rubber dam surfaces with a very low abundance and aligning with all previously mentioned study advocating its resistance to antimicrobial measures, it was proposed that the presence of *Enterococcus* in pre-obturation samples is attributed to the resistance to treatment, rather than external iatrogenic contamination.

Actinomyces

Actinomyces has long been implicated in a number of endodontic presentations. Besides recovering *Actinomyces* from primary infected root canal spaces (Siqueira *et al.*, 2002), resisting chemomechanical preparation (Sjögren *et al.*, 1997), and failed root canal treated cases (Sundqvist and Reuterving, 1980, Happonen, 1986, Sundqvist *et al.*, 1998), a special interest of this taxon is its association with extra-radicular infection and its presence in the periradicular tissue and periapical abscesses (Tronstad *et al.*, 1987, Sunde *et al.*, 2000b).

High-throughput sequencing studies also detected this genus among the most predominating in primary infected root canal spaces accounting for 11% of the reads (Özok *et al.*, 2012, Persoon *et al.*, 2017), and secondary endodontic infection with around 7-8% abundance (Persoon *et al.*, 2017, Bouillaguet *et al.*, 2018, Zandi *et al.*, 2018). Our results are in line with earlier reports in which *Actinomyces* was detected in initial samples and disproportionally persisted after treatment.

The virulence of *Actinomyces* was suggested to arise from its arrangement in cohesive filaments or chains, and thus resisting antimicrobial agents and phagocytosis (Figdor *et al.*, 1992). It is still to be determined whether it can cause disease as a mono-infection (Figdor *et al.*, 1992).

Actinomyces was recovered from the rubber dam surfaces, and files in our pilot study explicated in Chapter 2 with an abundance of 2% - 3.5%, and also present but less abundantly in instruments and gloves samples. One study revealed that at least ten different *Actinomyces*

species were recovered from half of the glove samples collected during endodontic treatment (Niazi *et al.*, 2016). The relative abundance of *Actinomyces* was significantly higher in the pre-obturation samples belonging to the SP when compared to their counterparts in the EP. In fact, some members were completely absent in the EP such as *A. radicidentis*, *A. viscosus*, and *A. naeslundii*. Our qualitative molecular findings suggest that the implemented protocol reduced the risk of introducing *Actinomyces* to the root canal space during treatment. The selective enrichment of *Actinomyces* was exhibited through the increased abundance in the SP but not in the EP pre-obturation samples.

Cutibacterium

Members of Gram-positive facultative anaerobic *Cutibacterium* are part of the skin microflora, conjunctiva and ear canal (Brook and Edith, 1991). Skin microflora is dominated by *C. acnes* (Tancrède, 1992). *Cutibacterium*, although not typical oral commensal, has been recovered from primary and secondary endodontic infections (Munson *et al.*, 2002, Chávez de Paz, 2004), frequently recovered in post-instrumentation samples (Sjögren *et al.*, 1997, Siqueira *et al.*, 2007b), and contributed to post-treatment endodontic diseases among other Gram-positive facultative anaerobes or anaerobes bacteria such as *Streptococci*, *Pseudoramibacter*, and *Parvimonas* (Sundqvist *et al.*, 1998, Siqueira and Rôças, 2004). Even though open-end molecular studies revealed that more diverse convoluted mixture of bacteria is associated with post-treatment disease (Sakamoto *et al.*, 2006, Sakamoto *et al.*, 2008), *Cutibacterium* was still recovered among the most abundant genera in primary and secondary endodontic infected cases using advanced molecular sequencing studies (Anderson *et al.*, 2013, Hong *et al.*, 2013, Persoon *et al.*, 2017). This genus was also associated with extra-radicular infections (Tronstad *et al.*, 1987, Sunde *et al.*, 2000b) and akin to *Actinomyces*, has the ability to resist treatment by cohesive arrangements (Figdor *et al.*, 1992). *C. acnes* were among the bacteria persisted metabolically active after chemomechanical preparation when detected with rRNA-based

assays and suggesting their persistence to contribute to persistent infection (Nardello *et al.*, 2020b). Findings of a meta-analysis showed that a significantly greater prevalence of the genus *Cutibacterium* was noted in secondary endodontic infections compared to primary. A higher prevalence of *C. acnes* was noted compared to *C. propionicum* confirming its association to persistent infections (Dioguardi *et al.*, 2020).

Members of *Cutibacterium* were recovered from all rubber dam surfaces samples in our pilot study (8/8) and was the most abundant genus (17.5%) recovered from most files (9/11) and to a lesser extent, in gloves and instruments. Furthermore, three species of this genera were recovered from all sampled gloves with a mean proportion of 17.6% (Niazi *et al.*, 2016), and were among the identified taxa in the rubber dam surfaces (Rorslett Hardersen *et al.*, 2019). Gomes *et al*. showed one-third of the gutta-percha cones after glove manipulation were contaminated with *Cutibacterium* (Gomes *et al.*, 2005). Moreover, in a study testing endodontic materials, it was the most common isolated genus (42%) in items that are commonly handled by practice staff after removal from the package, such as gutta-percha points, rubber dams, paper mixing pads, caulking agents, and endodontic instrument sponges (Saeed *et al.*, 2017). An increase in levels of contamination was noted after storage (Saeed *et al.*, 2017). It was suggested to be associated with nosocomial endodontic refractory cases (Niazi *et al.*, 2010).

In our study, the relative abundance of *Cutibacterium* was generally decreased after chemomechanical preparation. But *C. acnes* in particular were significantly identified more in the SP S2 samples compared to EP. Our data suggest that this selectively deposited taxa, associated with secondary and extra-radicular infections, can be easily eliminated with the enhanced protocol.

Haemophilus

Haemophilus is a Gram-negative, pleomorphic, coccobacilli bacteria and is among the fastidious bacterial species only detected in endodontic diseases using molecular methods (Siqueira *et al.*, 2000c). The checkerboard DNA-DNA hybridisation technique detected *Haemophilus* in 25% of the primary infected root canal spaces (Siqueira *et al.*, 2000c). It was also detected in sequencing studies associated with periradicular infections, periodontal lesions of endodontic origin, and symptomatic and asymptomatic secondary endodontic infections (Anderson *et al.*, 2013, Gomes *et al.*, 2015b, Qian *et al.*, 2019).

In our pilot study, this genus was prevalent in gloves and half of the rubber dam surfaces sampled with a relative abundance of 4.5% and 1.6%, respectively. *Haemophilus parainfluenzae* was found in rubber dam surfaces and gloves during treatment (Niazi *et al.*, 2016, Rorslett Hardersen *et al.*, 2019). Our data showed that *H. parainfluenzae* was identified in 12% of the pre-obturation samples in the SP compared to 2% of the cases in the EP. This difference might suggest the possible contribution of this genus in the iatrogenic contamination during treatment.

Greater variations in the detected spectrum of the microbiome and differences in taxonomic composition between studies of molecular and open-ended approaches are notable. This can be attributed to many factors, such as variations included the histopathological status of affected teeth and sampling techniques, and variations in the molecular methodologies used including DNA extractions and 16S rDNA amplification, as well as computational analysis. Moreover, geographic locations were shown to affect endodontic microbial communities and thus reflect the diversity of previous studies (Tzanetakis *et al.*, 2015). Yet, there was an overall agreement in the microbial changes and commonly found members with previous molecular studies which certainly validates the approach we used in our study.

Concerns about contamination

One of the major concerns in studies examining microbial contamination, as contamination during endodontic treatment in the present study, are contaminations arising from the sample collections and downstream processing. Sequencing is known to be highly sensitive and relatively unbiased. But, with this highly accurate remarkable method, challenges come with the reliability of the results processed. Contamination is a known unavoidable limitation of this application. In our study design, methodologies and analysis, the reduction of contamination risks was taken into consideration as follows: first, the meticulous inclusion of matching qPCR and sequencing control samples was essential to avoid bias arising from the risk of overestimating diversity (Kunin *et al.*, 2010). In our pilot study, control samples were included initially, but these control samples did not fulfil the submission criteria of the previous sequencing provider and amplicon generation was not possible. For these reasons, all control samples were not processed for sequencing analysis and none of the control samples were qualitatively investigated in Chapter 2. To overcome this issue, control samples were processed for the Illumina MiSeq platform (Integrated Microbiome Resource) and instructions were

given to run all samples, including those with low yields that fail the PCR step to identify possible sources of contaminations. Although most control samples yielded very low read number (less than 100 reads), they were all included in the downstream analysis. Second, the introduction of MDA amplification step, although it has its own shortcomings, was beneficial in terms of reducing the signal to noise ratio by increasing the amount of DNA in samples. Finally, during analysis, specific strains shown in the negative control samples were removed. Removing contaminants is a concern with any sequencing technique mitigated by the database. We removed contaminants from both groups thus, even if there is an error, it would be equally distributed. Examples of removed taxa were: *Alkalibacterium, Exiguobacterium, Halomonas, Filifactor, Bosea* and *Lactococcus*. The aggressive removal of contaminants during analysis was to avoid contaminants mainly affecting samples with low DNA, such as in our case on intracanal samples. Many algorithms were adopted to recognise and remove contaminants from downstream analysis of such data (de Goffau *et al.,* 2018). Since an overlap between endodontic microbiome and known contaminants is expected, a complete removal of all contaminants was not reasonable. Instead, the removal of contaminants was done manually where care was taken to avoid misinterpreting the existing community using two independent assessors and a cautious interpretation of the findings was obligatory. The risk is raised when contaminants are not properly eliminated with such methods resulting in jeopardising the microbiologic discoveries. In a rat model study, genera such as *Bradyrhizobium* and *Halomonas* were attributed to have a role in endodontic pathogenesis (Park *et al.*, 2020) although these are known contaminants (Laurence *et al.*, 2014, Weyrich *et al.*, 2019).

Limitations associated with paper point sampling have been previously addressed. Another aspect is discussed here in regard to contamination of pre-packed sterile paper points. In agreement with a previous study evaluating contamination arising from paper point (van der

Horst *et al.*, 2013), *Exiguobacterium* and *Enterococcus* were among the predominant taxa in our sterile paper points control samples. Moreover, the microbial profile of our control paper points included *Enterococcus casseliflavus*, *Paracoccus yeei*, *Pseudomonas fluorescens*, *Lactobacillus paracasei*, *Filifactor alocis*, and *Enterococcus italicus*. It is important to note that the magnitude of amplification from these samples was not enough to contribute to signals. With the exception of one paper point, most samples yielded very low read numbers but were forced to be subjected to taxonomic identification. Still, the presence of such taxa does not imply these taxa are viable as one of the drawbacks of the current approach not providing the viability of detected organisms.

Regarding the contamination control samples, these samples were taken after one minute disinfection with NaOCl from the target tooth surface. Still, microorganisms were detected in some samples, which is not a surprising finding with molecular approaches (Figdor and Brundin, 2016). Most found taxa in these CC samples were typically salivary bacteria such as *Peptostreptococcus stomatis*, *Parvimonas micra*, and *Rothia dentocariosa* and possibly resisting NaOCl, which have also frequently been recovered from endodontic samples.

The procedural, environmental contamination might arise at any step from extraction to sequencing. Thus, negative extraction control samples were included to address this aspect. Our NEC however had very low read numbers, predominated with typical contamination taxa such as *Pseudomonas fluorescens* (*Halomonas*), and *Afipia* sp. *genotype 4*. Other taxa were detected such as some typical endodontic microorganisms: *Moraxella osloensis*, *Peptostreptococcus* sp., *Streptococcus* sp., and *Prevotella* sp., and *Rothia* sp.

The effect of MDA on microbial diversity

To avoid samples failing the required amount of DNA concentration, this mandated us to include an MDA step to increase the amount of DNA and to allow for thus more precise amplification. Having DNA below the threshold of sequencing does not indicate the absence of relevant bacteria, especially in root canal systems where limitations with sampling are inevitable. Moreover, to minimise the higher risk of contamination originating when lower DNA was present (van der Horst *et al.*, 2013), MDA was carried out in all clinical samples. To further establish the validity of MDA and to ensure that downstream contamination is reduced, matching non-MDA amplified control samples were included. Matching samples were subjected to the same sequencing protocol and bioinformatic analysis. The abundance and proportion of microbiome has not changed dramatically. In fact, we noticed that MDA reduces contamination arising from NGS by having more DNA concentration to start with (van der Horst *et al.*, 2013). Moreover, diversity indices from the matching data showed similar values were shown in matching samples.

6.5. Conclusion

Using high-throughput sequencing approach, our findings showed a trend of reduced diversity observed in pre-obturation samples of teeth treated within the enhanced protocol compared to standard protocol. The comparison of the microbial composition of pre-obturation samples revealed a decrease in or absence of contaminants typically arising from saliva, skin or endodontic root canal spaces, and further supporting the reason why clinical outcomes have been shown to be improved in the EP, unlike the SP. Examples of contaminants as *Actinomyces, Cutibacterium*, and *Haemophilus* were significantly reduced in their abundance in the EP pre-obturation samples. Together with the previous chapter, it appears that the implemented protocol has both a quantitative reduction and specific reduction of some taxa believed to contribute to contamination. Improvement in the treatment protocols is essential to prevent the disease development as reflected by improved treatment outcomes, as well as quantitative and qualitative microbial reduction.

Chapter Seven: Summary and Suggestions for Future Work

7.1. Summary

The aim of this PhD was to determine if an improved, aseptic technique could improve treatment outcomes. The randomised clinical trial aimed to assess the impact of implementing an enhanced infection control protocol as a prognostic variable for favourable outcome in non-surgical root canal treatment of molars after one-year follow-up, as well as the microbiological status of these teeth.

Clinically, all efforts within different treatment phases are directed towards the elimination of existing bacteria. Yet, it is of paramount importance that bacteria do not gain access to the root canal space during treatment (Siqueira *et al.*, 1998, Schirrmeister *et al.*, 2007). It has been reported that iatrogenic contamination of the root canal space may occur during root canal treatment arising from microorganisms from the patient's saliva, gloves worn by the dentist, operating surfaces, the use of non-sterile materials and instruments, bacteria in the surrounding environment or bacterial leakage in between visits (Williams *et al.*, 2003, Niazi *et al.*, 2010, Niazi *et al.*, 2016, Saeed *et al.*, 2017). Thus, a substantial effort should be devoted to maintaining aseptic conditions during treatment. In the current dental literature, there is a lack of evidence supporting the effectiveness of including additional aseptic measures in root canal treatment outcomes.

To establish a protocol to be implemented prior to commencing a full trial, a pilot study was conducted to assess the presence and level of bacterial contamination during root canal treatment from different sites, throughout treatment of teeth presenting with irreversible

pulpitis, and thus to generate proof-of-concept data to determine the feasibility of implementing an EP protocol in root canal treatments.

Findings of the pilot study showed that rubber dam surfaces, initial files, gloves, instruments and rulers were contaminated with bacteria prior to obturation. Higher bacterial numbers were significantly more likely in intracanal samples before canal filling when instruments were contaminated. The most abundant genera in contaminated objects were *Streptococcus, Rothia, Granulicatella, Propionibacterium, Lactobacillus, Sphingomonas, Veillonella,* and *Fusobacterium.* This highlighted the risk of contamination during root canal treatment after chemomechanical preparations, mainly from repetitive use of same patency file, rubber dam surfaces, gloves and instrument used at time of obturation.

Only vital teeth were included to examine the iatrogenic microbial introduction in our pilot study, in which the presence of bacteria within the root canal space before the treatment is minimal due to the pulpal immune protective response. We further investigated the quantitative findings, characterisation, and diversity of endodontic microflora in cases diagnosed with irreversible pulpitis; therefore, bridging the gap in understanding this temporary stage of IP before necrosis as well as pulpal disease progression and potential therapeutic interventions. Findings highlighted the evidence of vital teeth diagnosed as IP harbouring considerable bacterial loads and comprised enrichment of genera reflective of established endodontic pathology, and offered insights into the initial events preceding pulpal necrosis. The most abundant genera observed among the vital cases were *Veillonella*, *Streptococcus*, *Corynebacterium*, *Cutibacterium*, and *Porphyromonas*.

The enhanced infection control protocol employed in the present study was set to follow the standardised root canal treatment protocol (European Society of Endodontology, 2006), with

changes after the completion of instrumentation and irrigation based on findings of a pilot study as well as the available data in the literature reports (Ng *et al.*, 2003, Williams *et al.*, 2003, Niazi *et al.*, 2016, Rorslett Hardersen *et al.*, 2019). These changes included replacing rubber dam, gloves, instruments and surface barriers to reduce the chances of iatrogenic contaminations at the time of obturation.

The clinical study included 144 teeth; at one-year follow-up, 115 teeth were analysed (54 in SP and 61 in EP). The clinical and radiographic findings revealed that the percentage of successful outcomes assessed by CBCT was 85.2% in the EP and 66.7% in the SP. The probability of 12-months success was three times higher in the EP group compared to the SP group.

The overall number of favourable outcomes was significantly lower when assessed with CBCT (76%) compared to PA radiographs (92%). More new lesions were detected with CBCT, which was in agreement with other studies where up to 20% more post-treatment lesions were detected with CBCT and confirming the superior diagnostic capabilities of CBCT compared with the two-dimensional radiographs (Patel *et al.*, 2012a, Liang *et al.*, 2013, van der Borden *et al.*, 2013, Al-Nuaimi *et al.*, 2018).

Furthermore, all other confounding variables possibly affecting the outcome of primary root canal treatment were studied. Besides the treatment protocols, other prognostic factors that remained significant and affected the outcome based on CBCT were absence of pre-operative periapical radiolucencies, and absence of cracks. Additionally, PA did not reveal any prognostic factor affecting the outcome including the treatment protocol used.

In addition to the evidence of improved clinical outcomes with the EP, quantitative microbial analysis showed that the EP was also associated with substantially less detectable bacterial gDNA in pre-obturation samples than the SP. This highlights the significant influence of EP on microbial load during molar root canal treatment, and ascertaining that implementing an enhanced infection control protocol has a greater potential of reducing bacteria prior to obturation.

The debate about bacterial presence prior to obturation impacting the outcome is well documented (Sathorn *et al.*, 2007), and the literature lacks studies examining this association using highly sensitive molecular methods. Our findings showed that cases with favourable outcomes at one-year follow-up had significantly lower bacterial numbers in S2 samples compared to unfavourable outcomes; further supporting the role of bacteria in endodontic diseases in which cleaner canals prior to obturation were associated with absence of disease/healing at one-year follow-up and thus validating the use of qPCR.

Endodontic infections harbour complex communities of bacteria that are considered to be the most significant agent in the development and progression of apical periodontitis. It is important to identify phylogenetic profiles of microbiota associated with endodontic infections, to identify the shift in bacteria after chemomechanical preparation. A non-culture-based high-throughout sequencing microbiological analysis was conducted to investigate the effectiveness of our EP on specific microbial taxa by comparing the pre-obturation samples of the two groups. Findings showed that our sequence data detected substantially more OTU, richness and diversity indices in the pre-obturation samples of the standard group compared to the enhanced protocol. Some taxa were detected significantly with a higher relative abundance in the SP

compared to EP such as *Actinomyces, Haemophilus, Peptostreptococcaceae, Porphyromonas, Cutibacterium, Bacteroidetes, Prevotella,* and *Veillonella*.

Together with qPCR findings, it appeared that the implemented protocol has both a quantitative reduction and specific reduction of some taxa believed to contribute to contamination. Improvement in the treatment protocols is essential to prevent the disease development as reflected by improved treatment outcomes as well as quantitative and qualitative microbial reduction.

In conclusion, the results of this thesis provided clinical, radiographic and microbiological evidence for the improved effectiveness of the enhanced infection control protocol compared to a standard protocol. The evidence from this study suggests that, even when best practice is followed, a scope of improvement can be achieved. The contribution of this study may allow clinicians to incorporate simple adjustments to current everyday best-practice protocols in endodontic treatment, resulting in a major success when a dentist improves sterility by changing gloves, rubber dam, and instruments.

7.2. Suggestions for future work

Longer-term follow-up of patients in the clinical trial, which include:

1. Clinical and radiographic assessment of patients for a period of 3-5 years, which involves using CBCT scans to assess the long-term implications of the implemented protocol.
2. Assessment of the microbiological profile of failed cases clinically/radiographically in future follow-ups of this trial that are undergoing retreatment; thus, investigating the bacterial presence, load and types associated with diseased root canal systems.

Further studies might also include:

1. Effect of implemented protocol on retreatment cases.
2. Investigating fungal role in endodontic infections and contamination.
3. RNA sequencing or integrated DNA- and rRNA-based may address the limitation of molecular methods by revealing the functional activity of the microbiota and active bacterial communities, although technically difficult, and raise the risk of contamination (Nardello *et al.*, 2020a, Nardello *et al.*, 2020b).
4. Investigating the full length of the 16S rRNA gene as we demonstrated that targeting of 16S variable regions with short-read sequencing platforms cannot achieve the taxonomic resolution afforded by sequencing the entire (~1500 bp) gene (Johnson *et al.*, 2019).

Appendices

Appendix 1: List of publications in international peer-reviewed journal

Zahran S, Witherden E, Mannocci F, Koller G. Characterization of Root Canal Microbiota in Teeth Diagnosed with Irreversible Pulpitis. J Endod. 2021 Mar;47(3):415-423. doi: 10.1016/j.joen.2020.12.009. Epub 2020 Dec 23. PMID: 33359531.

Zahran S, Patel S, Koller G, Mannocci F. The impact of an enhanced infection control protocol on molar root canal treatment outcome - a randomized clinical trial. Int Endod J. 2021 Aug 5. doi: 10.1111/iej.13605. Epub ahead of print. PMID: 34352123.

Appendix 2: Comparing bacterial composition, richness and diversity between initial and pre-obturation intracanal samples

An average of 56.07 OTUs per sample observed in initial samples reduced to an average of 20.9 OTUs in pre-obturation samples. The Chao1 nonparametric measure of richness showed that after chemomechanical preparation, the number of OTUs was reduced from 94.5 to 30.2. A significant reduction in bacterial richness (observed and Chao1) was noted (Wilcoxon rank-sum test, p=0.015 and p=0.018 respectively).

The microbial diversity (richness and evenness) was calculated with Shannon estimator of diversity which takes into consideration the presence of OTUs in a sample as well as their abundance in samples. The Shannon index was averaged at 2.9 in the S1 samples and reduced to 0.86 in the S2 samples. Significantly lower diversity in the pre-obturation samples when compared to initial intra canal samples (Wilcoxon rank-sum test, p=0.03). Table 2.1 and Figure 2.1 illustrate different measures of richness and diversity of the S1 and S2 samples.

Table 2.1: Comparison of diversity indices between S1 and S2 samples

		S1	**S2**	***p-value***
Observed	Mean (SD)	56.07 (41.6)	20.9 (6.3)	0.015*
Chao1 richness estimator	Mean (SD)	94.5 (57.01)	30.22 (9.8)	0.018*
Shannon estimator	Mean (SD)	2.9 (1.1)	0.86 (0.4)	0.03*

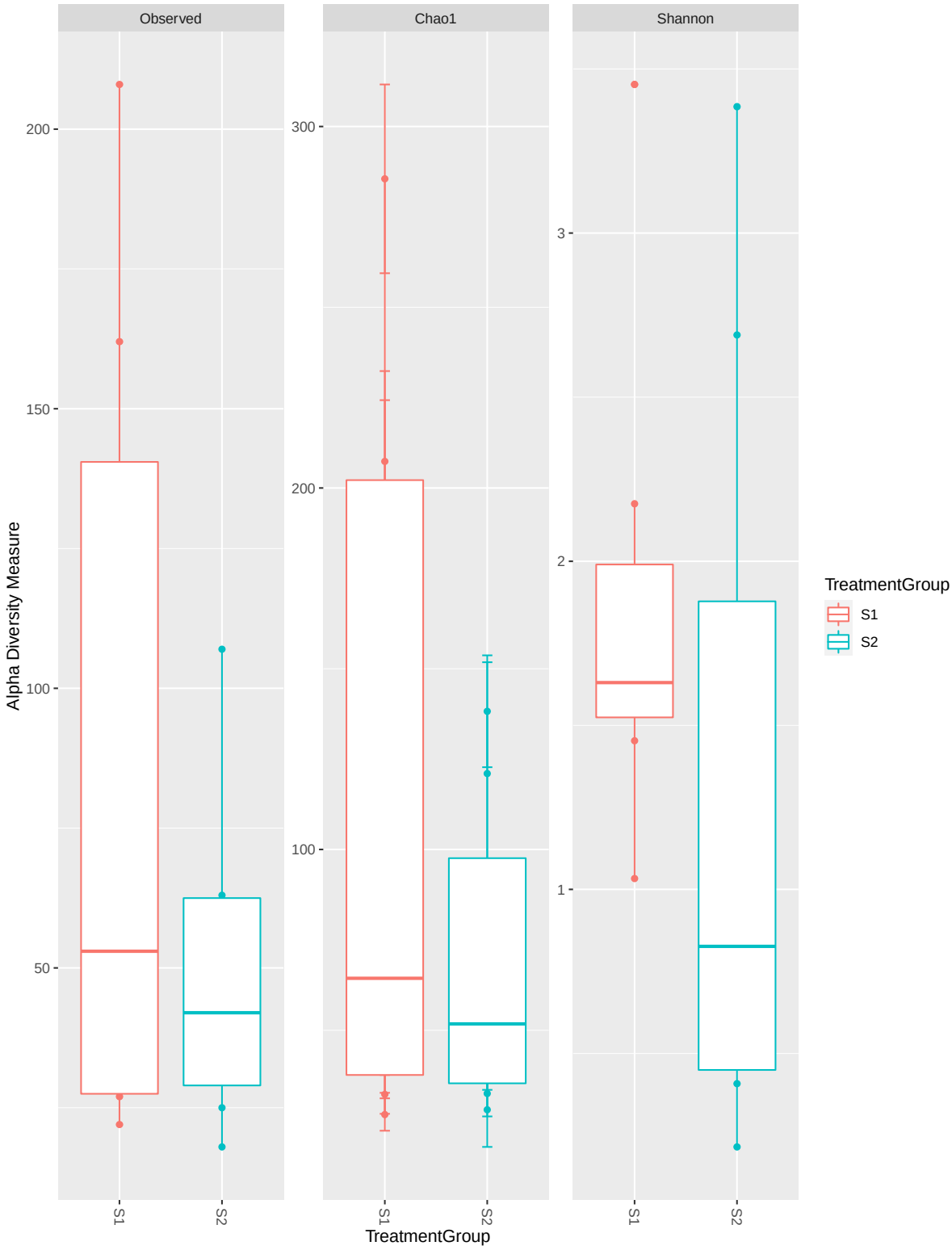


Figure 2.1: Microbial community richness (OUT and Chao1) and diversity (Shannon index) in the initial intra canal samples (S1) and pre-obturation samples (S2). Note a significant reduction in richness and diversity measures after chemomechanical preparations.

At the phyla level, Firmicutes was the most abundant phylum accounting for half of the OTUs in the S1 and 60% in the S2 samples. Following Firmicutes, the next most abundant phyla were Actinobacteria (25% and 17%) and Proteobacteria (5% and 21%) in S1 and S2 samples respectively, as shown in Figure A.2.

At the Genus level, some taxa were only detected in the pre-obturation samples but not present in the initial intracanal samples (cut-off 0.01); *Staphylococcus* 10%, *Bulleidia* 8.6%, *Haemophilus* 3.3%, *Mycobacterium* 1.8%, and *Sphingomonas* 2.3%.

On the other hand, some commonly found genera in both groups indicating persistence after chemomechanical preparation were *Streptococcus* (13% in S1,19% in S2), *Propionibacterium* (9.3%,11%), *Enterococcus* (2.8%,5.4%), *Exiguobacterium* (2.8%,3.6%), *Granulicatella* (1.8%,5.7%), and *Rothia* (2%,1.1%). Table 2.2 and Figure 2.2 summarize the different genera detected in each intra canal sample group.

Table 2.2: The relative abundance of most common genera detected in S1 and S2 samples (cut-off 0.01)

Genera	Relative abundance in S1	Relative abundance in S2
Alkalibacterium	4.3	Less than 0.01%
Bulleidia	Less than 0.01%	8.6
Corynebacterium	9.7	Less than 0.01%
Enterococcus	2.8	5.4
Exiguobacterium	2.8	3.6
Fusobacterium	4.3	
Granulicatella	1.8	5.7
Haemophilus	Less than 0.01%	3.3
Mycobacterium	Less than 0.01%	1.8
Porphyromonas	5.6	
Propionibacterium	9.3	11.4
Rothia	2	1.1
Sphingomonas	Less than 0.01%	2.3
Staphylococcus	Less than 0.01%	10.9
Streptococcus	13	19.7
Veillonella	16.2	Less than 0.01%

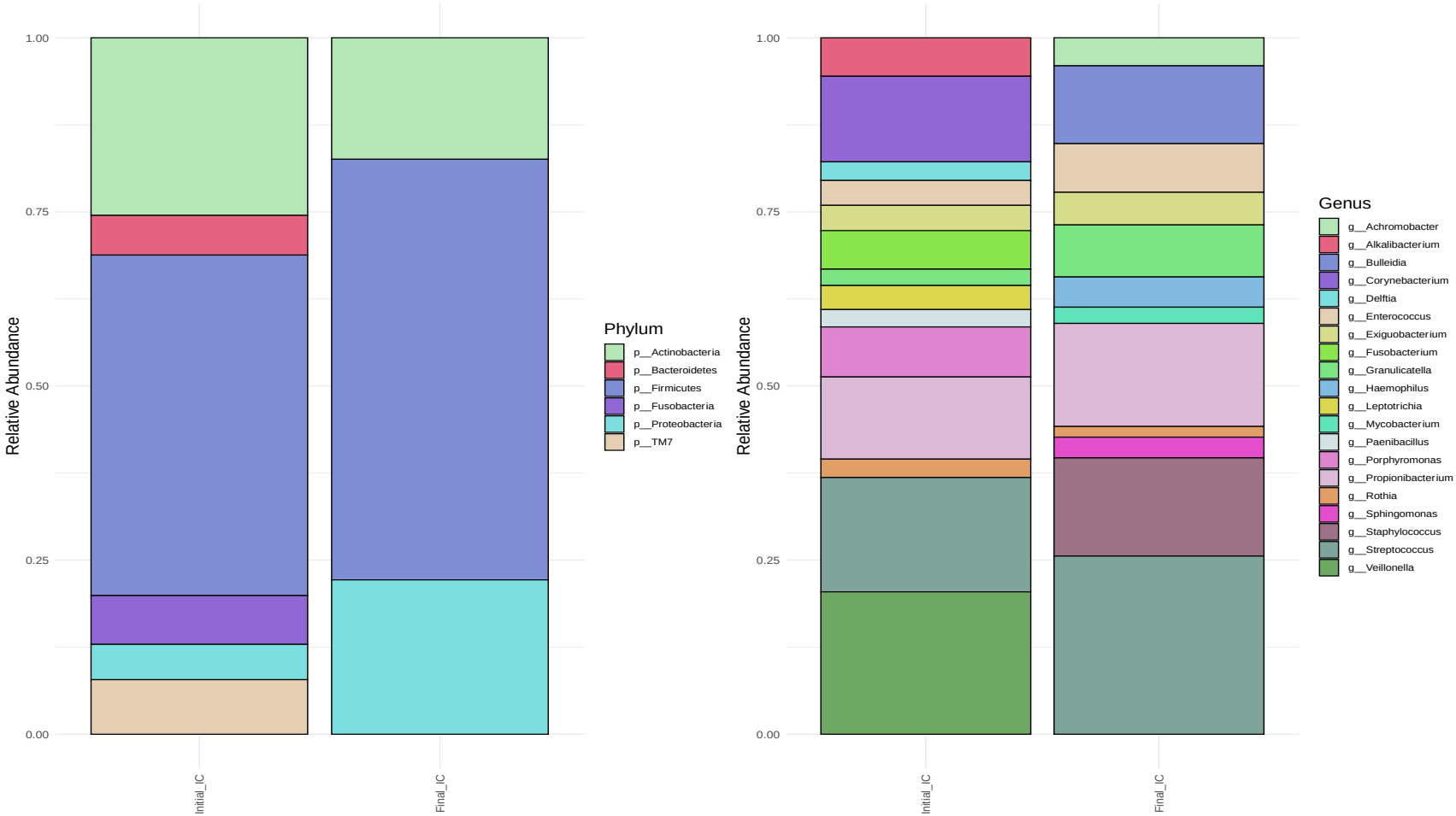


Figure 2.2 The most abundant phyla (left) and genera (right) identified in intra canal samples (S1 and S2).

Note the introduction of *Staphylococcus* 10%, *Bulleidia* 8.6%, *Haemophilus* 3.3%, *Mycobacterium* 1.8%, and *Sphingomonas* 2.3% at the S2 samples. Also, the persistence of *Streptococcus* , *Propionibacterium* , *Enterococcus* , *Exiguobacterium* , *Granulicatella*, and *Rothia* in S1 and S2 samples.

Appendix 3: The most abundant genera detected in different samples collected from gloves, rubber dam, files and instruments.

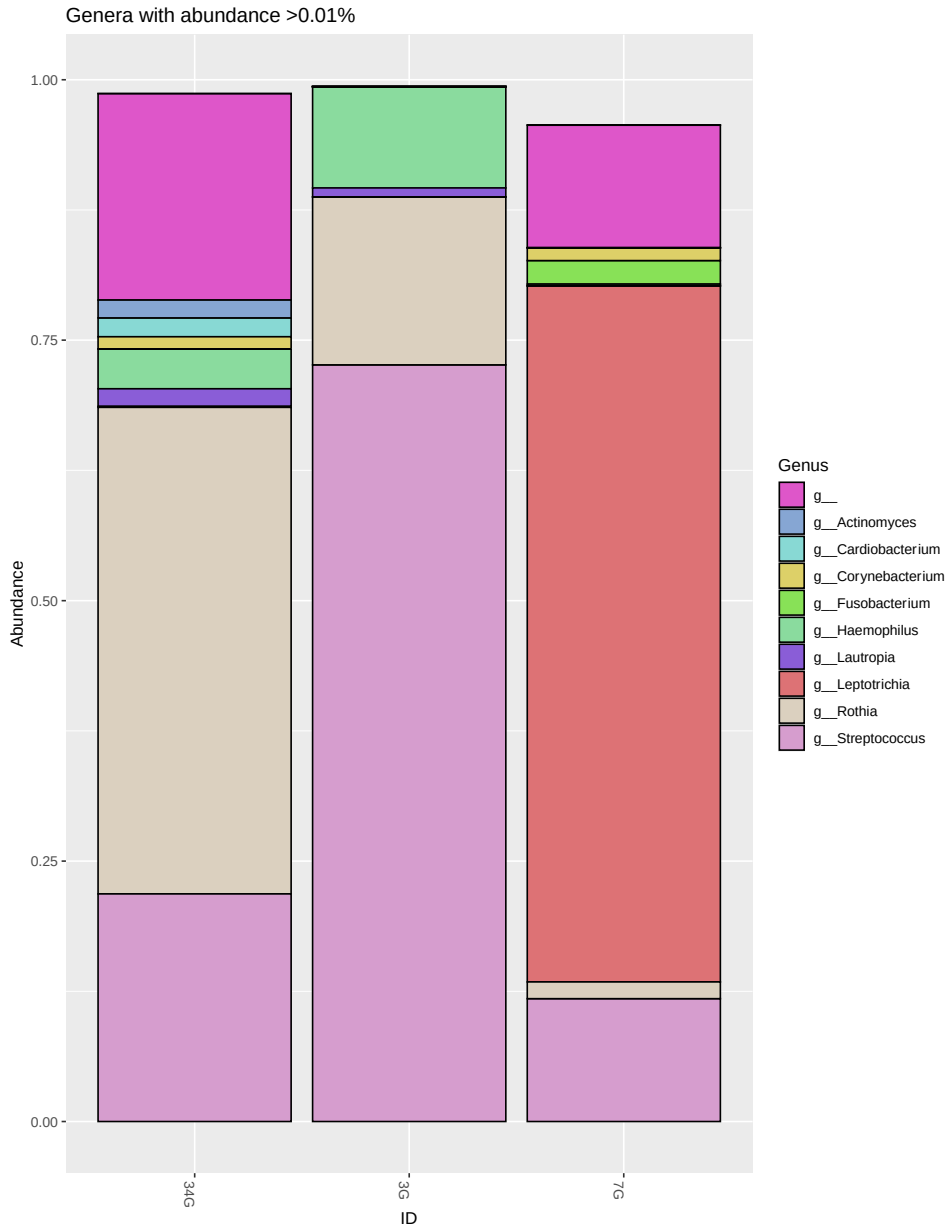


Figure 3.1: Abundant genera identified in gloves samples

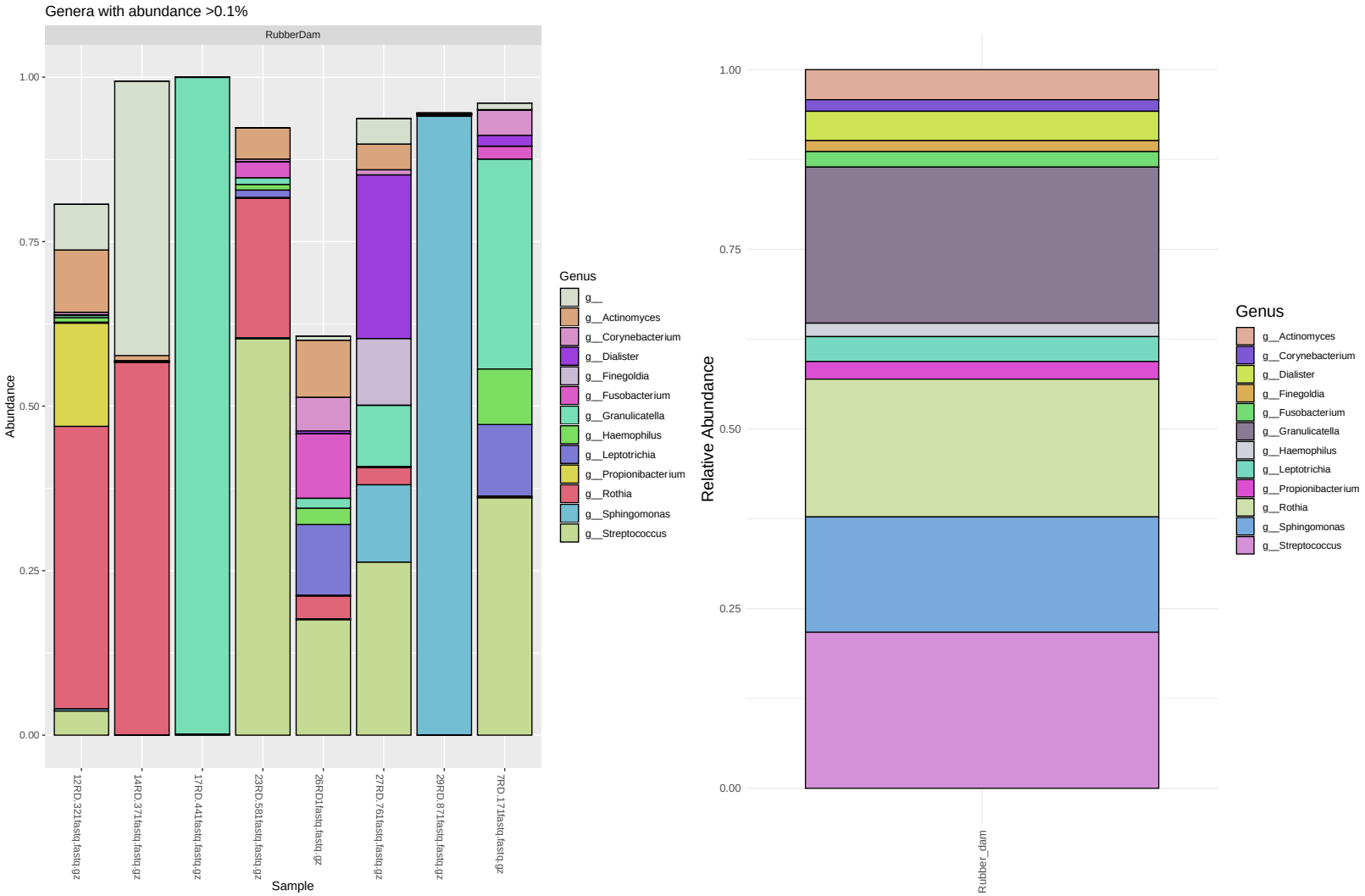


Figure 3.2: Top genera identified in rubber dam samples

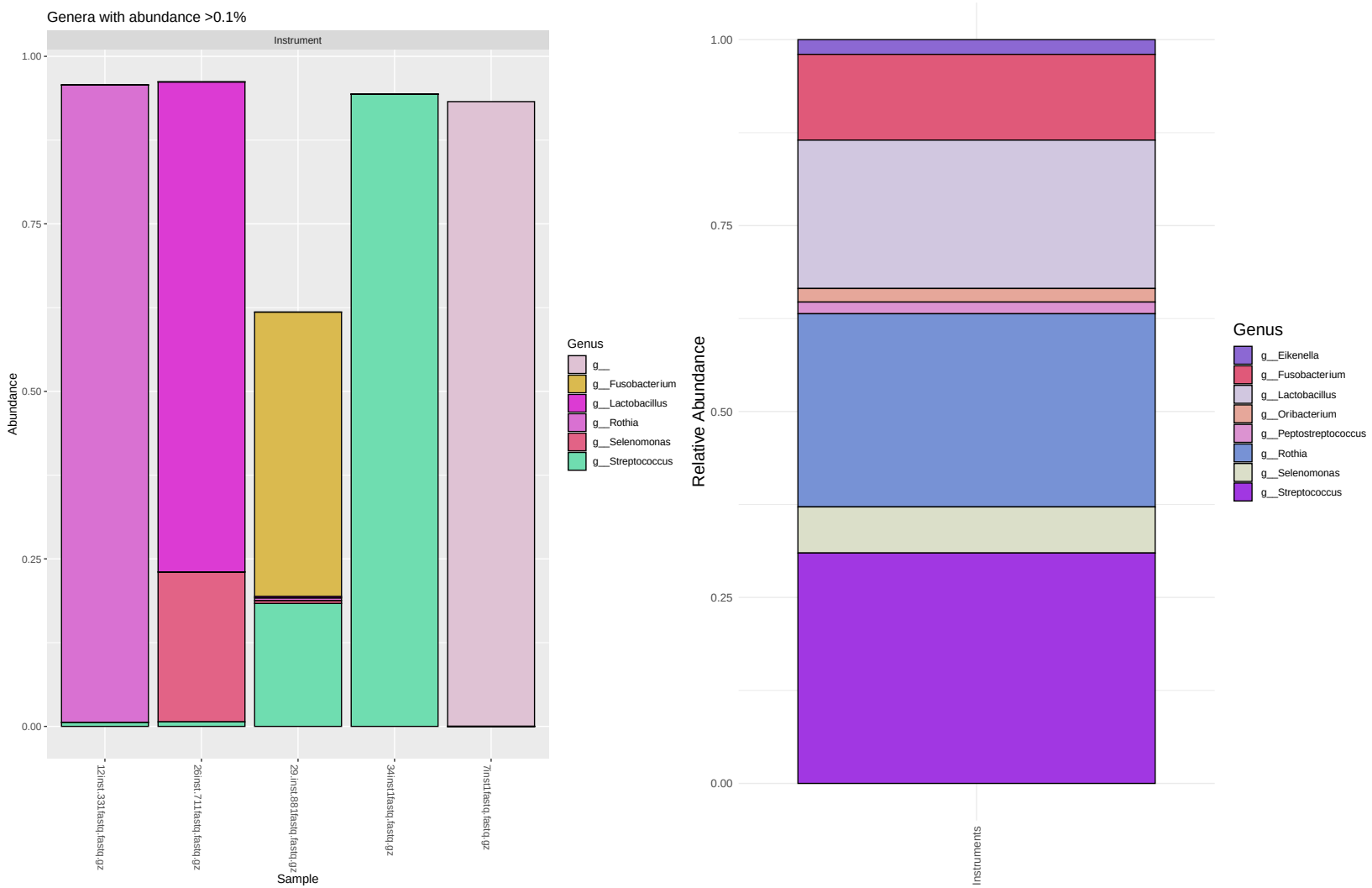


Figure 3.3: Genera abundantly expressed in instruments (Tip of the tweezers, DG-16 endodontic explorers, pluggers and flat plastic instruments) (cut-off 0.01).

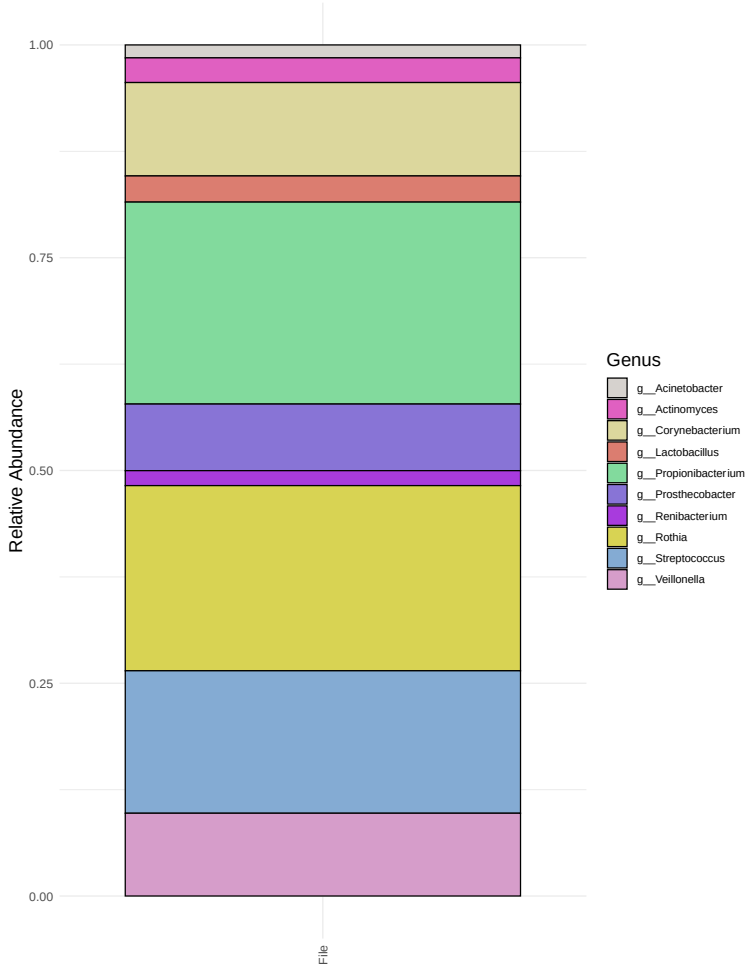


Figure 3.4: Abundant genera in files samples (cut-off 0.01).

Appendix 4: Health Research Authority (HRA) and Health and Care Research Wales (HCRW) Approval:

NHS
Health Research Authority

London - Surrey Research Ethics Committee
Whitefriars
Level 3, Block B
Lewins Mead
Bristol
BS1 2NT

Telephone: 0207 1048058

Please note: This is the favourable opinion of the REC only and does not allow you to start your study at NHS sites in England until you receive HRA Approval

23 October 2018

Prof Francesco Mannocci
King's College London Dental Institute
Floor 25, Tower Wing,Guy's Dental Hospital
London, UK
SE1 9RT

Dear Prof Mannocci

Study title:	**Impact of an Enhanced Sterile Protocol on Root Canal Treatment Outcome.**
REC reference:	**18/LO/1661**
Protocol number:	**n/a**
IRAS project ID:	**242774**

Thank you for your letter of 23 October, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information was considered in correspondence by a Sub-Committee of the REC.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to make a request to postpone publication, please contact hra.studyregistration@nhs.net outlining the reasons for your request.

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.

Conditions of the favourable opinion

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

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

Management permission should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements. Each NHS organisation must confirm through the signing of agreements and/or other documents that it has given permission for the research to proceed (except where explicitly specified otherwise).

Guidance on applying for HRA and HCRW Approval (England and Wales)/ NHS permission for research is available in the Integrated Research Application System, at www.hra.nhs.uk 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 management permissions from host organisations

Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database within 6 weeks of recruitment of the first participant (for medical device studies, within the timeline determined by the current registration and publication trees).

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g. when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non-clinical trials this is not currently mandatory.

If a sponsor wishes to request a deferral for study registration within the required timeframe, they should contact hra.studyregistration@nhs.net. The expectation is that all clinical trials will be registered, however, in exceptional circumstances non registration may be permissible with prior agreement from the HRA. Guidance on where to register is provided on the HRA website.

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).

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

The Committee has not yet completed any site-specific assessment (SSA) for the non-NHS research site(s) taking part in this study. The favourable opinion does not therefore apply to any non-NHS site at present. We will write to you again as soon as an SSA application(s) has been reviewed. In the meantime no study procedures should be initiated at non-NHS sites.

Approved documents

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

Document	*Version*	*Date*
Covering letter on headed paper [Clarification letter - patient identification]		10 September 2018
Covering letter on headed paper [Covering letter]		22 October 2018
Evidence of Sponsor insurance or indemnity (non NHS Sponsors only) [Insurance letter/ Certificate]	2	16 October 2018
IRAS Application Form [IRAS_Form_07092018]		07 September 2018
Letter from funder [financial guarantee tuition fees]		16 August 2018
Participant consent form [Consent form IRAS 242774 V1.0 16-08-18]	2	16 October 2018
Participant information sheet (PIS) [Patient information sheet IRAS 242774 V1.0 16-08-18]	2	16 October 2018
Research protocol or project proposal [Protocol IRAS 242774 V1.0 16-08-2018]	2	16 October 2018
Summary CV for Chief Investigator (CI) [CI CV]	1.0	16 August 2018
Summary CV for student [student cv]		
Summary CV for supervisor (student research) [CV Supervisor Patel]		
Summary CV for supervisor (student research) [Garrit Koller CV]		16 August 2018

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

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website:
http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/

HRA Training

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

18/LO/1661	**Please quote this number on all correspondence**

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

Yours sincerely

Pp

Chair

Email:nrescommittee.secoast-surrey@nhs.net

Appendix 5: Health and Care Research Wales (HCRW) approval

Ymchwil Iechyd a Gofal Cymru
Health and Care Research Wales

NHS
Health Research Authority

Professor Francesco Mannocci
King's College London Dental Institute
Floor 25, Tower Wing,Guy's Dental Hospital
London, UK
SE1 9RT

Email: hra.approval@nhs.net

23 October 2018

Dear Professor Mannocci

HRA and Health and Care Research Wales (HCRW) Approval Letter

Study title:	**Impact of an Enhanced Sterile Protocol on Root Canal Treatment Outcome.**
IRAS project ID:	**242774**
REC reference:	**18/LO/1661**
Sponsor	**King's College London**

I am pleased to confirm that **HRA and Health and Care Research Wales (HCRW) Approval** has been given for the above referenced study, on the basis described in the application form, protocol, supporting documentation and any clarifications received. You should not expect to receive anything further relating to this application.

How should I continue to work with participating NHS organisations in England and Wales?
You should now provide a copy of this letter to all participating NHS organisations in England and Wales, as well as any documentation that has been updated as a result of the assessment.

This is a single site study sponsored by a partner academic institution, under joint research governance arrangements. The Joint R&D Office will confirm to you when the study can start following issue of HRA and HCRW Approval.

It is important that you involve both the research management function (e.g. R&D office) supporting each organisation and the local research team (where there is one) in setting up

your study. Contact details of the research management function for each organisation can be accessed here.

How should I work with participating NHS/HSC organisations in Northern Ireland and Scotland?
HRA and HCRW Approval does not apply to NHS/HSC organisations within the devolved administrations of Northern Ireland and Scotland.

If you indicated in your IRAS form that you do have participating organisations in either of these devolved administrations, the final document set and the study wide governance report (including this letter) has been sent to the coordinating centre of each participating nation. You should work with the relevant national coordinating functions to ensure any nation specific checks are complete, and with each site so that they are able to give management permission for the study to begin.

Please see IRAS Help for information on working with NHS/HSC organisations in Northern Ireland and Scotland.

How should I work with participating non-NHS organisations?
HRA and HCRW Approval does not apply to non-NHS organisations. You should work with your non-NHS organisations to obtain local agreement in accordance with their procedures.

What are my notification responsibilities during the study?

The document "*After Ethical Review – guidance for sponsors and investigators*", issued with your REC favourable opinion, gives detailed guidance on reporting expectations for studies, including:

- Registration of research
- Notifying amendments
- Notifying the end of the study

The HRA website also provides guidance on these topics, and is updated in the light of changes in reporting expectations or procedures.

I am a participating NHS organisation in England or Wales. What should I do once I receive this letter?
You should work with the applicant and sponsor to complete any outstanding arrangements so you are able to confirm capacity and capability in line with the information provided in this letter.

The sponsor contact for this application is as follows:

IRAS project ID	242774

Name: Professor Reza Razavi
Tel: 02078483224
Email: reza.razavi@kcl.ac.uk

Who should I contact for further information?
Please do not hesitate to contact me for assistance with this application. My contact details are below.

Your IRAS project ID is **242774**. Please quote this on all correspondence.

Yours sincerely

Juliana Araujo
Assessor
Email: hra.approval@nhs.net

Copy to: *Sponsor Representative: Professor Reza Razavi, King's College London*
Lead NHS R&D Office Representative: Jennifer Boston, Guy's and St Thomas' NHS Foundation Trust

IRAS project ID	242774

List of Documents

The final document set assessed and approved by HRA and HCRW Approval is listed below.

Document	*Version*	*Date*
Covering letter on headed paper [Clarification letter - patient identification]		10 September 2018
Covering letter on headed paper [Covering letter]		22 October 2018
Evidence of Sponsor insurance or indemnity (non NHS Sponsors only) [Insurance letter/ Certificate]	2	16 October 2018
IRAS Application Form [IRAS_Form_07092018]		07 September 2018
Letter from funder [financial guarantee tuition fees]		16 August 2018
Participant consent form [Consent form IRAS 242774 V1.0 16-08-18]	2	16 October 2018
Participant information sheet (PIS) [Patient information sheet IRAS 242774 V1.0 16-08-18]	2	16 October 2018
Research protocol or project proposal [Protocol IRAS 242774 V1.0 16-08-2018]	2	16 October 2018
Summary CV for Chief Investigator (CI) [CI CV]	1.0	16 August 2018
Summary CV for student [student cv]		
Summary CV for supervisor (student research) [CV Supervisor Patel]		
Summary CV for supervisor (student research) [Garrit Koller CV]		16 August 2018
18 LO 1661 Favourable_opinion_on_further_information 23.10.18.pdf		23 October 2018
18 LO 1661_Application_valid 10.09.18.pdf		10 September 2018
242774 18 LO 1661 Ltr - provisional opinion 16.10.18.pdf		16 October 2018

IRAS project ID	242774

Summary of assessment

The following information provides assurance to you, the sponsor and the NHS in England and Wales that the study, as assessed for HRA and HCRW Approval, is compliant with relevant standards. It also provides information and clarification, where appropriate, to participating NHS organisations in England and Wales to assist in assessing, arranging and confirming capacity and capability.

Assessment criteria

Section	Assessment Criteria	Compliant with Standards	Comments
1.1	IRAS application completed correctly	Yes	No comments
2.1	Participant information/consent documents and consent process	Yes	No comments
3.1	Protocol assessment	Yes	No comments
4.1	Allocation of responsibilities and rights are agreed and documented	Yes	An agreement is not expected as Joint Research Office arrangements are in place between the sponsor and the participating NHS organisation.
4.2	Insurance/indemnity arrangements assessed	Yes	No comments
4.3	Financial arrangements assessed	Yes	The sponsor secured funding from the Royal Embassy of Saudi Arabia. A copy of the unconditional funding award letter was received.
5.1	Compliance with the Data Protection Act and data security issues assessed	Yes	No comments

Section	Assessment Criteria	Compliant with Standards	Comments
5.2	CTIMPS – Arrangements for compliance with the Clinical Trials Regulations assessed	Not Applicable	No comments
5.3	Compliance with any applicable laws or regulations	Yes	No comments
6.1	NHS Research Ethics Committee favourable opinion received for applicable studies	Yes	NHS Research Ethics Committee favourable opinion was confirmed by the London - Surrey Research Ethics Committee on 23 October 2018.
6.2	CTIMPS – Clinical Trials Authorisation (CTA) letter received	Not Applicable	No comments
6.3	Devices – MHRA notice of no objection received	Not Applicable	No comments
6.4	Other regulatory approvals and authorisations received	Not Applicable	No comments

Participating NHS Organisations in England and Wales

This provides detail on the types of participating NHS organisations in the study and a statement as to whether the activities at all organisations are the same or different.
This is a single site study; there is therefore one site type. The Chief Investigator or sponsor should share relevant study documents with participating NHS organisations in England and Wales in order to put arrangements in place to deliver the study. The documents should be sent to both the local study team, where applicable, and the office providing the research management function at the participating organisation. Where applicable, the local LCRN contact should also be copied into this correspondence. If chief investigators, sponsors or principal investigators are asked to complete site level forms for participating NHS organisations in England and Wales which are not provided in IRAS, the HRA or HCRW websites, the chief investigator, sponsor or principal investigator

should notify the HRA immediately at hra.approval@nhs.net or HCRW at Research-permissions@wales.nhs.uk. We will work with these organisations to achieve a consistent approach to information provision.

Principal Investigator Suitability

This confirms whether the sponsor position on whether a PI, LC or neither should be in place is correct for each type of participating NHS organisation in England and Wales, and the minimum expectations for education, training and experience that PIs should meet (where applicable).
A Principal Investigator should be in place at the participating NHS organisation in England. The Chief Investigator will take on this role at the sole participating site. GCP training is not a generic training expectation, in line with the HRA/HCRW/MHRA statement on training expectations.

HR Good Practice Resource Pack Expectations

This confirms the HR Good Practice Resource Pack expectations for the study and the pre-engagement checks that should and should not be undertaken
Where arrangements are not already in place, network staff (or similar) undertaking any of the research activities listed in A18 or A19 of the IRAS form (except for administration of questionnaires or surveys), would be expected to obtain an honorary research contract from one NHS organisation (if university employed), followed by Letters of Access for subsequent organisations. This would be on the basis of a Research Passport (if university employed) or an NHS to NHS confirmation of pre-engagement checks letter (if NHS employed). These should confirm enhanced DBS checks, including appropriate barred list checks, and occupational health clearance. For research team members only administering questionnaires or surveys, a Letter of Access based on standard DBS checks and occupational health clearance would be appropriate.

Other Information to Aid Study Set-up

This details any other information that may be helpful to sponsors and participating NHS organisations in England and Wales to aid study set-up.
The applicant has indicated that they do not intend to apply for inclusion on the NIHR CRN Portfolio.

Appendix 6: Patient information sheets:

Guy's and St Thomas' NHS
NHS Foundation Trust

KING'S College LONDON

Study Title: Impact of an Enhanced Sterile Protocol on Root Canal Treatment Outcome.

REC Ref No: 18/LO/1661

Dear Sir/Madam,

You were selected as a possible participant in this study because you are scheduled for a root canal treatment. Before you decide, please take the time to read following information carefully. Ask us if anything is not clear or if you would like more information. Take time to decide whether you wish to take part.
Thank you in advance.

What is the purpose of the study?
The study is a part of a PhD project named as Clinical Dentistry awarded by King's College London. It is investigating the effect of sterile protocol on attempting to reduce microbial numbers during root canal treatment and its outcome. The sterile protocol is aimed at reducing bacteria from the root canal system. If you agreed to participate, you will be randomly assigned to one of the two study groups. The first group will receive routine root canal treatment. In the second group, the dentist will perform root canal therapy following the routine treatment, in addition to more sterilisation steps of instruments and materials used throughout the treatment. We want to determine the effect of adopting this alternative protocol in removing/reducing bacteria as well as what this means in terms of outcome over the study period. We also want to study what types of bacteria are present in infected root canals using new advanced techniques aimed at cleaning materials and surfaces coming into contact with infected root canals or tooth tissues.

Dental X-rays (called periapical radiographs) are usually taken before, during and one year after root canal treatment. The amount of information gained from these x-rays is very limited because it is a 2D image of a 3D structure. More advance imaging technology is Cone Beam Computed Tomography (CBCT), which is commonly used for this indication. This 3D technology is more accurate in detecting disease in the bone around the root.

In this study, CBCT scans will be taken before and one year after root canal treatment. The additional radiation dose from this scan is minimal. Upon participating, total additional radiation is equal to 7% of annual background radiation which is equivalent to taking a flight from London to New York and back.

Why have I been invited?
We are inviting you to take part of this study because you have a tooth requires root canal treatment and you fulfil so called inclusion criteria, such as being over the age of 18. This makes you suitable for the study. We hope to recruit 176 volunteers. You are encouraged to ask any question you may have regarding your participation.

Do I have to take part?
No, your participation is entirely voluntary and your decision whether to participate will not affect the standard of care that you will receive. If you do decide to participate, you will be given a consent form to sign and you will be provided a copy. You are free to discontinue participation at any time without giving a reason. This will not affect the standard care you receive.
If after you consented and during the study you lost the capacity to consent, you will be automatically withdrawn from the study. Your Identifiable data or tissue already collected with consent would be retained and used in the study. No further data or tissue would be collected, or any other research procedures carried out on or in relation to you.

What will happen to me if I take part?
The root canal treatment that you have treatment planned to receive will be carried out as normal. The additional procedures as follow:

1. The collection of caries (Decay) which routinely removed during the treatment.
2. The collection of bacteria from the root that will be done by inserting absorbent paper points into the root canal.
3. One year follow up visit.
4. CBCT scans before the treatment and one year after completion.

Note: None of the procedures will cause pain and the length of the visits will not change because of these steps.

You will be randomly assigned to one of the two study groups:

One group will receive conventional routine root canal treatment following an established protocol which includes the following:

1. The affected tooth will receive local anaesthetic, a painkilling medication that numbs a specific area of the body.
2. A rubber dam (very similar to surgical drapes used in surgery to keep surgical sites sterile) will be placed around the affected tooth, a sheet of rubber is placed to ensure it is dry during treatment. Also prevents you from swallowing or breathing in any cleaning agents during the treatment.
3. Access opening will be done, a small opening in the crown of tooth is done to access the soft tissue at the centre of the tooth (pulp).
4. The length of the roots will be measured, using a so called an apex locator (electronic ruler) and conventional X-rays.
5. Cleaning and enlarging the root canal so it can be easily filled. This will be performed using series of small files to enlarge the canals.
6. A root canal filling will be inserted to fill the space created by the procedure. The material to fill the root canal is called "Gutta percha".
7. The access opening will be filled with an appropriate filling material.
   Note: Sterile dental instruments will be used throughout the procedure.

If you were selected in the second group; "The alternative sterile protocol":

The same procedure and steps will be followed. The exceptions as following:

1. At time of root canal filling (Step 6 above), all existing instruments will be replaced with a new sterile ones.
2. At time of filling, the rubber dam surface will be disinfected with an antibacterial solution (Sodium Hypochlorite) which is identical to the agent used already to clean the root canal space.
3. The dentist will change the gloves at the time of root canal filling, in addition to any time mandated by existing hygiene best practice.
4. The material used for obturation (Gutta percha) will be disinfected with the same sodium Hypochlorite solution (Antibacterial solution) given in step 2 of this alternative protocol.

What will I have to do?
Patients who agree to take part of the study will be required to attend 1 additional visit one year after treatment completion. During this visit, the treated tooth will be examined, and X-rays will be taken (Conventional and CBCT) to evaluate the success of root canal treatment. At that visit, if treatment failed or any intervention is needed, you will be referred accordingly.

What are the side effects, possible disadvantages and risks of taking part?
There are no side effects of taking part in this study other than those expected from routine dental care. Exposure to ionizing radiation carries a risk. However, due to low dose of radiation, from dental x-rays including CBCT, this risk is negligible. Periapical x-rays are normally taken for routine dental treatment and the effective dose from these conventional x-rays is equal to 0.19% of annual background radiation. In the current study, the dose from the CBCT is equal to 1.4 % annual background radiation. Taking two CBCT scans as a part of this study is equivalent to radiation on board aircraft for approximately 7 hours flight.

What are the possible benefits of taking part?
There is no direct benefit from participating in the study. Participants will benefit from the root canal treatment and the follow up appointment. The benefit from attending the recall is to determine if the treatment was successful or if any further treatment is required. The CBCT scans will allow us to accurately evaluate the success of the treatment as well as the anatomy of the root canal.

What if new information becomes available?
If we find new information this will not affect your course of treatment and you will be informed. If any abnormal findings in the scans identified, we will communicate with you and you will receive appropriate treatment as part of routine clinical management. If required, you may be referred to the appropriate clinical team or clinician for treatment.

What will happen to the results of the research study?
Results of this research will be published in dental and scientific journals. No personal information relating to you will be published. If you would like to know the results, please give the permission to the research team to access your contact information in the consent form. We will send you a summary of the results by email or post after the study been closed.

Who is organising and funding the research?
The sponsor of this study is King's College London.

Who has reviewed the study?
This study has been reviewed by the London-Surrey Research Ethics Committee.

What type of samples you are going to collect from me and how you are going to process it?
Experienced dentists undergoing their training to become specialist dentists (Endodontic MClinDent postgraduate students or PhD students who have the same experience) at KCL Guy's Hospital will collect samples of caries (Decay) which routinely removed during the treatment as well as bacteria from the root canal that will be done by inserting absorbent paper points into the root canal. These samples are residual materials and considered left over from routine root canal treatment.
The PhD student will transfer samples immediately to be stored in the lab then bacterial extraction and counting will be done. After that, disposal will be done in accordance with the Human Tissue Authority's Code of Practice.

Will my taking part in this study be kept confidential?
All information collected about you during the research will be kept strictly confidential and you will not be identified. You will be given a unique identification number; this number will be used during the study analysis and data collection. Your personal details and medical records will only be inspected by the research doctor. A linked spreadsheet that identifies your name and hospital number against your given study ID will be held separately in a secured locked filing cabinet accessed only by Principal Investigator on a strict-need-to-know basis only.

How long will my personal data be stored or accessed after the study has ended?
Your personal data will be accessed for 12 months after the study has ended. This to review any data collected during the research period.

How will the general data generated by the research will be stored after the study has ended?
The research data including the identification spread sheet will be stored securely in a locked cabinet in Guy's Hospital and can only be accessed by Prof. Francesco Mannocci. The electronic data of the research without any personal information will be archived in accordance with NHS data protection rules. This data will be stored for five years after the study has ended and disposed according to Data Protection Laws and King's College London policy.

New General Data Protection Regulations (2018)
King's College London and Guy's and St Thomas' NHS Foundation Trust as the co-sponsors for this study based in the United Kingdom. We will be using information from you and your medical records in order to undertake this study and will act as the data controller for this study. This means that we are responsible for looking after your information and using it properly. King's College London and Guy's and St Thomas' NHS Foundation Trust will keep identifiable information about you for 12 months after the study has finished.
Your rights to access, change or move your information are limited, as we need to manage your information in specific ways in order for the research to be reliable and accurate. If you withdraw from the study, we will keep the information about you that we have already obtained. To safeguard your rights, we will use the minimum personally-identifiable information possible.
You can find out more about how we use your information:
https://www.guysandstthomas.nhs.uk/research/patients/about.aspx

www.kcl.ac.uk/innovation/research/support/ethics/how-does-gdpr-affect-ethics/king's-college-london-statement-on-use-of-personal-data-in-research.aspx

King's College London and Guy's and St Thomas' NHS foundation trust will use your name, NHS number and contact details to contact you about the research study, and make sure that relevant information about the study is recorded for your care, and to oversee the quality of the study. Individuals from King's College London and Guy's and St Thomas' NHS foundation trust and regulatory organisations may look at your medical and research records to check the accuracy of the research study.
Guy's Hospital will pass these details to King's College London along with the information collected from you and your medical records. The only people in King's College London and Guy's and St Thomas' NHS foundation trust who will have access to information that identifies you will be people who need to contact you to set a follow up appointment or audit the data collection process. The people who analyse the information will not be able to identify you and will not be able to find out your name, NHS number or contact details.

What if something goes wrong?
If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions (Prof. Francesco Mannocci, Telephone: 020718881573, Email: francesco.mannocci@kcl.ac.uk). If you remain unhappy and wish to complain formally, you can do this through the Guy's and St Thomas' Patients Advice and Liaison Service (PALS) on 020 7188 8801, pals@gstt.nhs.uk. The PALS team are based in the main entrance on the ground floor at St Thomas' Hospital and on the ground floor at Guy's Hospital in the Tower Wing.
In the event that something does go wrong and you are harmed during the research you may have grounds for legal action for compensation against Guy's and St Thomas' NHS Foundation Trust and/or King's College London but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you (if appropriate).

Summary:
You are invited to participate in this PhD project study because you have a tooth that needs root canal treatment. Bacterial samples will be collected from your tooth during the treatment. One year after completion of treatment, you will be invited to follow up appointment. In this appointment, your tooth will be evaluated, and radiographs will be taken using CBCT.

Thank you for taking the time to read this and to take part in this study. If you have any questions about this research, please contact Shatha Zahran.

Contact for further Information
Shatha Zahran
King's College London Dental Institute
Floor 18, Tower Wing, SE1 9RT
Tel: 07403077970
Email:shatha.zahran@kcl.ac.uk

Appendix 7: Patients Informed written consent

Guy's and St Thomas' NHS
NHS Foundation Trust

KING'S College LONDON

REC Study Number: 18/LO/1661

Patient Identification Number for this trial:

CONSENT FORM FOR RESEARCH STUDY

Title of project: Impact of an Enhanced Sterile Protocol on Primary Root Canal Treatment Outcome.

Name of Researchers: Shatha Zahran, Prof. Francesco Mannocci, Garrit Koller, Shannon Patel.

	Please initial box
1- I confirm that I have read and understood the information sheet (Version 2, Date16/10/2018) for the above study and have had the opportunity to ask questions.	☐
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. I also understand that my participation to the study is confidential.	☐
3- I understand that sections of my medical notes may be looked at by responsible individuals from King's College London, Guy's and St Thomas' NHS Foundation Trust where it is relevant to me taking part in research. I give permission for these individuals to have access to my records.	☐
4- I understand that samples will be taken from my root canal and tooth decay and will be used for this research.	☐
5- I agree to take part in the above study.	☐
6- Optional: I would like to receive a summary of the results of the study. I consent to the researchteamusing my contact details to do this.	☐

.....	.....	.....
Name of patient	**Date**	**Signature**
.....	.....	.....
Name of person taking consent	**Date**	**Signature**

When completed: 1for participant; 1 for researcher site file; 1(Original) to be kept in medical notes.

Appendix 8: Factors affecting PA radiograph-based outcomes

Table 8.1: Outcome using PA by independent factors: total number of teeth, favourable outcome (%) and results of binary logistic regression: OR and 95%CI.

	Total	Favourable	OR	95%CI	*p-value*
Age (years)	37.6 ± 12.6	38.3 ± 12.6	1.00	0.98 – 1.06	0.990
Gender					
Male	49	45 (91.8)	1		
Female	66	61 (92.4)	1.08	0.28– 4.27	0.908
Tooth type					
1st molar	73	64 (87.7)	1		
2nd molar	41	41 (100)	--	--	--
3rd molar	1	1 (100)	--	--	--
Arch					
Upper	47	44 (93.6)	1		
Lower	68	62 (91.2)	1.71	0.71 – 2.97	0.633
Pulpal diagnosis					
Irreversible pulpitis	26	23 (88.5)	1		
Pulpal necrosis	19	17 (89.5)	1.11	0.17 – 7.38	0.915
Previously initiated	70	66 (94.3)	2.15	0.45 – 10.3	0.339
Pre-operative PARL (PA)					
No	65	60 (92.3)	1		
Yes	49	45 (91.8)	0.94	0.24 – 3.69	0.926
Pre-operative PARL (CBCT)					
No	50	47(94.0)	1		
Yes	65	59 (90.8)	0.63	0.15 – 2.64	0.526
Pre-operative cracks					
No	103	96 (93.2)	1		
Yes	12	10 (83.3)	0.37	0.07 – 2.00	0.245
Unfilled canals					
No	110	102 (92.7)	1		
Yes	5	4 (80.0)	0.31	0.03 – 3.15	0.325
Perforation					
No	112	103 (92.0)	1		
Yes	3	3 (100)	--	--	--
Obturation length					
Adequate	109	101 (92.7)	1		
Short	5	4 (80.0)	0.40	0.04 – 3.81	0.423
Long	1	1 (100)			
Obturation quality					
Inadequate	10	8 (80.0)	1		
Adequate	105	98 (93.3)	3.5	0.62 – 19.7	0.156
Restoration type					

Permanent coronal coverage	75	69 (92.0)	1		
Temporary (CORECEM)	40	37 (92.5)	1.07	0.25 – 4.54	0.924
Restoration quality					
Inadequate	16	15 (93.8)	1		
Adequate	99	91 (91.9)	0.76	0.09 – 6.51	0.801

*p<0.05; **p<0.01; ***p<0.001

Appendix 9: Prognostic factors effecting the completely healed outcome (CBCT and PA based outcomes)

Table 9.1: Outcome using CBCT by independent factors: total number of teeth, healed PARL (%) and results of binary logistic regression: OR and 95%CI.

	Total	Healed	OR	95%CI	*p-value*
Age (years)	37.6 ± 12.6	39.1 ± 13.3	1.03	0.99 – 1.06	0.131
Gender					
Male	49	30 (61.2)	1		
Female	66	38 (57.6)	0.86	0.40 – 1.83	0.694
Treatment group					
SP	54	25 (46.3)	1		
EP	61	43 (70.5)	2.7	1.2 – 5.9	**0.009****
Tooth type					
1st molar	73	39 (53.4)	1		
2nd molar	41	28 (68.3)	1.88	0.84 – 4.19	0.124
3rd molar	1	1 (100)	--	--	--
Arch					
Upper	47	25 (53.2)	1		
Lower	68	43 (63.2)	1.51	0.71 – 3.22	0.282
Pulpal diagnosis					
Irreversible pulpitis	26	20 (76.9)	1		
Pulpal necrosis	19	5 (26.3)	0.11	0.03 – 0.42	**0.005****
Previously initiated	70	43 (61.4)	0.48	0.17 – 1.34	
Pre-operative PARL (PA)					
No	65	48 (73.8)	1		
Yes	49	19 (38.8)	0.22	0.10 – 0.50	**<0.001*****
Pre-operative PARL (CBCT)					
No	50	43 (86.0)	1		
Yes	65	25 (38.5)	0.10	0.04 – 0.26	**<0.001*****
Pre-operative cracks					
No	103	63 (61.2)	1		
Yes	12	5 (41.7)	0.45	0.14 – 1.53	0.202
Unfilled canals					
No	110	66 (60.0)	1		
Yes	5	2 (40.0)	0.44	0.07 – 2.77	0.385
Perforation					
No	112	67 (59.8)	1		
Yes	3	1 (33.3)	0.34	0.03 – 3.82	0.379
Obturation length					
Adequate	109	66 (60.6)	1		
Short	5	1 (20.0)	0.33	0.06 – 1.86	0.207
Long	1	1 (100)			

Obturation quality					
Inadequate	10	2 (20.0)	1		
Adequate	105	66 (62.9)	6.77	1.37 – 33.5	**0.019***
Restoration type					
Permanent coronal coverage	75	49 (65.3)	1		
Temporary (CORECEM)	40	19 (47.5)	0.75	0.22 – 1.05	0.066
Restoration quality					
Inadequate	16	8 (50.0)	1		
Adequate	99	60 (60.6)	1.54	0.53 – 4.47	0.426

*p<0.05; **p<0.01; ***p<0.001

Table 9.2: Outcome using PA by independent factors: total number of teeth, healed PARL (%) and results of binary logistic regression: OR and 95%CI.

	Total	Healed	OR	95%CI	*p-value*
Age (years)	37.6 ± 12.6	38.8 ± 13.0	1.04	0.99 – 1.08	0.118
Gender					
Male	49	39 (79.6)	1		
Female	66	47 (71.2)	0.63	0.26 – 1.52	0.308
Treatment group					
SP	54	38 (70.4)	1		
EP	61	48 (78.7)	1.5	0.6 – 3.6	0.3
Tooth type					
1st molar	73	51 (69.9)	1		
2nd molar	41	34 (82.9)	2.10	0.81 – 5.44	0.129
3rd molar	1	1 (100)	--	--	--
Arch					
Upper	47	37 (78.7)	1		
Lower	68	49 (72.1)	0.70	0.29 – 1.68	0.420
Pulpal diagnosis					
Irreversible pulpitis	26	22 (84.6)	1		
Pulpal necrosis	19	12 63.2)	0.31	0.08 – 1.28	0.107
Previously initiated	70	52 (74.3)	0.53	0.16 – 1.73	0.290
Pre-operative PARL (PA)					
No	65	60 (92.3)	1		
Yes	49	25 (51.0)	0.09	0.03 – 0.25	**<0.001*** **
Pre-operative PARL (CBCT)					
No	50	46 (92.0)	1		
Yes	65	40 (61.5)	0.14	0.05 – 0.43	**0.001** **
Pre-operative cracks					
No	103	78 (75.7)	1		
Yes	12	8 (66.7)	0.64	0.18 – 2.31	0.497
Unfilled canals					
No	110	82 (74.5)	1		
Yes	5	4 (80.0)	1.37	0.15 – 12.7	0.784
Perforation					
No	112	83 (74.1)	1		
Yes	3	3 (100)	--	--	--
Obturation length					
Adequate	109	82 (75.2)	1		
Short	5	3 (60.0)	0.66	0.11 – 3.80	0.640
Long	1	1 (100)			
Obturation quality					
Inadequate	10	8 (80.0)	1		
Adequate	105	78 (74.3)	0.72	0.14 – 3.61	0.692

Restoration type					
Permanent coronal coverage	75	61 (81.3)	1		
Temporary (CORECEM)	40	25 (62.5)	0.38	0.16 – 0.91	**0.029***
Restoration quality					
Inadequate	16	10 (62.5)	1		
Adequate	99	76 (76.8)	1.98	0.65 – 6.04	0.229

*p<0.05; **p<0.01; ***p<0.001

Appendix 10: Other factors affecting microbial load in pre-obturation samples

10.1: Factors affecting Bacterial reduction/increase

Other clinical variables: age, gender, pulpal diagnosis, arch, tooth type, initial bacterial load at S1, and presence of PARL were evaluated to detect variables predicting bacterial reduction/increase using multiple regression model. Besides the treatment protocol, the only significant variable was the microbial initial load at S1 as shown in Table 10.1. Increased bacterial load at initial samples (S1) increased the probability of bacterial reduction (OR=1, p=0.035, CI: 1.0-1.0) i.e., cases with lower microbial load initially are at a higher risk of introducing bacteria during root canal treatment (Figure 10.1). After the multivariate analysis, cases in the EP group were almost four folds more likely to have a bacterial reduction than cases in the standard group (OR=3.8; p=0.003).

Table 10.1: Multiple logistic regression model of all predictors affecting bacterial reduction from S1 to S2.

Change of microbial load after chemomechanical preparation using bacterial reduction: Total number of teeth, number of cases with reduced microbial load (%) and results of multiple binary logistic regression: adjusted OR and 95%CI.

	Total	Bacterial reduction	OR	95%CI	p
Age (years)	36.5±12.2	36.5±12.2	0.9	0.96–1.03	0.8
Gender					
Male	61	49 (80.3)	1		
Female	80	57 (71.3)	0.6	0.25 – 1.5	0.31
Tx protocol					
Standard	71	45 (63.4)	1		
EP	70	61 (87.1)	3.8	1.5– 9.6	**0.003****
Tooth type					
1st molar	99	74 (74.7)	1		
2nd molar	41	31 (75.6)	0.98	0.3 – 2.5	0.96
3rd molar	1	1 (100)	--	--	--
Arch					
Upper	59	39 (66.1)	1		
Lower	82	67 (81.7)	1.9	0.8 – 4.7	0.13
Pulpal diagnosis					
Irreversible pulpitis	30	23 (76.7)	1		
Pulpal necrosis	27	22 (81.5)	2.08	0.38 – 11.1	0.39
Previously initiated	84	61 (72.6)	0.94	0.28 – 3.1	0.92
Pre-operative PARL (CBCT)					
No	55	44 (80)	1		
Yes	86	62 (72.1)	0.52	0.918– 1.5	0.24
Bacterial initial gene copy number (S1)					
	2.294×10^3	3.3×10^3	1.0	1.0-1.0	**0.035***

*p<0.05; **p<0.01; ***p<0.001

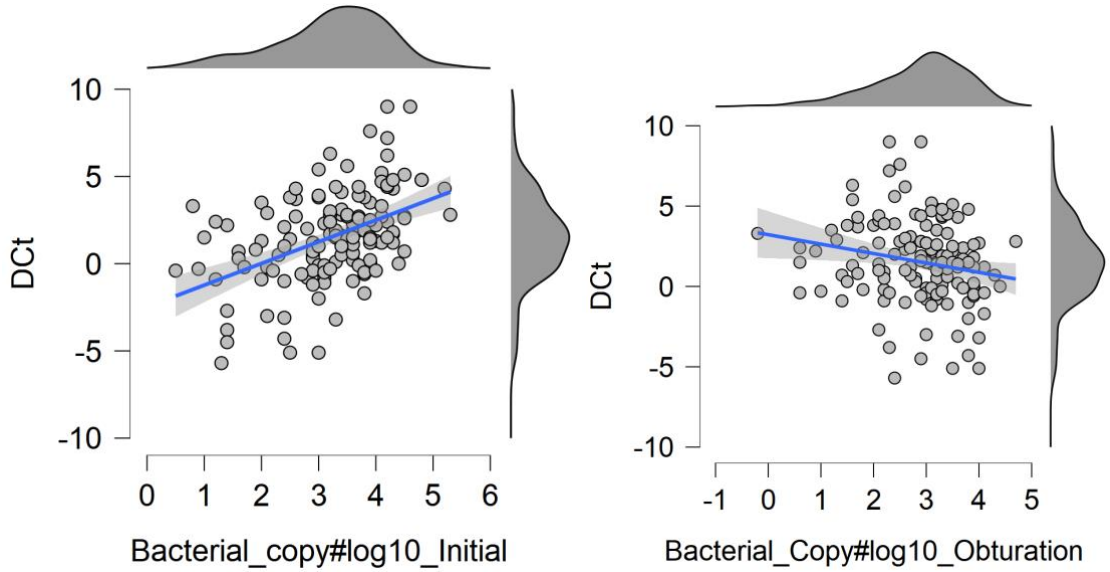


Figure 10.1: Scatterplot highlighting the correlation between cleaning efficiency and initial/ final bacterial counts.

Effective cleaning reflected by greater bacterial reduction (higher δCt) was associate with increased bacterial count initially (left). This was not reflected by the bacterial counts prior to obturation, suggesting contamination (right).

10.2:Factors affecting the presence/ absence of bacteria gDNA prior to obturation

Other clinical measures were also evaluated to check any significant association to this outcome measure (presence/absence of bacteria at S2). The significant variables other than treatment protocol affecting the presence/absence in pre-obturation samples were age, initial bacterial load at S1 and teeth diagnosed with pulpal necrosis (Table 10.2). Cases in the EP group had a higher probability of negative bacteria in pre-obturation samples than standard protocol (OR=43.16, p=0.001). Cases diagnosed with pulpal necrosis had a lower probability of negative bacteria in pre-obturation samples than cases with irreversible pulpitis (OR=0.022, p=0.043). Cases with higher microbial load initially (S1) reduces the probability of having bacteria-free samples prior to obturation (OR=0.95, p=0.002). The strongest trend with residual bacterial numbers at obturation was noted for bacterial initial copy numbers (Figure 10.2).

Table 10.2: Multiple logistic regression model of all predictors affecting the absence of bacterial gDNA in pre-obturation samples.

Total number of teeth, number of cases with negative bacteria (%) and results of multiple binary logistic regression: adjusted OR and 95%CI.

	Total	Negative bacteria S2	OR	95%CI	p
Age (years)	36.5±12.2	44±13.1	1.07	1.006–1.14	**0.032***
Gender					
Male	61	9 (14.8)	1		
Female	80	11 (13.8)	1.6	0.26 – 10.7	0.58
Tx protocol					
Standard	71	5 (7)	1		
EP	70	15 (21.4)	43.16	4.3– 427.7	**0.001****
Tooth type					
1st molar	99	13 (13.1)	1		
2nd molar	41	7 (17.1)	1.1	0.2 – 6.7	0.85
3rd molar	1	0 (0)	--	--	--
Arch					
Upper	59	8 (13.6)	1		
Lower	82	12 (14.6)	1.2	0.19 – 8.2	0.79
Pulpal diagnosis					
Irreversible pulpitis	30	5 (16.7)	1		
Pulpal necrosis	27	2 (7.4)	0.022	0.001 – 0.883	**0.043***
Previously initiated	84	13 (15.5)	0.19	0.017 – 2.29	0.194
Pre-operative PARL (CBCT)					
No	55	12 (21.8)	1		
Yes	86	8 (9.3)	11.3	0.9 – 132.2	0.052
Bacterial initial gene copy number (S1)					
	2.294×10^3	6.7×10^1	0.995	0.992-0.998	**0.002****

*p<0.05; **p<0.01; ***p<0.001

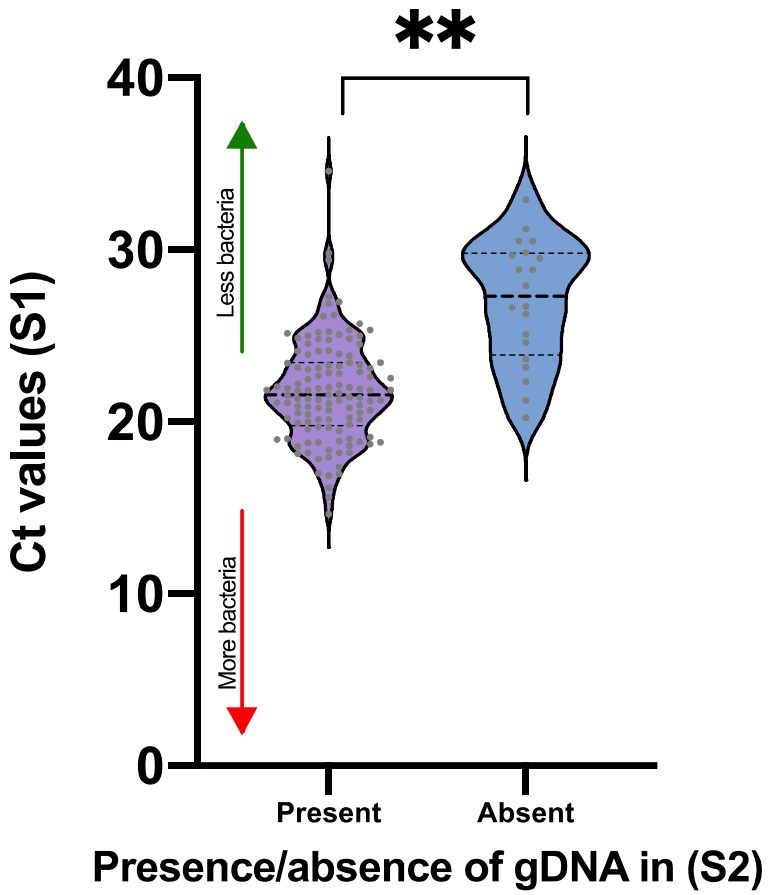


Figure 10.2: Violin plot of initial samples Ct values and presence/absence of bacterial gDNA amplification prior to obturation (S2).

Showing that presence of gDNA amplification in pre-obturation samples was associated with greater bacterial count initially (lower Ct values in S1 samples). Dashed line indicate the mean while top and bottom dotted lines indicate the 25th and 75th quartiles.

Appendix 11: Microbial load in paper points and files

Initial intracanal samples were taken with paper points (S1) and files (F). The relation between pairs of initial paper points and files of intracanal samples was determined with Pearson's correlation. The Pearson coefficient was equal to 0.2 indicating a weak positive correlation. This correlation was significant (p=0.018). In order to assess the two methods (paper point and file) agreement on bacterial load measurement from the canal, Bland- Altman statistical method was used as shown in Figures 11.1, 11.2.

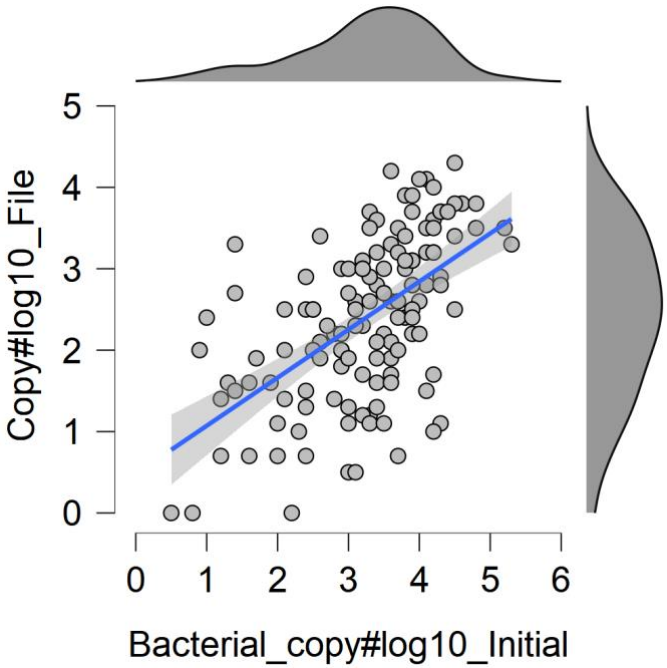


Figure 11.1: Scatterplot showing agreement in bacterial count between paper points and file sampling.

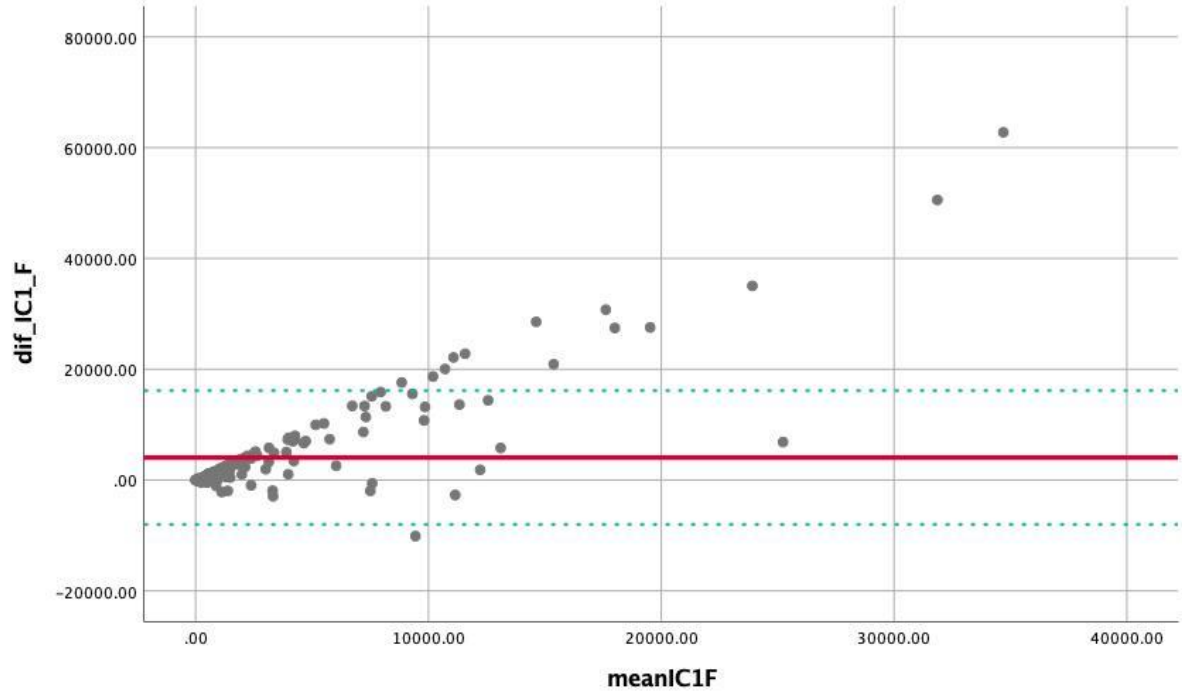


Figure 11.2: Bland Altman plot for initial intra canal samples (S1) and initial file samples (F1). The red line represents the mean difference in initial bacterial load between paper points and files. The top dotted green line represents the mean difference + 2SD while the bottom dotted line represents the difference -2SD. The plot shows a quite good agreement at the lower end when smaller values of bacterial load measured. As the bacterial load increases, the difference between the two measures also increases and the discrepancy between the two measure increases. Majority of the difference was found to be positive, indicating higher bacterial load detected in paper points (S1) than files (F1).

Appendix 12: Microbiological profile of control samples

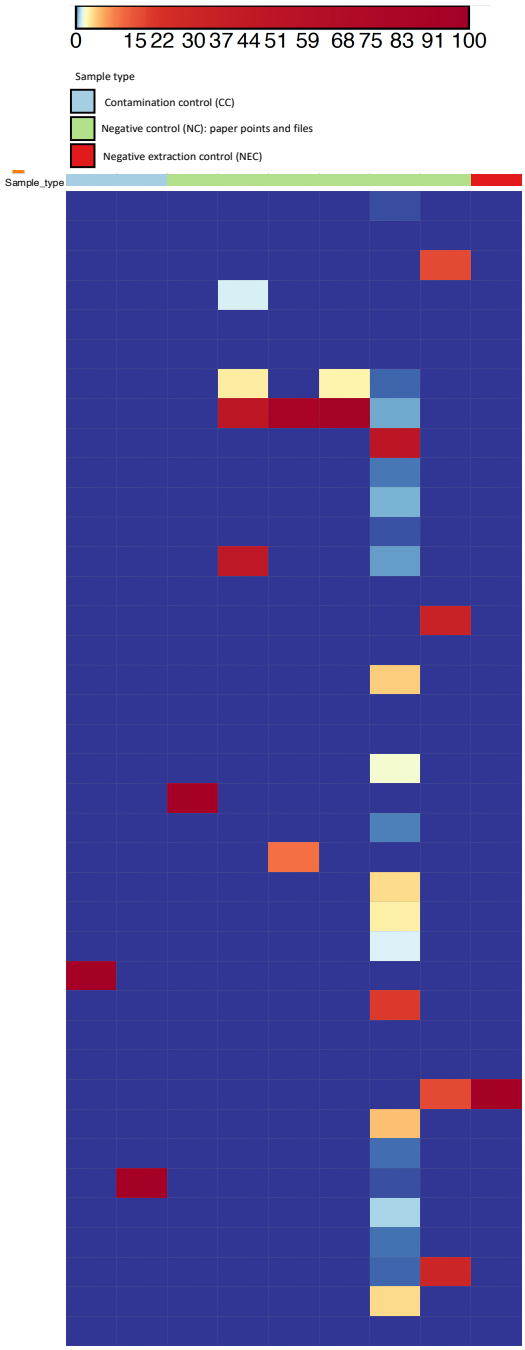
Table 12.1:List of genera and species detected in different control samples included in the study.

Sample	Genus	Species	Relative abundance (%)
Contamination control samples			
CC-1	*Enterococcus*	*E.italicus*	100.0
CC-2	*Lachnospiraceae*	*L.bacterium HMT 86*	66.7
	Pyramidobacter	*P.piscolens*	33.3
CC-3	*Pseudoramibacter*	*P.alactolyticus*	2.8
	Streptococcus	*S.anginosus*	11.1
	Bacteroidaceae	*B.bacterium HMT 272*	2.8
	Peptostreptococcaceae	*P.brachy*	2.8
	Rothia	*R.dentocariosa*	2.8
	Pseudomonas	*P.fluorescens*	2.8
	Peptoniphilus	*P.lacrimalis*	8.3
	Parvimonas	*P.micra*	2.8
	Streptococcus	*S.mutans*	2.8
	Streptococcus	*S.oralis subsp. tigurinus clade 71*	5.6
	Atopobium	*A.parvulum*	2.8
	Dialister	*D.pneumosintes*	8.3
	Atopobium	*A.rimae*	5.6
	Parvimonas	*P.sp. HMT 110*	2.8
	Prevotella	*P.sp. HMT 313*	2.8
	Peptostreptococcus	*P.stomatis*	19.4
	Streptococcus	*S.vestibularis*	5.6
	Paracoccus	*P.yeei*	8.3
CC-4	*Enterococcus*	*E.casseliflavus*	87.2
	Enterococcus	*E.italicus*	1.1
	Pseudomonas	*P.pseudoalcaligenes*	4.6
	Pseudomonas	*P.stutzeri*	1.0
	Agrobacterium	*A.tumefaciens*	1.2
CC-5	*Rothia*	*R.dentocariosa*	71.4
	Streptococcus	*S.oralis subsp. tigurinus clade 71*	14.3
	Streptococcus	*S.sp. HMT 74*	14.3
Paper points			
PP-1	*Enterococcus*	*E.casseliflavus*	81.1
	Paracoccus	*P.yeei*	10.4
	Staphylococcus	*S.pasteuri*	2.4
	Lactococcus	*L.lactis*	1.2

	Ochrobactrum	*O.anthropi*	1.2
PP-2	*Pseudomonas*	*P.fluorescens*	21.5
	Streptococcus	*S.vestibularis*	13.0
	Streptococcus	*S.parasanguinis clade 411*	6.5
	Rothia	*R.mucilaginosa*	6.1
	Streptococcus	*S.oralis subsp. tigurinus clade 71*	6.1
	Streptococcus	*S.mutans*	5.3
	Rothia	*R.dentocariosa*	4.0
	Peptostreptococcaceae [XI][G-6]	*P.minutum*	4.0
	Filifactor	*F.alocis*	3.6
	Peptostreptococcus	*P.stomatis*	2.8
	Streptococcus	*S.cristatus clade 578*	2.0
	Streptococcus	*S.sp. HMT 74*	2.0
	Fusobacterium	*F.nucleatum subsp. animalis*	1.6
	Lactobacillus	*L.buchneri*	1.2
	Streptococcus	*S.lactarius*	1.2
PP-3	*Enterococcus*	*E.casseliflavus*	45.1
	Lactobacillus	*L.paracasei*	43.6
	Enterococcus	*E.italicus*	3.4
	Actinomyces	*cardiffensis*	1.5
	Enterococcus	*E.durans*	1.2
PP-4	*Filifactor*	*F.alocis*	47.9
	Porphyromonas	*P.gingivalis*	19.1
	Pseudoramibacter	*P.alactolyticus*	5.7
	Megasphaera	*T.sp. HMT 123*	5.0
	Tannerella	*T.forsythia*	4.4
	Peptostreptococcaceae [XI][G-4]	*P.bacterium HMT 369*	4.4
	Peptostreptococcaceae [XI][G-6]	*P.nodatum*	3.4
	Mogibacterium	*M.timidum*	2.3
	Peptostreptococcaceae [XI][G-6]	*P.minutum*	1.6
PP-5	*Enterococcus*	*E.casseliflavus*	93.9
	Enterococcus	*E.italicus*	3.1
Files			
F-1	*Moraxella*	*M.osloensis*	19.4
	Pseudomonas	*P.fluorescens*	11.1
	Streptococcus	*S.vestibularis*	11.1
	Peptostreptococcaceae [XI][G-1]	*P.bacterium HMT 383*	8.3
	Enterococcus	*E.casseliflavus*	8.3
	Streptococcus	*S.oralis subsp. tigurinus clade 71*	8.3
	Kocuria	*K.palustris*	8.3
	Bergeyella	*B.sp. HMT 422*	8.3
	Paracoccus	*P.yeei*	8.3

	Pseudoramibacter	*P.alactolyticus*	2.8
	Peptostreptococcaceae [XI][G-4]	*P.bacterium HMT 369*	2.8
	Actinomyces	*A.sp. HMT 172*	2.8
F-2	*Streptococcus*	*S.oralis subsp. tigurinus clade 71*	37.1
	Streptococcus	*S.vestibularis*	9.0
	Streptococcus	*S.cristatus clade 578*	6.9
	Streptococcus	*S.parasanguinis clade 411*	4.0
	Streptococcus	*S.sp. HMT 74*	3.3
	Streptococcus	*S.lactarius*	3.0
	Streptococcus	*S.sinensis*	2.3
	Streptococcus	*S.sp. HMT 66*	2.3
	Streptococcus	*S.sp. HMT 56*	2.1
	Leptotrichia	*S.sp. HMT 417*	1.9
	Streptococcus	*S.cristatus clade 886*	1.8
	Streptococcus	*S.mutans*	1.6
	Veillonella	*V.dispar*	1.5
	Rothia	*R.mucilaginosa*	1.3
	Prevotella	*R.melaninogenica*	1.3
	Streptococcus	*S.australis*	1.2
	Porphyromonas	*P.endodontalis*	1.2
	Veillonella	*V.atypica*	1.1
	Actinomyces	*A.sp. HMT 180*	1.0
F-3	*Atopobium*	*A.rimae*	40.0
	Filifactor	*F.alocis*	20.0
	Prevotella	*P.oris*	20.0
	Actinomyces	*A.sp. HMT 180*	20.0
F-4	*Cutibacterium*	*C.acnes*	37.5
	Pseudomonas	*P.fluorescens*	25.0
	Filifactor	*F.alocis*	12.5
	Lactobacillus	*L.paracasei*	12.5
	Oribacterium	*O.sp. HMT 102*	12.5
F-5	*Lawsonella*	*L.clevelandensis*	13.5
	Streptococcus	*S.oralis subsp. tigurinus clade 71*	12.2
	Pseudomonas	*P.fluorescens*	6.8
	Actinomyces	*A.graevenitzii*	6.8
	Granulicatella	*G.adiacens*	5.4
	Rothia	*R.mucilaginosa*	5.4
	Streptococcus	*S.parasanguinis clade 411*	5.4
	Paracoccus	*P.yeei*	5.4
	Dialister	*D.invisus*	4.1
	Prevotella	*P.melaninogenica*	4.1
	Pseudomonas	*P.pseudoalcaligenes*	4.1
	Actinomyces	*A.sp. HMT 180*	4.1

	Streptococcus	*S.vestibularis*	4.1
	Campylobacter	*C.concisus*	2.7
	Tannerella	*T.forsythia*	2.7
	Prevotella	*P.multiformis*	2.7
	Gemella	*G.sanguinis*	2.7
	Cutibacterium	*C.acnes*	1.4
	Veillonella	*V.atypica*	1.4
	Prevotella	*P.histicola*	1.4
	Peptostreptococcaceae	*P.minutum*	1.4
	Porphyromonas	*P.pasteri*	1.4
	Yersinia	*Y.pestis*	1.4
Negative extraction control			
NEC-1	*Pseudomonas*	*P.fluorescens*	45.5
	Haemophilus	*H.parainfluenzae*	36.4
	Peptostreptococcaceae [XI][G-1]	*P.infirmum*	9.1
	Streptococcus	*S.oralis subsp. tigurinus clade 71*	9.1
NEC-2	*Afipia*	*A.sp. genotype 4*	50.0
	Peptostreptococcus	*P.stomatis*	50.0
NEC-3	*Fusobacterium*	*F.nucleatum subsp. animalis*	25.0
	Streptococcus	*S.oralis subsp. tigurinus clade 71*	25.0
	Veillonella	*V.dispar*	12.5
	Prevotella	*P.melaninogenica*	12.5
	Rothia	*R.mucilaginosa*	12.5
	Streptococcus	*S.vestibularis*	12.5
NEC-4	*Moraxella*	*M.osloensis*	100.0



id	Genus
408_8636 Acinetobacter sp._HMT_408	Acinetobacter
172CT047 Actinomyces sp._HMT_172	Actinomyces
866F0530 Actinomyces graevenitzii	Actinomyces
850F0333 Actinomyces cardiffensis	Actinomyces
118BS095 Dialister invisus	Dialister
604_8293 Enterococcus faecalis	Enterococcus
803_6902 Enterococcus italicus	Enterococcus
801_9899 Enterococcus casseliflavus	Enterococcus
539_6962 Filifactor alocis	Filifactor
420_5404 Fusobacterium nucleatum_subsp._animalis	Fusobacterium
200_R002 Fusobacterium nucleatum_subsp._vincentii	Fusobacterium
084N058A Kocuria palustris	Kocuria
716_9212 Lactobacillus paracasei	Lactobacillus
804N000B Lactococcus lactis	Lactococcus
173N105C Lawsonella clevelandensis	Lawsonella
417_8621 Leptotrichia sp._HMT_417	Leptotrichia
123_9314 Megasphaera sp._HMT_123	Megasphaera
123_9043 Megasphaera sp._HMT_123	Megasphaera
087N061A Micrococcus luteus	Micrococcus
042W9173 Mogibacterium timidum	Mogibacterium
711_5304 Moraxella osloensis	Moraxella
078F263b Oribacterium sp._HMT_078	Oribacterium
104N072A Paracoccus yeei	Paracoccus
369_1212 Peptostreptococcaceae_[XI][G-4] bacterium_HMT_369	Peptostreptococcaceae_[XI][G-4]
694_6274 Peptostreptococcaceae_[XI][G-6] nodatum	Peptostreptococcaceae_[XI][G-6]
673_3037 Peptostreptococcaceae_[XI][G-6] minutum	Peptostreptococcaceae_[XI][G-6]
112A21H2 Peptostreptococcus stomatis	Peptostreptococcus
619_3964 Porphyromonas gingivalis	Porphyromonas
311_6474 Prevotella oris	Prevotella
740_6666 Pseudomonas pseudoalcaligenes	Pseudomonas
612_4476 Pseudomonas fluorescens	Pseudomonas
538CK057 Pseudoramibacter alactolyticus	Pseudoramibacter
188_3025 Rothia aeria	Rothia
587_9055 Rothia dentocariosa	Rothia
686_3965 Streptococcus mutans	Streptococcus
758_3928 Streptococcus sanguinis	Streptococcus
071_7062 Streptococcus oralis_subsp._tigurinus_clade_071	Streptococcus
613_6495 Tannerella forsythia	Tannerella
653_3040 Treponema lecithinolyticum	Treponema

Figure 12.1: Heat map of negative control samples included in the study.

Taxa as *Enterococcus italicus*, *Enterococcus casseliflavus*, *Paracoccus yeei*, *Filifactor alocis*, and *Pseudomonas fluorescens* were among the common taxa found in control samples. To obviate the impact of potential contamination, specific taxa were judiciously removed from the analysis of the clinical samples.

Appendix 13: Microbial profile of initial intra canal samples (S1)

Table 13.1: List of species detected in all initial samples regardless of treatment protocol or diagnosis. Cut-off at 0.001 relative abundance.

Species	Relative abundance (%)
Peptostreptococcus stomatis	*7.24*
Streptococcus oralis subsp. tigurinus clade 71	*4.92*
Rothia dentocariosa	*3.54*
Bacteroidaceae [G-1] bacterium HMT 272	*3.20*
Streptococcus anginosus	*3.04*
Dialister invisus	*3.01*
Streptococcus vestibularis	*2.93*
Oribacterium sp. HMT 102	*2.72*
Atopobium rimae	*2.51*
Streptococcus sanguinis	*2.40*
Enterococcus italicus	*2.27*
Parvimonas sp. HMT 110	*2.07*
Parvimonas micra	*2.07*
Rothia aeria	*1.96*
Fusobacterium nucleatum subsp. animalis	*1.79*
Peptostreptococcaceae [XI][G-1] infirmum	*1.74*
Atopobium sp. HMT 416	*1.67*
Shuttleworthia satelles	*1.66*
Mycoplasma salivarium	*1.64*
Prevotella denticola	*1.56*
Fusobacterium nucleatum subsp. vincentii	*1.55*
Peptostreptococcaceae [XI][G-1] bacterium HMT 383	*1.48*
Filifactor alocis	*1.36*
Pseudoramibacter alactolyticus	*1.27*
Streptococcus parasanguinis clade 411	*1.13*
Peptoniphilus lacrimalis	*1.13*
Prevotella sp. HMT 376	*0.97*
Prevotella oris	*0.94*
Catonella sp. HMT 164	*0.87*
Lactobacillus gasseri	*0.85*
Streptococcus mutans	*0.79*
Peptostreptococcaceae [XI][G-9] brachy	*0.79*
Enterobacter hormaechei	*0.72*
Veillonella dispar	*0.65*
Corynebacterium matruchotii	*0.61*
Porphyromonas endodontalis	*0.60*
Solobacterium moorei	*0.54*
Lactobacillus pentosus	*0.53*

Enterococcus faecalis	*0.52*
Corynebacterium pilbarense	*0.47*
Campylobacter gracilis	*0.47*
Tannerella forsythia	*0.46*
Rothia mucilaginosa	*0.45*
Enterococcus saccharolyticus	*0.44*
Cutibacterium acnes	*0.35*
Olsenella uli	*0.34*
Agrobacterium tumefaciens	*0.34*
Actinomyces gerencseriae	*0.34*
Lactococcus lactis	*0.32*
Prevotella baroniae	*0.31*
Enterococcus durans	*0.30*
Aggregatibacter sp. HMT 458	*0.30*
Dialister pneumosintes	*0.30*
Acinetobacter sp. HMT 408	*0.28*
Lactobacillus casei	*0.28*
Anaeroglobus geminatus	*0.27*
Streptococcus sp. HMT 74	*0.26*
Oribacterium sp. HMT 78	*0.25*
Lactobacillus buchneri	*0.25*
Atopobium sp. HMT 199	*0.24*
Streptococcus sp. HMT 66	*0.23*
Neisseria mucosa	*0.22*
Peptostreptococcaceae [XI][G-6] minutum	*0.22*
Prevotella melaninogenica	*0.22*
Peptostreptococcaceae [XI][G-7] yurii subspp. yurii & margaretiae	*0.22*
Actinomyces graevenitzii	*0.21*
Actinomyces odontolyticus	*0.20*
Micrococcus luteus	*0.20*
Lactobacillus paracasei	*0.19*
Peptostreptococcaceae [XI][G-6] nodatum	*0.19*
Prevotella nigrescens	*0.19*
Oribacterium sinus	*0.19*
Campylobacter showae	*0.18*
Actinomyces sp. HMT 172	*0.18*
Prevotella histicola	*0.18*
Bacteroidales [G-2] bacterium HMT 274	*0.18*
Leptotrichia sp. HMT 417	*0.17*
Veillonella parvula	*0.17*
Mogibacterium timidum	*0.16*
Staphylococcus caprae	*0.16*
Streptococcus cristatus clade 578	*0.16*

Veillonella atypica	*0.15*
Bacteroidetes [G-3] bacterium HMT 281	*0.14*
Prevotella oralis	*0.14*
Treponema denticola	*0.14*
Selenomonas flueggei	*0.14*
Streptococcus constellatus	*0.14*
Granulicatella adiacens	*0.13*
Mogibacterium neglectum	*0.13*
Actinomyces sp. HMT 180	*0.12*
Selenomonas sp. HMT 146	*0.12*
Bulleidia extructa	*0.12*
Pyramidobacter piscolens	*0.11*
Streptococcus lactarius	*0.11*
Prevotella multisaccharivorax	*0.10*
Prevotella sp. HMT 306	*0.10*
Streptococcus sp. HMT 57	*0.10*
Bacteroidetes [G-7] bacterium HMT 911	*0.10*

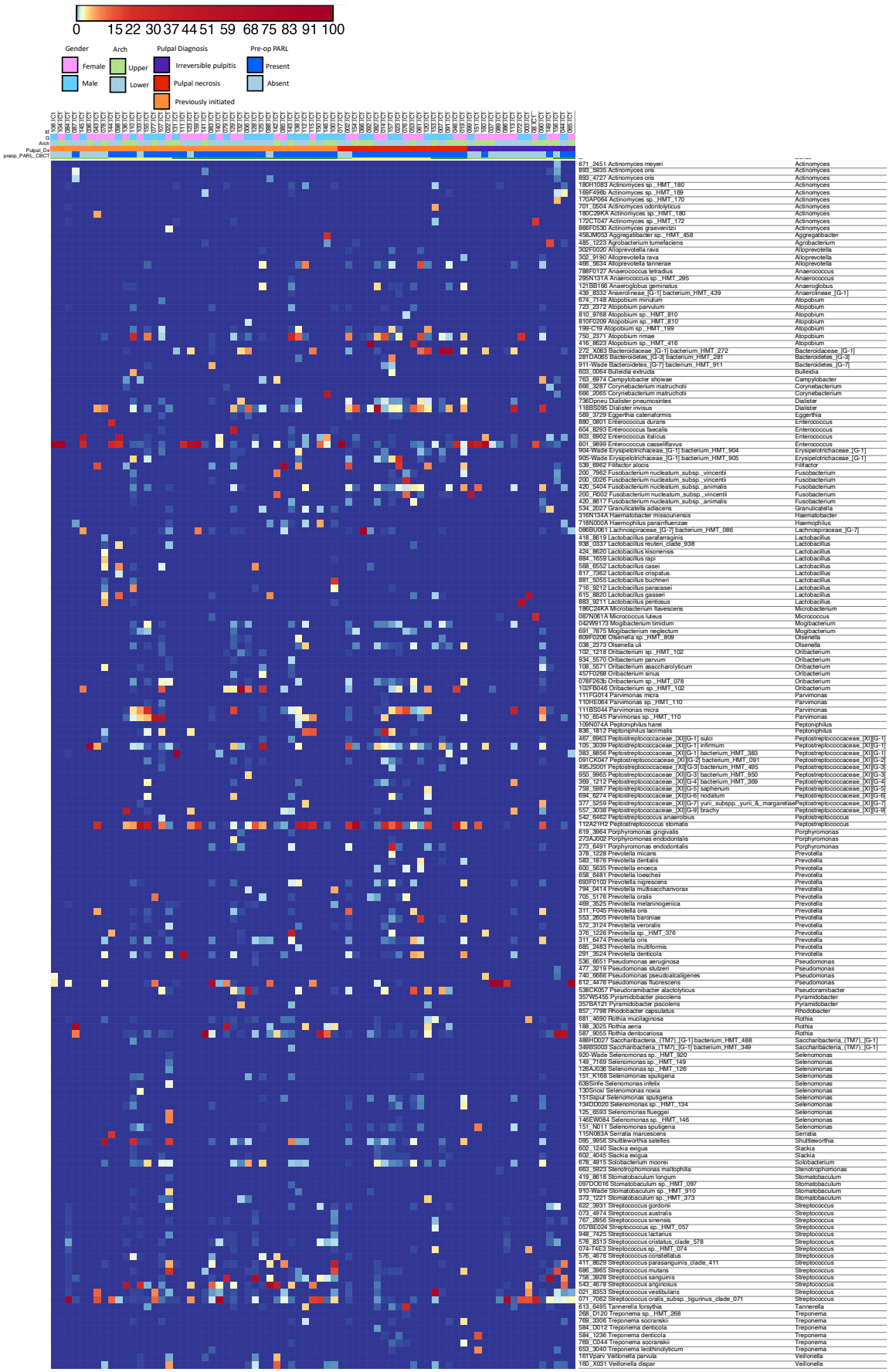


Figure 13.1: Heat map of the initial intra canal samples of two treatment groups.
Showing different taxa found in each sample.

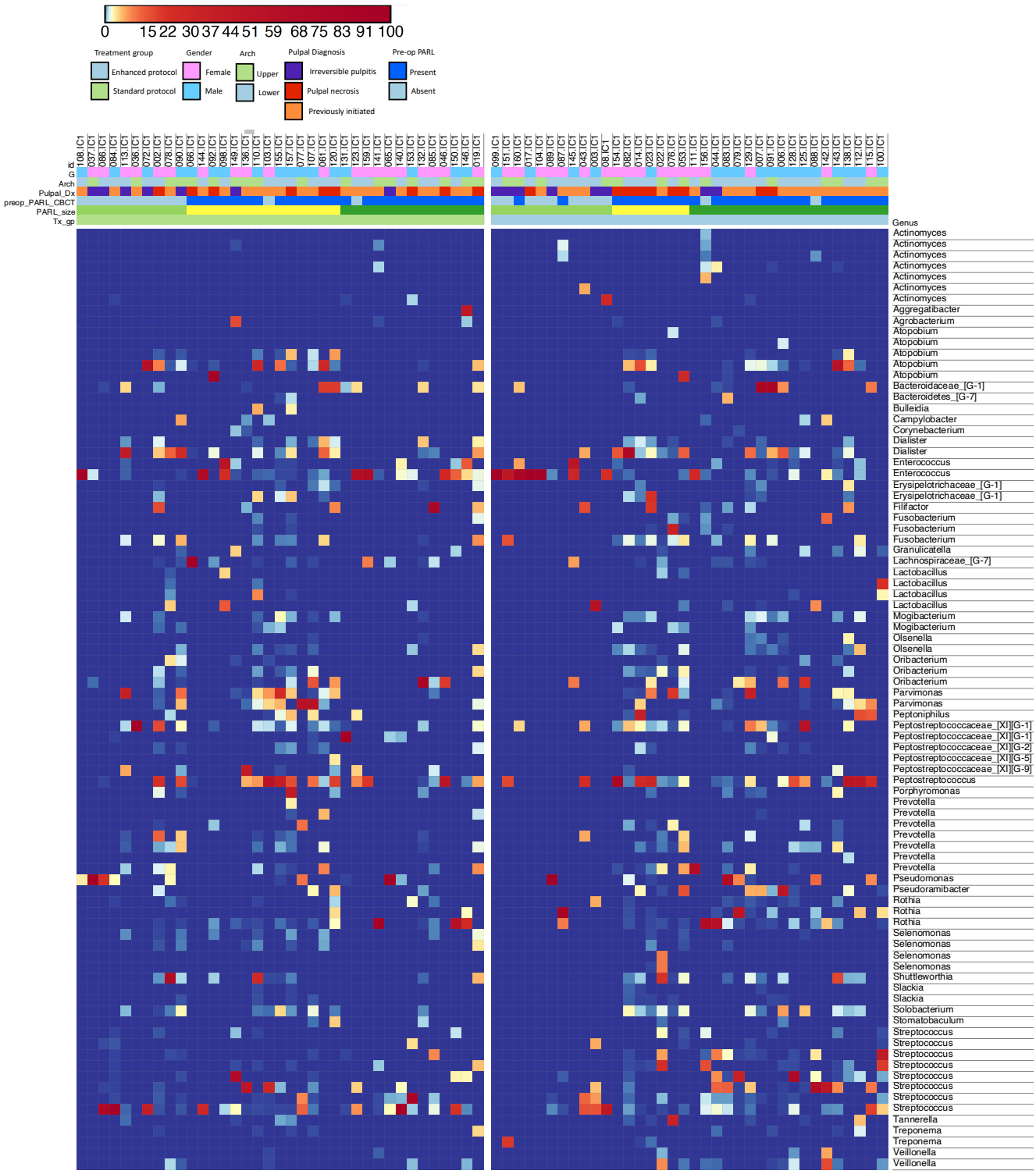


Figure 13.2: Heat map of the initial intra anal samples grouped by treatment protocol (EP and SP).
Showing a comparable microbial profile between the two groups in their initial samples.

Table 13.2: Richness and diversity indexes of bacterial community in initial samples of both treatment groups. Results of Wilcoxon signed-rank test. Values of the mean and range. Showing similar values of diversity indexes in both groups' initial samples.

Indicator	S1-SP (n=25)		S1-EP (n=24)		*p-value*
Number of OTUs per canal	62.12	(23-127)	63.58	(16-110)	0.78
Chao1 estimator of richness	76.96	(30.2-148.4)	82.75	(19.75-166.6)	0.85
ACE estimator of richness	79.55	(32.5-153.7)	84.18	(20.31-143.61)	0.98
Shannon index of diversity	1.55	(0.004-2.9)	1.59	(0.088-2.62)	0.90
Simpson index of diversity	0.58	(0.0008-0.91)	0.61	(0.022-0.88)	0.90

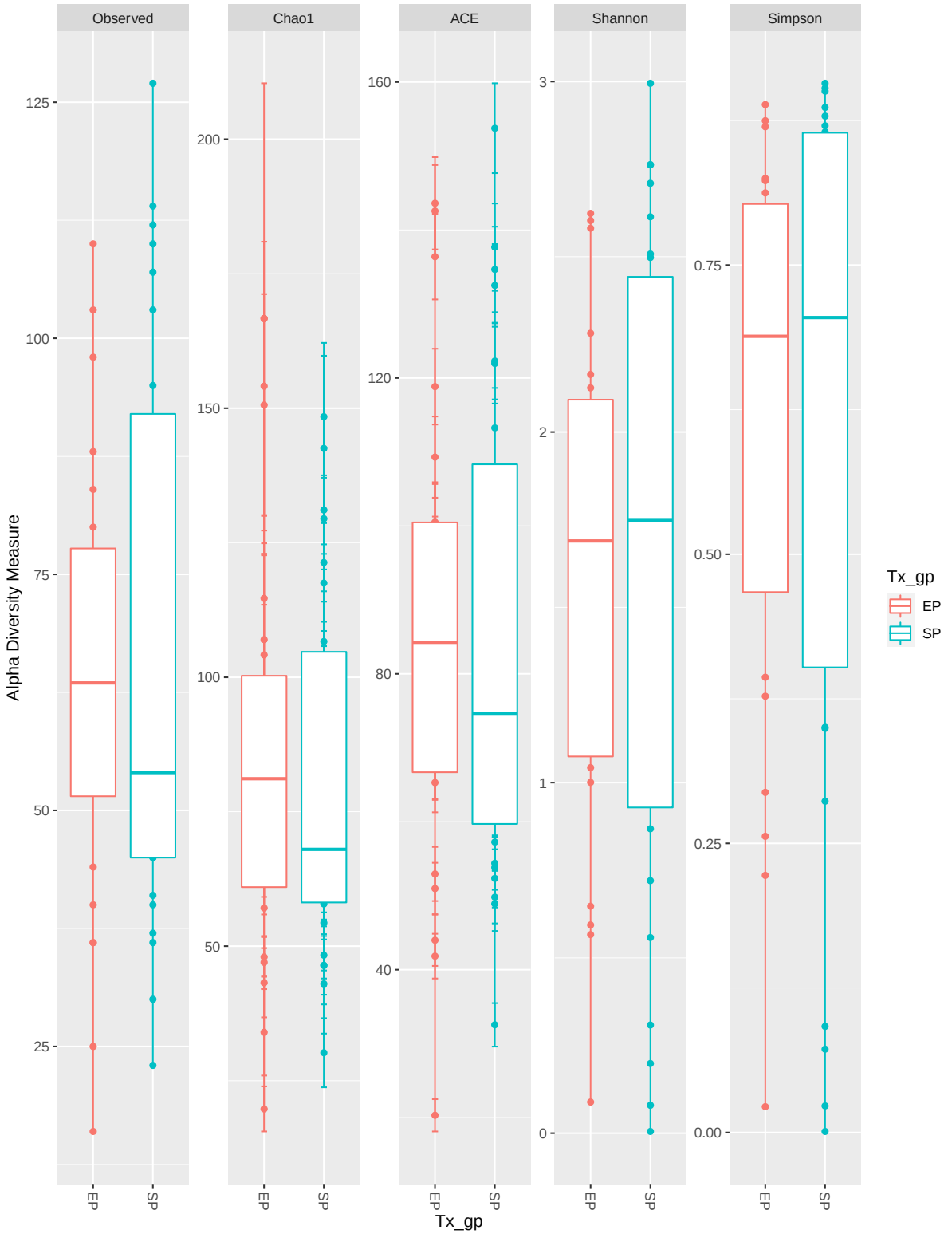


Figure 13.3: Richness and diversity indices of bacterial community in primary infected root canal system initial samples (S1) pf the two groups.

Species richness measures: observed species richness, Chao1, and ACE. Diversity measured with Shannon diversity index were all comparable between the two groups initial samples indicating proper randomisation.

Appendix 14: Microbiological profile change after chemomechanical preparations

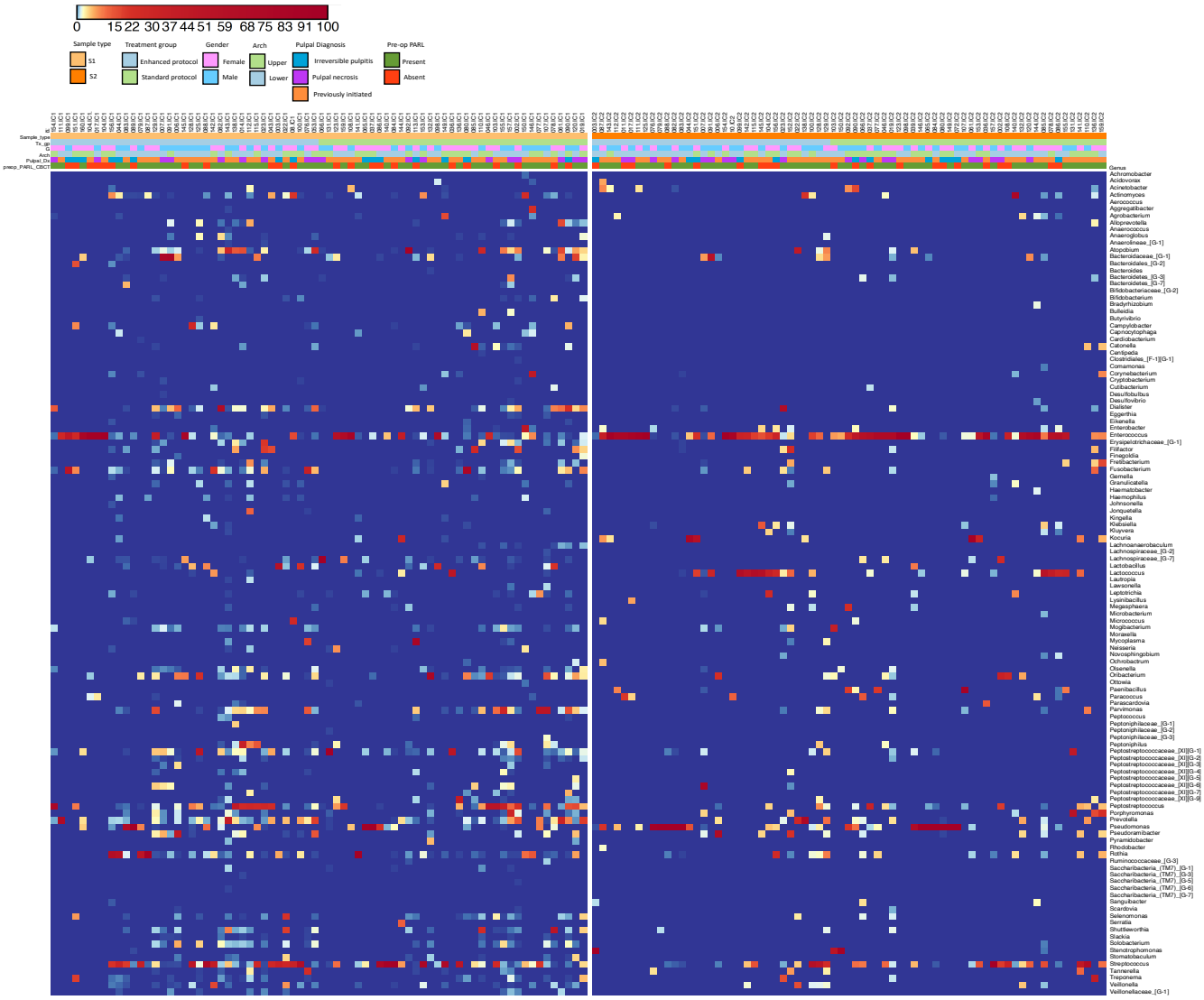
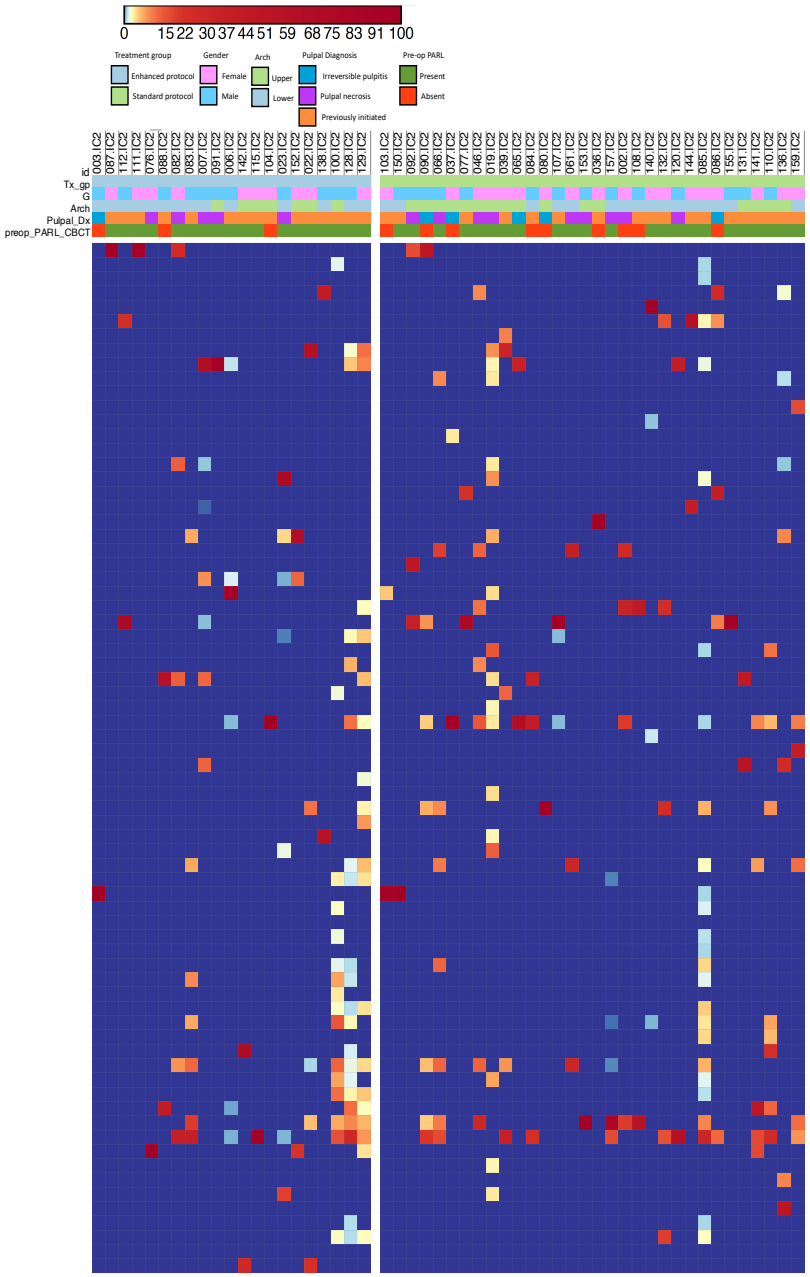


Figure 14.1: Heat map presenting different genera in each intra canal sample of S1 and S2.

Appendix 15: Microbiological profile of pre-obturation samples



id	Genus
408_8636 Acinetobacter sp._HMT_408	Acinetobacter
180H1083 Actinomyces sp._HMT_180	Actinomyces
180C29KA Actinomyces sp._HMT_180	Actinomyces
850F0333 Actinomyces cardiffensis	Actinomyces
171BL008 Actinomyces sp._HMT_171	Actinomyces
485_1223 Agrobacterium tumefaciens	Agrobacterium
199-C19 Atopobium sp._HMT_199	Atopobium
750_2371 Atopobium rimae	Atopobium
272_X083 Bacteroidaceae_[G-1] bacterium_HMT_272	Bacteroidaceae_[G-1]
365_7777 Bacteroidetes_[G-3] bacterium_HMT_365	Bacteroidetes_[G-3]
666_3287 Corynebacterium matruchotii	Corynebacterium
595F235b Corynebacterium durum	Corynebacterium
666_2065 Corynebacterium matruchotii	Corynebacterium
530N000A Cutibacterium acnes	Cutibacterium
552_3055 Cutibacterium avidum	Cutibacterium
736Dpneu Dialister pneumosintes	Dialister
118BS095 Dialister invisus	Dialister
802_1004 Enterococcus saccharolyticus	Enterococcus
880_0801 Enterococcus durans	Enterococcus
604_8293 Enterococcus faecalis	Enterococcus
539_6962 Filifactor alocis	Filifactor
086BU061 Lachnospiraceae_[G-7] bacterium_HMT_086	Lachnospiraceae_[G-7]
123_9043 Megasphaera sp._HMT_123	Megasphaera
042W9173 Mogibacterium timidum	Mogibacterium
078F263b Oribacterium sp._HMT_078	Oribacterium
102FB046 Oribacterium sp._HMT_102	Oribacterium
048_5540 Paenibacillus sp._HMT_048	Paenibacillus
111BS044 Parvimonas micra	Parvimonas
110_6545 Parvimonas sp._HMT_110	Parvimonas
836_1812 Peptoniphilus lacrimalis	Peptoniphilus
105_3039 Peptostreptococcaceae_[XI][G-1] infirmum	Peptostreptococcaceae_[XI][G-1]
383_8856 Peptostreptococcaceae_[XI][G-1] bacterium_HMT_383	Peptostreptococcaceae_[XI][G-1]
091CK047 Peptostreptococcaceae_[XI][G-2] bacterium_HMT_091	Peptostreptococcaceae_[XI][G-2]
112A21H2 Peptostreptococcus stomatis	Peptostreptococcus
279CW034 Porphyromonas pasteri	Porphyromonas
273AJ002 Porphyromonas endodontalis	Porphyromonas
273BB134 Porphyromonas endodontalis	Porphyromonas
306DO027 Prevotella sp._HMT_306	Prevotella
693F0103 Prevotella nigrescens	Prevotella
469_3525 Prevotella melaninogenica	Prevotella
376_1226 Prevotella sp._HMT_376	Prevotella
311_6474 Prevotella oris	Prevotella
291_3524 Prevotella denticola	Prevotella
681_4690 Rothia mucilaginosa	Rothia
188_3025 Rothia aeria	Rothia
663_5923 Stenotrophomonas maltophilia	Stenotrophomonas
886_9095 Streptococcus cristatus_clade_886	Streptococcus
056AY020 Streptococcus sp._HMT_056	Streptococcus
066FN051 Streptococcus sp._HMT_066	Streptococcus
622_3931 Streptococcus gordonii	Streptococcus
073_4974 Streptococcus australis	Streptococcus
767_2856 Streptococcus sinensis	Streptococcus
057BE024 Streptococcus sp._HMT_057	Streptococcus
948_7425 Streptococcus lactarius	Streptococcus
578_8313 Streptococcus cristatus_clade_578	Streptococcus
074-T4E3 Streptococcus sp._HMT_074	Streptococcus
576_4676 Streptococcus constellatus	Streptococcus
411_8629 Streptococcus parasanguinis_clade_411	Streptococcus
686_3965 Streptococcus mutans	Streptococcus
758_3928 Streptococcus sanguinis	Streptococcus
543_4678 Streptococcus anginosus	Streptococcus
021_8353 Streptococcus vestibularis	Streptococcus
071_7062 Streptococcus oralis_subsp._tigurinus_clade_071	Streptococcus
613_6495 Tannerella forsythia	Tannerella
769_3306 Treponema socranskii	Treponema
584_D012 Treponema denticola	Treponema
769_C044 Treponema socranskii	Treponema
653_3040 Treponema lecithinolyticum	Treponema
524_3631 Veillonella atypica	Veillonella
160Vdisp Veillonella dispar	Veillonella
161Vparv Veillonella parvula	Veillonella
160_X031 Veillonella dispar	Veillonella

Figure 15.1: Heat map of the pre-obturation samples of two treatment groups.
Showing different genera found in each sample. Note the overall increase in frequency of taxa in the SP compared to EP.

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