Insights into the bacterial and fungal ecology of endodontic infections
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Nativity façade of the Templo Expiatorio de la Sagrada Familia by Gaudi in Barcelona, Spain.

The Nativity façade was the first side of the church to be completed, depicting the birth of Christ. Similarly, this chapter is the beginning of this thesis.
Chapter 1

When the coronal tooth structures are compromised by caries, trauma or failing restorations, microorganisms can invade the tooth. In the interior of the tooth lies the dental pulp or endodontium, which is composed of connective tissue, blood vessels, nerve endings and dentin-forming cells, odontoblasts. As the dental pulp is unable to oppose the microbial invasion, the immune system will react with an inflammatory response; this is called pulpitis. Unfortunately, microorganisms often kill the outcompeted pulp and infect the root canal system. Subsequently, the periapical tissues will respond with an inflammation which is unable to eliminate the microorganisms and their metabolites from the root canal system; this is called apical periodontitis (Kakehashi et al. 1965).

Epidemiological studies show that 5 ± 6% of all teeth present with apical periodontitis and that 10 ± 6% of teeth have received root canal treatment (Pak et al. 2012). Regrettably, 36 ± 10% of the teeth having received root canal treatment also have apical periodontitis (Pak et al. 2012), either because of a persistent infection or reinfection of the root canal system (Sundqvist et al. 1998). Especially root canal treatment of teeth with persistent infection or reinfection has lower success rates than treatment of teeth with primary infections, 85% versus 77% respectively (Ng et al. 2007; Ng et al. 2008). Retreatment may be complicated by procedural errors of the previous treatment or by a persisting infection within the apical delta requiring surgical resection of the root apex (Sundqvist et al. 1998; Ng et al. 2008). Despite all research and adaptations of treatment procedures during the past five decades, reported success rates of root canal treatment have not significantly improved (Ng et al. 2007). Apical periodontitis and treatment thereof are the main reasons for emergency dental care (Quiñonez et al. 2009) and tooth extraction (McCaul et al. 2001). This tooth loss has a major economic impact, which can be attributed to the direct costs of dental care as well as the indirect costs due to productivity losses (Listl et al. 2015). Thus, there is ample space for improving apical periodontitis treatment and general patient comfort. Since current treatment strategies are insufficient, research should again focus on the principles of root canal infections. More insight into the microbiological organization of root canal infections can aid in improving root canal treatment and prevent the need for retreatment.

A polymicrobial community is continuously present in the oral cavity, usually living in symbiosis with the host (Levy et al. 2015). This commensal microbiome contributes to health. When environmental conditions change, the balance shifts towards a dysbiotic community and causes disease within the host, such as caries and marginal periodontitis (Lamont & Hajishengallis 2015). Contrary to other parts of the oral cavity, the root canal system is sterile and without a commensal microbial community. Thus, each microorganism may pose a threat to the host and
General introduction

A microbial community is essential for developing apical periodontitis (Kakehashi et al. 1965; Möller et al. 1981). Since the root canal system is inoculated from the oral cavity, it is infected with a polymicrobial derivative of the oral microbiome (Möller et al. 1981; Hsiao et al. 2012). Saliva contains over $2.9 \times 10^8 \pm 3.0$ bacteria per mL (Cousido et al. 2008), but only $5.2 \times 10^6 \pm 3.1 \times 10^6$ bacteria can be isolated from a root canal system (Akpata 1974). Saliva is very diverse with a single study identifying 5600 bacterial phylotypes, which are equivalent to species (Keijser et al. 2008). In root canal infections, 128 traditional studies have distinguished 468 separate bacterial phylotypes, of which 317 using molecular techniques and 258 using cultivation (Siqueira & Rôças 2009). Per root canal only 20 different bacteria (range 7 - 30; Munson et al. 2002) can be detected. Despite the high diversity of the total isolated bacteria, the number of species per root canal is limited. The root canal system has low oxygen tension and offers nutrients that are rich in peptides and low in carbohydrates (Matsumiya & Kitamura 1960). Therefore, primary infections are dominated by obligately anaerobic Gram-positives and Gram-negatives (Sundqvist 1976; Möller et al. 1981), while persistent infections and reinfections mainly include facultatively and obligately anaerobic Gram-positives in equal proportions (Sundqvist et al. 1998; Cheung & Ho 2001).

Still, this is not a precise distinction. Thus far, no single species has been identified to be the cause of a specific phase of endodontic pathology or certain symptoms, although some associations have been made (Sundqvist 1976; Siqueira & Rôças 2009). Especially the influence of a certain microorganism on the healing of apical periodontitis after treatment would be of interest. In the past, negative cultures of root canal samples taken using paper or charcoal points were a prerequisite before the root canal treatment could be finished (Sathorn et al. 2007). Nevertheless, a negative root canal culture does not preclude presence of microorganisms in the root canal system (Akpata 1976). More sensitive techniques on samples of the entire root canal system should facilitate full coverage of the present microorganisms and allow a better comprehension of the microbial community in root canal infections. Moreover, a negative root canal culture after preparation and irrigation of the root canal does not guarantee periapical healing and, vice versa, a positive culture does not preclude periapical healing (Peters & Wesselink 2002).

Of all microorganisms responsible for apical periodontitis, bacteria have been studied most extensively. More current research is also focusing on microorganisms from other kingdoms, such as archaea (Vianna et al. 2006), bacteriophages (Stevens et al. 2009), fungi (Morse & Yates 1941) and viruses (Sabeti et al. 2003). Studies into general health and disease have shown that microorganisms from separate kingdoms interact with each other and with the host. In health, these interactions appear to be
essential in modulating the immune system for homeostasis with the commensal microbiota and protection from disease, but they also contribute to efficient human metabolism (Levy et al. 2015; Nobbs & Jenkinson 2015). However, in disease, interactions can increase virulence and decrease treatment effectiveness leading to increased morbidity and mortality, especially when the host is immunocompromised (Levy et al. 2015; Nobbs & Jenkinson 2015; Schlecht et al. 2015). Thus far, evidence of the extent and significance of the microbiota appears to increase daily and the once so distinct separation between health and disease seems to fade away.

Nonetheless, apical periodontitis can be truly considered as a detrimental condition, although the contribution of kingdoms other than bacteria still needs elucidation. Much of the in vitro research studying antimicrobials and other materials for root canal treatment focuses on the fungus Candida albicans besides frequently isolated bacteria. Fungi are considered to have an effect on root canal infections and treatment thereof, although their true relevance is still uncertain. Similar to influences on general health and disease, fungal-bacterial collaboration could enhance virulence of root canal infections and the following immune responses. Also, treatment of bacteria is not always similarly effective on fungi, which could stimulate fungal multiplication and persistence. Ultimately, this could lessen treatment success. Therefore, research into the combined bacterial-fungal contribution to root canal infections is required.

In root canal infections, most microorganisms aggregate and adhere to the root canal wall as biofilms (Nair et al. 2005; Ricucci & Siqueira 2010). Biofilms are omnipresent and consist of microorganisms that are adhered to a surface and enclosed in their extracellular matrix (Hall-Stoodley et al. 2004). The extracellular matrix protects the microbial cells from environmental challenges. Also, the close proximity of microorganisms stimulates cell-cell contact, which assists in nutrient exchange, gene transfer and activation of virulence factors (Hall-Stoodley et al. 2004). These interactions support a dynamic environment that can adapt rapidly to internal and external fluctuations (Möller et al. 1981; Dahlén et al. 1982; Fabricius et al. 1982). Despite these dynamics, the metabolic rate can be strongly reduced creating less targets for antimicrobials (Hall-Stoodley et al. 2004). Biofilm formation also triggers the emergence of a resistant subpopulation that is less susceptible to antimicrobials (Hall-Stoodley et al. 2004). Thus, biofilms enable microorganisms to establish a community that assists in their persistence and protects against the host response and antimicrobials.

Enterococcus faecalis is a Gram-positive coccus that is commensal to the gastrointestinal tract (Noble 1978). E. faecalis is suggested to occur in the oral cavity as a transient microorganism, but can colonize the specific ecological niche of the
previously treated root canal if this is present (Zehnder & Guggenheim 2009). The bacterium is present in 16 - 77% of the persistent infections and reinfections (Molander et al. 1998; Siqueira & Rôças 2004; Vidana et al. 2011) and in only 0 - 16% of primary infections (Sundqvist 1976; Lana et al. 2001; Dumani et al. 2012). This could be attributed to the many virulence factors of E. faecalis, such as production of biofilm, aggregation substance, surface adhesin, bacteriocin, cytolysin, gelatinase (Sedgley et al. 2005) and extracellular superoxide (Huycke et al. 2002). Also, it is resistant to antibiotics (Sedgley et al. 2004), fluctuations in osmolality, pH and temperature, and nutrient deprivation (Hartke et al. 1998). Because of these characteristics E. faecalis is a virulent bacterium that appears to be appropriate to mimic root canal infections within a laboratory setting (Fux et al. 2005). Also, its cultivation poses little difficulty which facilitates reproducible and reliable research.

Candida albicans is a highly prevalent fungus in the oral cavity, being isolated in 19 - 32% of salivary samples (Egan et al. 2002; Munguia-Pérez et al. 2012) and 0.5 - 55 % of root canal infections (Egan et al. 2002). Although in the past Candida was mainly associated with disease (Dagistan et al. 2009; Brown et al. 2012), recently a role in health has been uncovered as well (Ghannoum et al. 2010; Munguia-Pérez et al. 2012; Cui et al. 2013). C. albicans is a dimorphic fungus that can occur both as yeast and hyphal filament. The hyphal phenotype is capable of invading host tissues and secretes Candidalysin which activates the immune system and damages host cells (Moyes et al. 2016). Other virulence factors are production of adhesins, secreted aspartyl proteases and phospholipases (Calderone & Fonzi 2001). Thus, C. albicans is also used often in laboratory studies.

Root canal treatment aims at disinfecting the root canal system and removing any irritant remnants. Also, it should prevent reinfection of the root canal system (European Society of Endodontology 2006). Unfortunately, sterilization of the root canal system is not possible so far (Nair et al. 2005), although apical periodontitis still heals in 85% of the cases (Ng et al. 2007). Thus, root canal treatment has several limitations. These can be related to the host, the infection or the therapy. Microorganisms can penetrate the root canal system to its full extent, as they colonize the many root canals, shapes of canals, anastomoses (Vertucci 1984; Wolf et al. 2016) and dentinal tubules of the root canal wall (Peters et al. 2001). In this complex morphology, the microorganisms cannot be reached by traditional instrumentation and disinfectants. Especially the apical delta is very complex and in as much as 87.5% of the teeth microorganisms can still be detected after treatment (Nair et al. 2005). Another reason for retention of microorganisms is biofilm formation, both within the root canal system and on the periapical surface (Ricucci & Siqueira 2010). As discussed before, polymicrobial biofilm communities can possess intrinsic
resistance or develop resistance to toxic external influences, such as applied during treatment (Hall-Stoodley et al. 2004).

Current root canal treatment consists of mechanical preparation of merely the main root canals (Byström & Sundqvist 1981) and further chemomechanical cleansing using disinfecting irrigants (Byström & Sundqvist 1985). The most commonly applied irrigant is sodium hypochlorite (NaOCl; Byström & Sundqvist 1985), which has a broad antimicrobial spectrum and can dissolve organic tissues (McDonnell & Russell 1999). Another frequently applied irrigant is chlorhexidine, which has less antimicrobial efficacy, adheres to the root canal wall (substantivity) and has no tissue-dissolving properties (Ringel et al. 1982). Exposure time to antimicrobials can be extended by dressing the root canal between two appointments with another disinfectant, such as calcium hydroxide (Peters & Wesselink 2002). This is only a mild antimicrobial which is not very effective against biofilms and does not increase apical healing (Peters & Wesselink 2002; Abdullah et al. 2005). Despite proper activity of antimicrobials in vitro, the root canal environment poses more challenges and renders antimicrobials less effective. The organic and the mineral component of dentin, the necrotic pulp tissue and the biofilm significantly inactivate different available antimicrobials (Wang & Hume 1988; Portenier et al. 2001). The anatomical restrictions of the root canal system allow only a small volume to be inserted, which hinders fluid dynamics and diffusion into the dentinal tubules and biofilm (Wang & Hume 1988; Chávez de Paz et al. 2010; Gulabivala et al. 2010). These limitations require new treatment strategies to successfully treat the complete tooth-pulp-periapical complex.

Emerging techniques attempt to improve root canal disinfection. These can be either novel methods, including antimicrobial photodynamic therapy and laser assisted root canal disinfection, or new antimicrobial substances, such as ozone, nanoparticles, natural plant extracts and enzymes (Kishen 2010). Local production of an antimicrobial substance by enzymes can overcome limitations such as the decreased shelf life of antimicrobials and the inability to reach the microorganisms (Gulabivala et al. 2010; Van der Waal et al. 2014). The fungus *Curvularia inaequalis* produces the enzyme vanadium chloroperoxidase (VCPO; Van Schijndel et al. 1994). This enzyme uses freely available substrates to produce the antimicrobials hypochlorous acid, hypochlorite and singlet oxygen. Antimicrobial activity against the cariogenic *Streptococcus mutans* has already been established (Hoogenkamp et al. 2009). Interappointment dressing of the root canal with VCPO offers an innovative strategy where disinfectants can be produced locally using freely available substrates.
SCOPE AND OUTLINE OF THE THESIS

The works presented in this thesis aimed to give more insight into the complexity of root canal infections and interactions within the infection itself and those with the host, especially focusing on the bacterial and fungal kingdoms. A better understanding of the aetiopathogenesis of apical periodontitis should aid in preventing it and developing successful treatment strategies.

Previous research has established that bacteria are the main cause of apical periodontitis. Microbial communities were only identified through laborious traditional techniques on paper or charcoal point samples. The samples did not fully represent the infection of root canal systems and gave superficial coverage that yielded little species diversity. To obtain more depth of coverage, roots with primary infections were pulverized and diversity was explored using next-generation sequencing (chapter 2a). Among the identified bacteria, several are capable of forming spores. In a follow-up pilot-study, root canal infections were examined for the actual presence of this treatment-resistant phenotype (chapter 2b). Besides bacteria, fungi have been identified within root canal infections as well. Because the literature appeared contradictory and diverse, a systematic review and meta-analysis were done to summarize the diversity and prevalence of fungi found in previous studies (chapter 3). To study the full bacterial and fungal contribution to root canal infections, the microbiome and mycobiome of primary root canal samples were analysed using next-generation sequencing (chapter 4). The interaction of both these kingdoms with the host’s immune system was explored in a pilot in vitro study using *E. faecalis* and *C. albicans* (chapter 5).

Contemporary root canal treatment is unable to remove the full microbial community, which appears to be more diverse and complex than ever considered before. Root canal disinfection has to be improved using new techniques. Therefore, a novel strategy was tested in vitro where the VCPO enzyme is tested on *E. faecalis* biofilms (chapter 6). The enzyme was further optimized for the local pH of the root canal system (chapter 7).

The thesis ends with a discussion, considerations for further research (chapter 8) and a summary of the findings and conclusions.