Insights into the bacterial and fungal ecology of endodontic infections
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General discussion

*Le Penseur* by Rodin in Glasgow, Scotland.

The Burrell Collection contains a cast of this famous bronze statue. Similarly, this chapter contemplates the findings of the thesis.
This thesis aimed to give more insight into the complexity of root canal infections. Bacteriome profiling revealed an immense diversity of up to 184 OTUs per root segment. The ecology of the infections is even more complex because of the contribution of the mycobiome. Fungi were present in 57% of the sampled teeth and had a significant correlation with the composition of the bacteriome. In vitro studies showed that the co-occurrence of bacteria and fungi can influence their effect on the host.

Although these results help to improve the understanding of root canal infections, certain methodological considerations should be kept in mind. Firstly, the type of sample and the sampling technique can influence the validity of the results. Extracted teeth were chosen as samples within the studies. When pulverized, these samples are likely to better reflect the microbiome of the whole root canal system, whereas paper point sampling only samples the microorganisms residing in the main root canal (Akpata 1976). Also, on the one hand, sterile paper points can be a source of DNA contamination in community-profiling of clinical samples (Van der Horst et al. 2013). On the other hand, it has been suggested that extraction forces create a fluid influx, which could contaminate the root canal community, although the number of contaminating bacteria in relation to the microbial community has not been studied (Kapalas et al. 2011). Secondly, sample size and population can influence to which extent conclusions can be generalized to the entire population (Goodrich et al. 2014). Even though the studies in this thesis had a sample size above 20, it is uncertain whether the samples accurately represent the nature of these infections within the total population (Kelly et al. 2015). Lastly, the cross-sectional design of the study and the analysis of the functional potential of the microbiome have only mapped part of the infection process and only allow correlations to be made; no statements of cause and effect can be made (Franzosa et al. 2015). Analysis of RNA (transcriptomics), proteins (proteomics) or metabolites (metabolomics) is based on functional activity and, when assessed over time, can offer further insight into the aetiopathogenesis of apical periodontitis and give targets for intervention strategies (Franzosa et al. 2015).

Apart from methodological limitations, microbiome profiling also comes with technical limitations. Microbiome research has come a long way by applying culture-independent techniques instead of cultivation (Anderson et al. 2012). Still, many steps are involved in DNA analysis, bioinformatical and statistical processing, where each step can introduce bias, especially when it comes to the relatively unexplored analysis of fungal DNA (Bokulich & Mills 2013; Goodrich et al. 2014; Franzosa et al. 2015). For instance, DNA isolation can be hindered because of containment within tough microbial cells or binding to dentin (Brundin et al. 2014). Also, library preparation for sequencing requires primers and polymerase chain
reactions. Although this should allow universal amplification, small but significant variation can exist for different DNA templates. For instance, variation in nucleotide sequence can influence the affinity of the primer. Similarly, GC-content and length of the DNA template can influence PCR efficacy (Kennedy & Oswald 2011; Bokulich & Mills 2013; Op De Beeck et al. 2014). This can result in preferential sequencing of certain DNA over other DNA. Moreover, all reads generated by microbiome sequencing have to be filtered, normalized, clustered, taxonomically assigned and statistically analysed. For each step, many different methods are possible, and each is accompanied by its own hurdles and bias (Goodrich et al. 2014; Franzosa et al. 2015). Studies using current techniques are likely to underestimate the diversity of the microbiome. Development of technologies can help to improve microbiome analysis. For instance, whole metagenome shotgun sequencing has an increased resolution when compared to amplicon sequencing, and functional profiling gives more information than compositional profiling (Franzosa et al. 2015). Therefore, as technologies and methods are improving, many of the obstacles will be overcome and the conclusions of the studies in this thesis can possibly be affirmed.

Despite these considerations, it is clear that bacteria and fungi are present in complex communities and interact with the host. This can have important implications for our views on root canal infections and their treatment. The microbial composition of root canal infections greatly resembles that of the oral cavity, which is the source of inoculation of the infection (Möller 1966; Hsiao et al. 2012). The pathway through the dental coronal structures, the nutrient and oxygen availability and the host response dictate the ecological niche of the root canal and select for the root canal specific microbiome (Sundqvist 1976; Lamont & Hajishengallis 2015). The microbiome of root canal infections can differ compositionally, but despite the differences in composition all are capable of sustaining disease. Likewise, in marginal periodontitis, compositionally different microbial communities can demonstrate similar functions and sustain disease (Jorth et al. 2014). The disease process is even more complex than the interactions between the microorganisms, since the microbial-host interface is a dynamic environment where the host and the infecting microbes co-exist in relative homeostasis. During an exacerbation of apical periodontitis, it is debatable whether a microbiological shift is responsible for the host’s response, or that the microorganisms react to a change in the host. Even without root canal treatment, apical periodontitis can subside, although not disappear (Whitworth 2000). When the root canal is treated, homeostasis is severely disturbed. It can be questioned whether the inflammation goes down because of less microbial stimulation, or that the subsiding inflammation causes the microorganisms to die and express less virulence factors (Matsumiya & Kitamura 1960).
The role of fungi in complex root canal communities is still unclear, but studies have shown that fungi may be involved in many processes. *Candida* can coaggregate with streptococci, which may enhance fungal colonization of oral surfaces (Jenkinson *et al.* 1990). Bacteria and fungi can also show nutrient interdependence (Willems *et al.* 2016). *Candida* can consume lactate that is secreted by *Streptococcus mutans*, and in turn increase the pH which is a more suitable growth environment for both (Willems *et al.* 2016). Also, bacterial-fungal interaction can modify the virulence of both microorganisms (Xu *et al.* 2014; Schlecht *et al.* 2015; thesis chapter 5). In mice, co-infection of *Streptococcus oralis* and *Candida albicans* can lead to augmented colonization of both, increased oral thrush and deep organ infection (Xu *et al.* 2014). In another mice model, *Candida* infection assisted deep organ infection of *Staphylococcus aureus* (Schlecht *et al.* 2015). Additionally, the bacterial-fungal interaction can influence the immune response of the host (Fallarino & Puccetti 2006; Ifrim *et al.* 2014). Immune cells that are primed with fungal or bacterial fragments can be trained to respond with immunity or tolerance to a subsequent stimulation (Ifrim *et al.* 2014). Immunosuppression is possible after Toll-like receptor activation, which in turn can stimulate tryptophan catabolism. Tryptophan catabolites promote tolerance of microorganisms by the immune system (Fallarino & Puccetti 2006). Thus, interactions between bacteria, fungi and the host are complex and the processes involved in root canal infections need to be studied further.

Complex microbial communities interacting with the host can be of significance in multiple areas. In this thesis, teeth with asymptomatic apical periodontitis were analysed. Possibly, mixed-kingdom infections with special virulence traits are more prone to cause certain symptoms. Several bacteria have already been linked to specific symptoms of apical periodontitis (Sundqvist 1976; Siqueira & Rôças 2009). Moreover, conventional root canal treatment may be more complicated in mixed-kingdom communities. The increased diversity and interactions can lead to more resilience and treatment resistance (Adam *et al.* 2002; Harriott & Noverr 2010). Because bacteria and fungi are morphologically and physiologically different organisms, different treatment strategies may be required. Furthermore, chronic inflammation of apical periodontitis is correlated to systemic disease, although a cause-effect relationship has not yet been established (Segura-Egea *et al.* 2015). The interface between the root canal and the periapical tissues also provides a portal of entry for microorganisms to cause local and possibly systemic disease. These microorganisms can colonize and infect the host if it is more susceptible, such as in the elderly or immunocompromised patients (Bodineau *et al.* 2009; Brown *et al.* 2012). Since dental and general health care continue to improve, more people will retain their dentition longer in life and more people will be at risk. Accordingly,
antimicrobial treatment efficacy of mixed-kingdom root canal infections needs to be established, especially when taking a possible systemic effect and risk population into consideration.

The complex ecology of root canal infections could be one of the causes for little improvement of success rates of root canal treatment over the years (Ng et al. 2007). Therefore, novel methods and new antimicrobial substances have been studied. This thesis aimed to explore a new treatment approach using vanadium chloroperoxidase (VCPO). The enzyme converts available substrates into antimicrobial products, which proved to have good antimicrobial properties in vitro. These preliminary results on VCPO are promising. Nevertheless, these are based on in vitro studies using biofilm models with laboratory microbial strains. Laboratory studies permit well-controlled simulation of a certain aspect of the clinical situation where only a single variable is altered (McBain 2009). Even though the used strain was isolated from a patient, years of cultivation in a laboratory setting may have altered its properties (Fux et al. 2005). For several studies within this thesis, *E. faecalis* was the model organism. Because of all its virulence traits and its ease of handling in a laboratory setting, it seems appropriate for in vitro studies. However, it is questionable whether the used strains reflect the properties making them virulent in root canal infections and whether *E. faecalis* actually significantly contributes to the infections. Microbiome analyses in this thesis revealed only minor presence in primary root canal infections (chapter 2a: 0.2% abundance in 18/23 teeth; chapter 4: no Enterococcus found). Therefore, direct translation of the in vitro efficacy to the clinical situation is not possible. Further studies are required to test the efficacy of VCPO against more complex microbial communities, also within the restrictions of the root canal system. Before clinical application is an option, some other hurdles have to be overcome. The medicament has to be proven to be safe to the patient. Since VCPO generates products similar to NaOCl, similar complications as with extrusion beyond the apical foramen may exist, such as tissue necrosis (Hulsmann & Hahn 2000). Remnants of a medicament in the root canal can influence the sealing of the root canal system, so, ideally, a medicament should be completely removed before the filling procedure (Kim & Kim 2002). Immobilizing the enzyme on magnetic beads could facilitate insertion and removal of the medicament from the root canal system and prevent extrusion into the periapical tissues (Krogh et al. 1999). Thus, although VCPO is a promising new approach to root canal treatment, further studies are required before implementation in clinical practice.

The results of this thesis have contributed to uncovering the full extent of the complexity of root canal infections. Over the past years, research has focused on enhancing the technical aspects of root canal treatment, although no improvement
of success rates is apparent (Ng et al. 2007). It is unlikely that the root canal system can be rendered sterile using current treatment techniques. However, when the root canal system and especially the apical delta has a low enough microbial load in both quantity and quality, it is compatible with size reduction and possible elimination of the apical inflammation (Matsumiya & Kitamura 1960; Peters & Wesselink 2002). Unfortunately, the cut-off level for compatibility of the microbial load to the host is unknown and impossible to determine within the limitations of current sampling techniques. Therefore, instead of attempting to decrease the microbial load, root canal treatment could aim at lowering the influx of proteinaceous inflammatory exudate. This could be done by modulating the host response. In marginal periodontitis, several host modulatory agents have been suggested to intervene with the immune response causing bone degradation. Such agents can block specific inflammatory mediators or enzymes, possibly in conjunction with conventional antimicrobial therapy (Gokhale & Padhye 2013). Further studies should examine whether such strategies could prevent additional loss of the periodontal and periapical tissues and possibly resolve apical periodontitis.

In a completely different strategy dentists could aim to care for patients and manage their teeth with apical periodontitis, instead of aiming to cure the inflammation. Many of the teeth with apical periodontitis give no or mild symptoms. Despite the relatively high prevalence of 36% in root canal treated teeth with apical periodontitis (Pak et al. 2012), the 10-year survival of root-treated teeth is about 80% (Kirkevang et al. 2014). Therefore, intervention may not be necessary for tooth survival and comfort of the patient. This is in line with changing views on all aspects of oral health and comprehensive oral health care. In this light, much of the dental intervention can be considered overtreatment and promoting tooth loss, rather than improving oral well-being (Qvist et al. 1992; Fejerskov et al. 2013). However, taking into account the aforementioned possible effect of apical periodontitis on systemic health, the absence of such an effect has to be affirmed before deciding not to attempt to eliminate periapical inflammation. Future research should elucidate how the microbiome of root canal infections functions, and if and how it interacts with the host. If a systemic detrimental effect is evident, more effective strategies to eliminate or inactivate the infection and preserve oral function and well-being should be developed.