University of Glasgow

Candida albicans and *Enterococcus faecalis* interkingdom interaction: implications for management of endodontic infections

Om Alkhir Mohamad Alshanta BDS, MSc

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College of Medical, Veterinary and Life Sciences

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Abstract

Endodontic disease is inflammation or infection of the pulp tissue lying within the root canal space of teeth and the associated periapical tissue, also known as periapical periodontitis. It is estimated that around 52% of adults worldwide have at least one tooth with periapical periodontitis. Root canal infections are usually debilitating, associated with pain, discomfort and decreased quality of life. The role of microorganisms in the pathogenesis of periapical periodontitis is well established.

Root canal infections are characterised by biofilms, i.e. polymicrobial infections encased in polymers that are isolated from infection sites. *Candida albicans* and *Enterococcus faecalis* are common endodontic pathogens that have been linked with persistent endodontic infections. Deeper understanding of their response to endodontic procedures and how they interact with each other may enhance our understanding of their role and lead to better treatment outcomes.

Chapter 1: reviews the literature in relation to endodontic infections and their related microbiology, basics of endodontic treatment, *C. albicans* and *E. faecalis* as endodontic pathogens and *C. albicans* and *E. faecalis* in mixed biofilms.

Chapter 2: is presented in the traditional chapter format. It aimed at assessing a panel of *C. albicans* oral clinical isolates for their ability to form biofilms using the crystal violet assay (biomass). Following the selection of representative low and high biofilm forming isolates, the response of different *C. albicans* phenotypes to NaOCl was assessed using XTT (metabolism), crystal violet and microscopic imaging. *C. albicans* showed an ability to tolerate NaOCl treatment and was able to regrow when a nutrient source is provided. In addition, *C. albicans* heterogeneity influenced the response to NaOCl treatment, where low biofilm forming isolates showed increased tolerance compared with high biofilm forming isolates.

Chapter 3: is presented as a published journal article. It further analysed the response of single species of *C. albicans* biofilms to NaOCl. It also assessed the effect of sequential treatment with sodium hypochlorite followed by EDTA on *C.*

albicans biofilms viability using XXT and biofilm biomass using crystal violet assays. In this analysis, the immediate post treatment effect as well as the regrowth potential over 72h of re-incubation with fresh media were investigated. Treatment with NaOCl alone resulted in a significant regrowth of the treated population while regrowth was significantly inhibited with sequential treatment.

Chapter 4: is presented as a published journal article. In this chapter the complexity of the used model was increased by incorporating *E. faecalis* into the subsequent analysis. *C. albicans* and *E. faecalis* mono- and dual-species biofilms were assessed against sequential (NaOCl followed by EDTA) or continuous chelation protocols (NaOCl combined with HEDP) using qPCR and microscopic examination. Sequential treatment with NaOCl and EDTA were more effective in inhibiting *C. albicans* and *E. faecalis* regrowth compared with continuous chelation. In terms of the difference between mono- and dual-species, *C. albicans* was more susceptible to endodontic irrigants when co-cultured with *E. faecalis*, while *E. faecalis* remained unaffected. The regrowth of *C. albicans* was significantly inhibited when cocultured with *E. faecalis*.

Chapter 5: is presented as a published journal article. Firstly, the effect of *E. faecalis* on *C. albicans* biomarkers was assessed using crystal violet, microscopy and real time PCR. Afterwards, RNA seq was employed to investigate the molecular basis of the interaction between *C. albicans* and *E. faecalis*. The expression of *EntV* across twelve *E. faecalis* strains was also measured and correlated with the degree of *C. albicans* biofilm inhibition. Finally, the effect of pH modified and ultrafiltered *E. faecalis* supernatant on *C. albicans* hyphal morphogenesis and biofilm formation was assessed. The results show that *E. faecalis* inhibited *C. albicans* growth, hyphal morphogenesis and biofilm formation. The transcriptional analysis showed rapid and significant changes in the *C. albicans* transcriptome characterized by upregulation in amino acid biosynthesis and metabolism genes. This guided further analysis on EntV and pH dependent mechanisms where *C. albicans* biofilm inhibition were shown to be related to an *E. faecalis* induced environmental pH drop.

In conclusion, this thesis highlights the importance of considering the tolerance potential of the treated microorganisms and the role it may have in the aetiology

of disease persistence. It also contributes to our knowledge of the interaction between microorganisms in polymicrobial infections and how the ultimate outcome of this interaction may impact disease progression and prognosis.

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Author's Declaration

I declare that I have carried out the work described in this thesis unless otherwise acknowledged or cited, under the supervision of Professor Gordon Ramage and Dr. William McLean. I further declare that this thesis has not been submitted for any other degree at the University of Glasgow, or any other institution.

Om Alkhir Alshanta

List of publications

Alshanta, O.A., Shaban, S., Nile, C.J., McLean, W. and Ramage, G., 2019. *Candida albicans* biofilm heterogeneity and tolerance of clinical isolates: implications for secondary endodontic infections. Antibiotics, 8(4), p.204.

Abusrewil S, **Alshanta OA**, Albashaireh K, Alqahtani S, Nile CJ, Scott JA, McLean W. Detection, treatment and prevention of endodontic biofilm infections: what's new in 2020? Crit Rev Microbiol. 2020 Mar;46(2):194-212. doi: 10.1080/1040841X.2020.1739622. Epub 2020 Apr 1. PMID: 32233822.

Alshanta, O.A., Alqahtani, S., Shaban, S., Albashaireh, K., McLean, W. and Ramage, G., 2020. Comparison of three endodontic irrigant regimens against dual-species interkingdom biofilms: Considerations for maintaining the status quo. *Antibiotics*, *9*(9), p.634.

Young, T., **Alshanta, O.A.**, Kean, R., Bradshaw, D., Pratten, J., Williams, C., Woodall, C., Ramage, G. and Brown, J.L., 2020. *Candida albicans* as an essential "keystone" component within polymicrobial oral biofilm models?. *Microorganisms*, *9*(1), p.59.

Kalimuthu, S., **Alshanta, O.A.**, Krishnamoorthy, A.L., Pudipeddi, A., Solomon, A.P., McLean, W., Leung, Y.Y., Ramage, G. and Neelakantan, P., 2022. Small molecule based anti-virulence approaches against *Candida albicans* infections. *Critical Reviews in Microbiology*, pp.1-27. DOI: 10.1080/1040841X.2021.2025337.

Alshanta, O.A., Albashaireh, K., McKloud, E., Delaney, C., Kean, R., McLean, W. and Ramage, G., 2022. *Candida albicans* and *Enterococcus faecalis* biofilm frenemies: When the relationship sours. *Biofilm*, 4, p.100072. DOI: https://doi.org/10.1016/j.bioflm.2022.100072.

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Abbreviations

AP: Apical periodontitis

AS: Artificial saliva

BHI: Brain heart infusion

$Ca(OH)_2$: Calcium hydroxide

CBA: Columbia blood agar

CFE: Colony forming equivalent

CFU: Colony forming units

CHX: Chlorhexidine digluconate

Ct: Cycle threshold

CV: Crystal violet

EDTA: Ethylenediaminetetraacetic acid

FBS: Fetal bovine serum

GDH: Glasgow Dental Hospital

HBF: High biofilm former

HEDP: 1-hydroxyethane 1,1-diphosphonic acid

LBF: Low biofilm former

MHB: Muller Hinton broth

MTA: The mineral trioxide aggregate

NaOCl: Sodium hypochlorite

NGS: Next generation sequencing

PA: Periapical periodontitis

PBS: Phosphate buffered saline

PCA: Principal components analysis

PCR: Polymerase chain reaction

qPCR: Quantitative polymerisation chain reaction

RPM: Revolutions per minute

RPMI: Roswell Park Memorial Institute

SAB: Sabouraud's dextrose agar

SEM: Scanning electron microscopy

THB: Todd Hewitt broth

XTT: (2,3-bis[2-methyloxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide

YPD: Yeast peptone dextrose

1 General introduction

Oral diseases, especially those affecting primary and permanent dentitions represent a substantial health burden, affecting a large percentage of the global population. It is estimated that approximately 3.5 billion people worldwide are affected by oral conditions (World Health Organization, 2022). Oral diseases encompass various conditions including, but not limited to, dental caries, periodontitis, oral cancers and congenital defects. Oral conditions are usually debilitating, associated with pain, discomfort and decreased quality of life. Dental caries is the most common non-infectious disease worldwide affecting the permanent teeth of 2 billion individuals (World Health Organization, 2022). While dental caries is a preventable disease, it continues to be a major health concern and may result in serious consequences. Untreated caries eventually leads to endodontic disease marked by inflammation and potentially infection of the pulp tissue inside the root canals of teeth which may spread to affect the periapical tissue surrounding the tips of dental roots. Infections of the periapical tissues (also known as periapical or apical periodontitis) (AP) may result in apical abscess and may eventually lead to tooth extraction. Although the global prevalence of AP is poorly reported compared with other oral diseases such as dental caries and periodontitis, recent meta-analyses reported a high prevalence of AP with around 52% of adults worldwide having at least one tooth with AP (Tibúrcio-Machado et al., 2021, Miri et al., 2018).

Endodontic infections are biofilm-based and polymicrobial in nature with various organisms contributing to the disease. While bacteria appear to predominate within the diseased endodontic environment, most studies are bacterial based and use bacterial 16S gene analysis. However, other microorganisms are believed to be involved in the pathogenesis of AP such as archaea, viruses and fungi (Narayanan and Vaishnavi, 2010). Yeasts are common inhabitants of the oral cavity; therefore, it is very likely they represent an important yet underestimated population of organisms in the endodontic environment. A broader view of endodontic microbiology should be deemed essential. As reported for other infection sites of the human body such as gut and vagina, there is a potential for interkingdom interactions between bacteria and yeasts (McKloud et al., 2021, Garsin and Lorenz, 2013). This interaction is likely to impact the endodontic infection outcome and require different treatment strategies (Persoon et al., 2017a).

This chapter aims to provide a review of the literature surrounding endodontic infection as a biofilm-based disease in terms of microbiology, treatment strategies as well as the role played by *Candida albicans* and *Enterococcus faecalis* as model pathogens. Finally, it highlights the importance of interkingdom interaction in developing our understanding of the nature of root canal infections and how it impacts disease outcome and response to endodontic therapeutics.

Elements of this chapter have been previously published in:

Abusrewil S, **Alshanta OA**, Albashaireh K, Alqahtani S, Nile CJ, Scott JA, McLean W. Detection, treatment and prevention of endodontic biofilm infections: what's new in 2020? Crit Rev Microbiol. 2020 Mar;46(2):194-212. doi: 10.1080/1040841X.2020.1739622. Epub 2020 Apr 1. PMID: 32233822.

Kalimuthu, S., **Alshanta, O.A**., Krishnamoorthy, A.L., Pudipeddi, A., Solomon, A.P., McLean, W., Leung, Y.Y., Ramage, G. and Neelakantan, P., 2022. Small molecule based anti-virulence approaches against *Candida albicans* infections. *Critical Reviews in Microbiology*, pp.1-27. DOI: 10.1080/1040841X.2021.2025337.

1.1 Endodontic infections

Endodontics is the area of dentistry concerned with aetiology, diagnosis, treatment and prognosis of diseases of the soft pulp tissue inside the root canal of teeth and their surrounding periradicular or periapical tissue. Dental pulp is generally a sterile tissue containing nerves and vascular tissues that are connected to the surrounding periradicular tissues through a small apical opening, though recent studies appear to suggest the presence of bacterial DNA in the pulp of pristine, healthy teeth (Widmer et al., 2018). The source of bacterial DNA inside healthy teeth is not clear. However, it may indicate a transient bacteraemia in the pulp blood circulation or, more likely, a contamination from oral bacteria during microbiological sampling. The pulp is protected from the oral microbiome by layers of mineralised tissues (enamel, dentine and cementum). Breach of these tissues, as a result of dental caries, tooth fractures or cracks, leaking restorations, resorption or advanced periodontal disease can expose the dentine and provide routes for the oral microbiota to ingress towards the pulp (Yu and Abbott, 2007). Another possible route of pulp infection is the direct invasion of microorganisms to the pulp during initial root canal treatment if substandard clinical procedures are used. This microbial infection of pulp tissue then spreads to affect the periradicular tissues resulting in a periapical inflammatory reaction, host immunological response and tissue damage (Figure 1.1).

The essential role of microorganisms in the aetiology of AP is well established. The landmark paper by Kakehashi *et al* (Kakehashi et al., 1965) undoubtedly demonstrated that no apical periodontitis developed in germ-free rats whose molar pulps were left exposed to the oral cavity, whereas a massive periapical infection developed in the control rats with conventional oral microflora. Microorganisms including bacteria and fungi are known for their ability to form biofilms on abiotic and biotic surfaces. Biofilm-mediated endodontic infections are probably formed by the initial invasion and colonisation of radicular dentine by planktonic oral microorganisms at some time following hard tissue breakdown leading to biofilm formation (Svensäter and Bergenholtz, 2004).

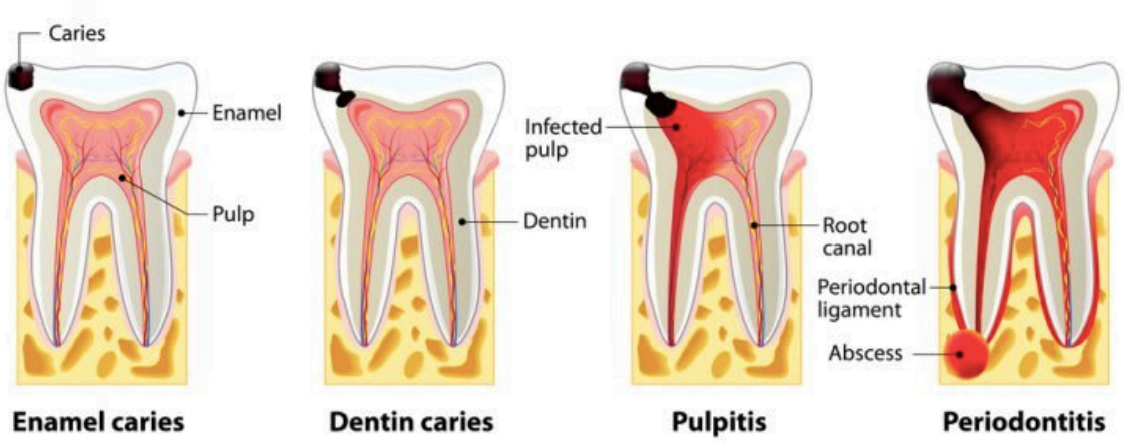


Figure 1.1: Stages of caries progression from enamel caries to periapical periodontitis. Taken from (Shutterstock, 2022).

When considering endodontic biofilms and their management, it seems that the sensible question to ask is whether endodontic biofilms are different from those of other human infection sites? Theoretically, the endodontic environment of the necrotic pulp is different as it is mainly an anaerobic environment, lacks blood supply and has radicular dentine as a unique substrate for biofilm formation. In addition, it is characterised by the presence of anatomical complexity such as dentinal tubules, lateral canals and isthmuses that favour microbial growth and provide physical protection of biofilms from antimicrobial disinfection. Therefore, it can be assumed that the unique nature of the endodontic environment may render endodontic biofilms distinct from other microbial biofilms. Interestingly, dental plaque, another oral biofilm at later stages of biofilm development is characterised by the precipitation of calcium phosphates within the plaque extracellular matrix forming a calcified biofilm (calculus) (Akcali and Lang, 2018). Similarly, the interaction of dentine with endodontic biofilms formed by *E. faecalis* results in bacterial mediated dentine dissolution with deposition of calcium and phosphate ions within biofilm matrix and biofilm calcification. Therefore, endodontic biofilms do share some features of other oral biofilms. Calcified biofilms require treatment strategies that are completely different from those applied for other human biofilms where mechanical disruption of the calcified biofilms is indispensable.

1.1.1 Microbiology of endodontic infections

1.1.1.1 Culture techniques

Primary (untreated) endodontic infections are caused by microorganisms initially invading and colonising the root canal and pulp tissue contained within. Secondary endodontic infections (endodontic treatment failure), on the other hand, are caused either by microorganisms that survived biochemical disinfection during primary endodontic treatment or those that have re-invaded the root canal via coronal leakage. The terms "secondary" and "persistent" endodontic infections are often used interchangeably in the literature. Endodontic microbiology has been traditionally studied by culture techniques. Early culture based microbiological analysis of endodontic infections shows that there is a clear difference in the microbial composition of primary and secondary endodontic infection. The microflora of untreated teeth is mainly a mixture of Gram-negative, Gram-positive, and mostly anaerobic microorganisms. On the other hand, canals with failed endodontic treatment are dominated by facultative anaerobic and Gram-positive bacteria (Gomes et al., 2004). Despite the broad range nature of culture techniques that allow for cultivation of a variety of microbial species, this methodological approach overlooks a large number of uncultivable, yet important species (Siqueira and Rôças, 2005a).

1.1.1.2 Molecular techniques

The introduction of molecular based techniques has helped revolutionise our understanding of endodontic microbiology. Molecular techniques such as polymerase chain reaction (PCR) have been used extensively to redefine the nature of endodontic infections. For example, it was shown that a group of uncultivable and unrecognised bacterial species were actually predominant in dentoalveolar abscess and inflammatory exudate samples of pulpal infections. Furthermore, *Fusobacterium nucleatum* and *Porphyromonas endodontalis* were reported to be largely underestimated in those samples (Wade et al., 1997). Siqueira and Rôças (2005b) showed a marked difference in the bacterial composition between primary symptomatic and asymptomatic cases. Overall, they showed that *Treponema Denticola*, *Porphyromonas endodontalis*, *Tannerella forsythia*, *Pseudoramibacter alactolyticus*, *Dialister pneumosintes*, *Filifactor alocis*, *Porphyromonas gingivalis*, *Pseudopropionibacterium propionicum*, and

Treponema socranskii are the most prevalent species in primary endodontic infections. Secondary infections are associated with less diverse bacterial species. Culture studies revealed that *Enterococcus faecalis* are found in significantly higher frequency in secondary infections (Sundqvist et al., 1998, Siqueira and Sen, 2004). These findings were further confirmed by various culture independent molecular studies (Siqueira Jr and Rôças, 2004, Rôças et al., 2004a, Rôças et al., 2004b) and in a systematic review of studies with both detection methods included (Zhang et al., 2015). An illustration of microorganisms associated with asymptomatic primary and persistent endodontic infections is presented in Figure 1.2.

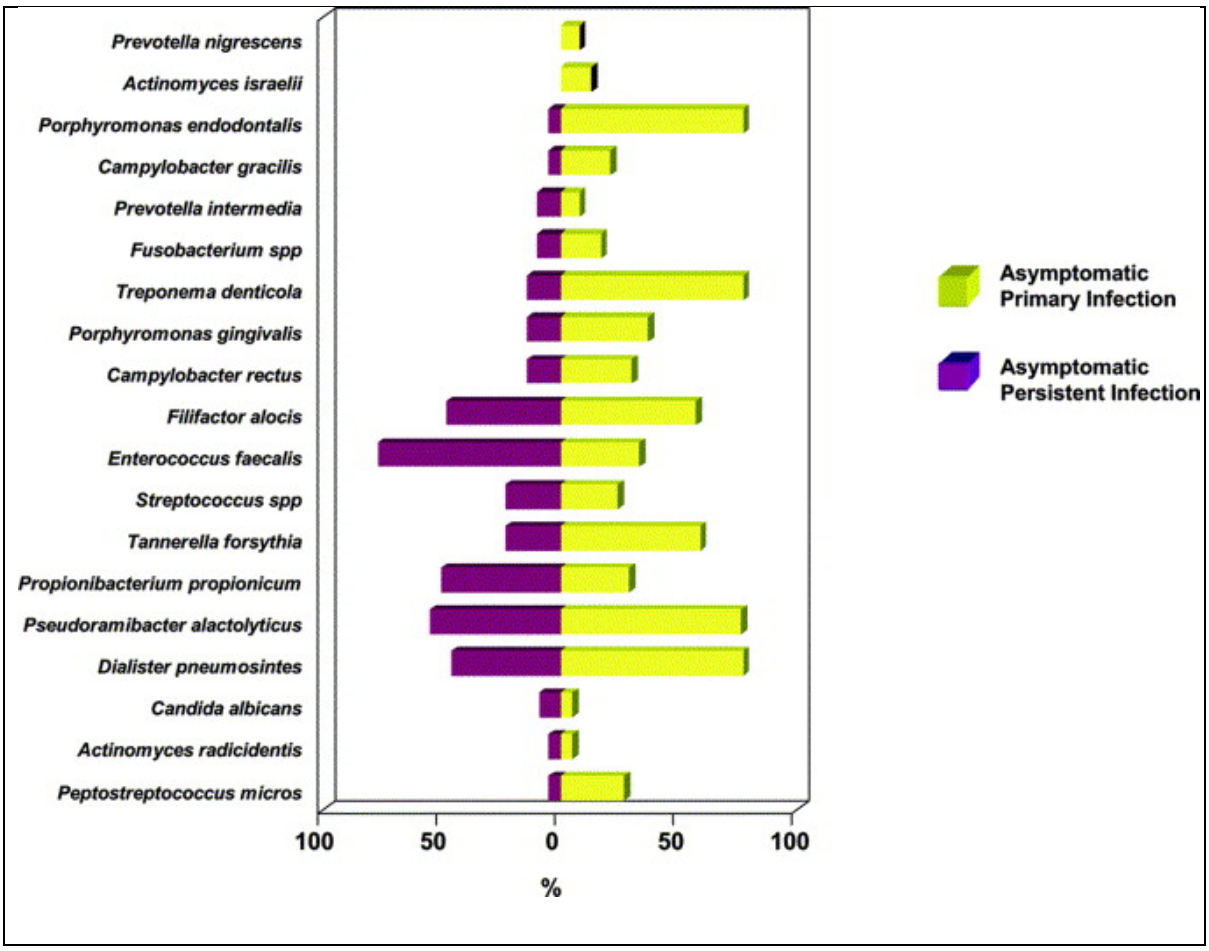


Figure 1.2: Comparison of microorganisms associated with asymptomatic primary and persistent infections. Taken from (Siqueira and Rôças, 2005b).

Unlike some human diseases where a single organism is responsible for disease causation, such as tuberculosis, endodontic infections are polymicrobial in nature where a variety of microbes can be involved (Siqueira JR and Rôças, 2014). Although most endodontic microbiological studies are bacteria focussed, the

existence of interkingdom contributions in root canal infection is a more plausible ecological scenario. Archaea, viruses and fungi have been implicated in endodontic disease (Narayanan and Vaishnavi, 2010). Fungi, predominantly *C. albicans*, have also been detected in infected root canals using culture and molecular techniques (Baumgartner et al., 2000, Waltimo et al., 1997). Similar to *E. faecalis*, *C. albicans* showed higher prevalence in persistent infections (Poptani et al., 2013, Siqueira and Sen, 2004). In addition, it is worth mentioning that the presence of fungi in root canal infections impacts upon the composition of the bacteriome (Persoon et al., 2017a). Due to their higher prevalence in secondary infections and being seldom found in primary infections, it was hypothesized that *E. faecalis* and *C. albicans* have been introduced in the root canal system at some time following the professional intervention (Siren et al., 1997). For a long time, *C. albicans* and *E. faecalis* were considered as the most commonly isolated fungi and bacteria from persistent infections, and are used extensively as model endodontic pathogens to evaluate endodontic therapeutics and treatment techniques (Poptani et al., 2013, Swimberghe et al., 2019).

1.1.1.3 Next generation sequencing

More recently, next-generation sequencing (NGS) has been used for profiling endodontic microbial communities which has led to a deeper understanding of the composition and relative abundance of microbial communities inside the root canal (Fouad, 2017). Li et al. (2010) conducted the first next generation sequencing study on endodontic infections. Study samples contained only one sample of persistent infection; therefore, it was not possible to compare primary and persistent infections on this occasion. However, they identified six phyla that were not previously reported in endodontic infections. The top five abundant phyla identified were *Bacteroidetes, Firmicutes, Actinobacteria, Fusobacteria*, and *Proteobacteria* in agreement with earlier studies (Munson et al., 2002, Vickerman et al., 2007). Subsequently, further NGS studies appeared in the literature. Manoil et al. (2020) performed a systematic review of the NGS studies published over the last decade. They identified *Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria,* and *Fusobacteria* as the most abundant phyla, regardless of the infection type. *Enterococcus*, as identified by NGS studies, is no longer considered as the most abundant genera in secondary endodontic infections as previously believed. However, it still presented amongst the highly abundant

genera in secondary infections as reported by various NGS studies (Anderson et al., 2013, Siqueira Jr et al., 2016, Zandi et al., 2018). Moreover, it was shown that although *E. faecalis* may be detected in only a limited number of samples, when present, it represented the most abundant species with high abundance ranging from 17% to 99.9% (Zandi et al., 2018, Bouillaguet et al., 2018). This is an indication that *E. faecalis*, when present, tends to dominate the endodontic microbiome in secondary endodontic infections. The main phyla, genera and species of primary and secondary endodontic infections are outlined in Table 1.1 and Table 1.2 respectively.

Mycobiome studies investigating fungi in endodontic infections are far less common. The first NGS investigation of the mycobiome in endodontic infections was conducted by Persoon et al. (2017a) who assessed the prevalence, abundance and diversity of fungi in primary endodontic infection. They also investigated the relationship between fungi and bacteria in these infections. Fungi were detected in 57 % of teeth and *Candida* (96.1 %) followed by *Malassezia* (4.1 %) were the most commonly isolated fungi. Interestingly, they outlined a significant difference in the associated microbiome in teeth with or without fungi. This is in alignment with previous reports where fungi, principally *C. albicans,* had a significant impact on microbiome composition (Janus et al., 2017, Zhai et al., 2020, Bertolini and Dongari-Bagtzoglou, 2019).

Table 1.1: Main phyla, genera and species of primary endodontic infections. Taken from (Manoil et al., 2020).

Study	Siqueira Jr et al. (2011)	Özok et al. (2012)	Persoon et al. (2017a)	Gomes et al. (2015)	Bouillaguet et al. (2018)
Main phyla (max. top 6)	Proteobacteria (43%) Firmicutes (25%) Fusobacteria (16%) Bacteroidetes (9%) Actinobacteria (5%) Synergistetes.	Firmicutes (48%) Actinobacteria (30%) Bacteroidetes (12%) Acidobacteria Chlamydiae	No assessment at the phylum level.	Firmicutes (75.09%) Proteobacteria (7.85%) Actinobacteria (7.01%) Bacteroidetes (6.77%)	Firmicute (36%) Bacteroidetes (23.8%) Actinobacteria (6.4%) Fusobacteria (16%) Synergistetes (9.9%) Proteobacteria (2.4%)
Main genera/species (max. top 12)	*Fusobacterium* (15%) *Pseudoramibacter* (8%) *Novosphingobium* (8%) *Ralstonia* (6%) *Bacterioides* (5%)	Lactobacillus (14.3%) Actinomyces (11.9%) Streptococcus (0.4%) unclassified Actinobacteria (6.9) Prevotella (6.1%) Parvimonas (3.4%) Pseudoramibacter (3%) Bacteroidales (2.7%) Veillonellaceae (2.5%) Fusobacterium (2%) Peptostreptococcus (2%) Porphyromonas (1.8%)	*Prevotella* (12.7 %) *Lactobacillus* (11.2%) *Actinomyces* (7.5 %) *Fusobacterium* (7.2%) *Atopobium* (6.9 %) *Streptococcus* (4.4%) *Leptotrichia* (4.3%) *Phocaeicola* (3.5 %) *Pyramidobacter* (2.9%) *Porphyromonas* (2.7%)	*Enterococcus faecalis* *Parvimonas micra* *Bacteroidaceae* Peptostreptococcaceae *Mogibacterium timidum* *Peptostreptococcus stomatis* *Filifactor alocis* *Fretibacterium fastidiosum.*	*Fusobacterium nucleatum (16%)* *Enterococcus faecalis (0.01%)* *Parvimonas micra (8%)* *Porphyromonas endodontalis (5.7 %)* *Streptococcus constellatus (0.6%)* *Slackia exigua (0.7%)* *Schwatzia AF287291 (1%)* *Dialister pneumosintes (3.4%)* *Prevotella oris (5.7%)*

Table 1.2: Main phyla, genera and species of secondary endodontic infections. Taken from (Manoil et al., 2020).

Study	Anderson et al. (2013)	Siqueira Jr et al. (2016)	Zandi et al. (2018)	Bouillaguet et al. (2018)
Main phyla (max. top 6)	Firmicutes (29.9%) Proteobacteria (26.1%) Actinobacteria (22.72%) Bacteroidetes (13.31%) Fusobacteria (4.55%)	Proteobacteria (46%) Firmicutes (18%) Fusobacteria (15%) Actinobacteria (8%)	Firmicutes (47%) Fusobacteria (14%) Bacteroidetes (12%) Proteobacteria (12%) Actinobacteria (9%) Synergistetes (4%)	Firmicute (48.4%) Bacteroidetes (9.5%) Actinobacteria (23.4%) Fusobacteria (5.6%) Synergistetes (4.5 %) Proteobacteria (4.8%)
Main genera/species (max. top 12)	*Streptococcus* (10.9%) *Prevotella* (8.21%) *Lactobacillus* (8.06%) *Kocuria* (5.17%) *Neisseria* (3.38%) *Enterococcus* (2.59%) *Acinetobacter* *Atopobium, Rothia* *Pseudomonas* *Propionibacterium* *Schlegelella*	*Fusobacterium* and *Pseudomonas* (15 %) *Klebsiella* *Stenotrophomonas* *Pseudoramibacter* *Pyramidobacter* *Enterococcus*	*Enterococcus* (13.9%) *Fusobacterium* (12.7%) *Streptococcus* (9.8%) *Actinomyces* (8.2%) *Desulfobulbus* (5.2%) *Fretibacterium* (3.6%) *Treponema* (2.3%) *Prevotella* (2%) *Alloprevotella* (0.01%)	*Fusobacterium nucleatum* (5.3%) *Enterococcus faecalis* (18.9%) *Parvimonas micra* (2.6%) *Porphyromonas endodontalis* (2%) *Streptococcus constellatus* (3.5%) *Slackia exigua* (1.3%) *Schwatzia* (3.5%) *Dialister pneumosintes* (1.2%) *Prevotella oris* (1.5%)

1.1.1.4 Limitations of endodontic microbiological studies

With all the advances brought by NGS, it has created more complexity. This has created challenges in our ability to model the biogeography of the oral cavity. This has necessitated the need to utilize relevant bioinformatic tools to interrogate the resultant data. It is important to recognize that NGS alone provides merely a list of implicated microorganisms. Although relative abundance can be determined, we cannot extrapolate from this a primary driving role in endodontic disease. Importantly, metagenomic approaches are unable to determine viability unless appropriate methodologies are applied. Therefore, unless studies are designed to exclude sequencing from dead bacteria or fungi then it is possible for example that our understanding of the microbiome in post-treatment disease may be founded on erroneous data. One must recognize that despite the power of metagenomics in determining composition, it is somewhat uni-dimensional, and some may say an exercise in "stamp collecting". Though there is a vast array of deeper understanding that is necessary and possible if alternative methodologies are embraced, such as metatranscriptomics, proteomics and metabolomics (Aguiar-Pulido et al., 2016). Embracing the "omics" will lead us to a deeper understanding of endodontic biofilm functionality and more importantly its response to environmental/treatment challenges.

Another underestimated yet important factor to be considered when interpreting endodontic microbiological studies is the sampling technique. Sampling root canals is achieved either by sterile paper point or pulverisation techniques. In the first technique, a sterile paper point is inserted passively inside the root canal with or without previous mechanical disruption of the endodontic biofilm using an endodontic file. The second technique involves cryogenic grinding of the whole or part of the tooth (Figure 1.3). It is important to emphasise the fact that despite the high detection rate using molecular techniques, they are generally based upon paper point sampling. Pulverisation results in higher detection compared to paper point sampling, however, its use is limited to extracted teeth or periapical sampling obtained during periradicular surgery (Tran et al., 2013). The passive sampling that occurs with paper points may not reflect accurately the type, number and diversity of the root canal flora because it does not include inaccessible areas such as lateral canals, or more importantly the adherent endodontic biofilm (Sundqvist and Figdor, 2003, Sathorn et al., 2007). Sampling

using culture and molecular techniques in microbiologically focussed endodontic studies is also highly prone to contamination. This may occur at the site of sample recovery from oral microorganisms that can easily contaminate the sample or during handling and laboratory procedures. The accuracy of current sampling methodologies is also affected by intracanal medicaments and cannot be used for patient follow up after canal obturation. Efforts should be directed towards enhancing the sampling techniques to be more representative of the microbial diversity of the root canal and more robust. Improved sampling may have a greater impact on enhancing our understanding of endodontic microbiology than molecular techniques alone.

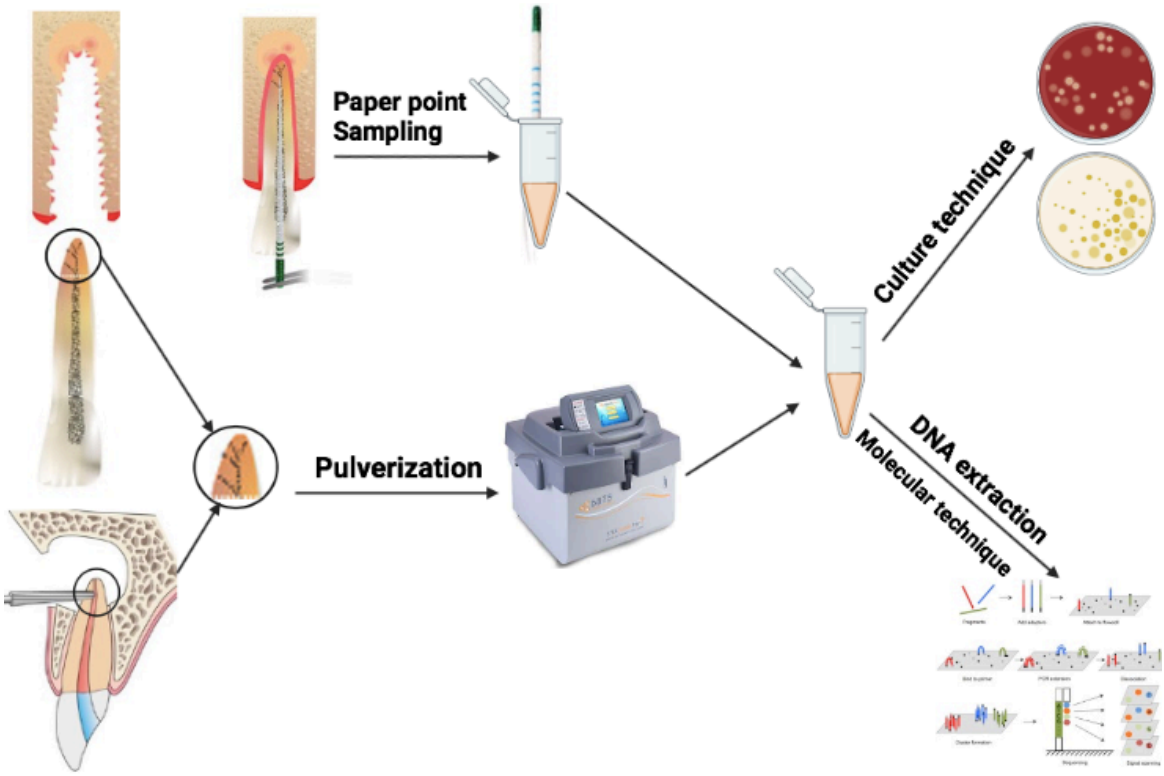


Figure 1.3: Sampling of root canals for microbiological identification. Sampling is achieved using paper point inserted passively into the root canal or through pulverisation. The root tip of the tooth is acquired from extracted teeth or during periradicular surgery. Diagram created with Biorender.com.

1.1.2 Endodontic treatment

Root canal treatment is the treatment of choice for endodontic infections. Root canal therapy aims to (1) eliminate microorganisms from the root canal system to a level that promotes the healing of periradicular tissues, (2) provide a three-

dimensional hermetic seal to prevent reinfection. Different hand and rotary instruments are used, during root canal therapy, to mechanically debride the root canal walls thus disturbing/removing biofilm. Nevertheless, the cross-sectional root canal configuration can pose a challenge for adequate debridement, as these instruments tend to leave untouched recesses in oval canals (Taha et al., 2010, Weiger et al., 2002). These instruments also create a smear layer that covers the root canal walls. The smear layer consists of organic (pulp tissue remnants) and inorganic (dentine chips) tissues (Gwinnett, 1984). This layer might act as a protective barrier to biofilms formed on root canal walls (Paque et al., 2009) and might encourage the adherence of microorganisms such as *C. albicans* (Sen et al., 2003). Furthermore, it may compromise the quality of root canal sealing (Kokkas et al., 2004). Therefore, the use of chemical irrigating solutions, to maximise the root canal debridement and to remove the smear layer, is vital for successful root canal treatment (Figure 1.4).

1.1.2.1 Chemical disinfection

Until the present day, the management of endodontic infections is based on the non-specific broad killing of endodontic microbes using broad spectrum antimicrobials, mainly sodium hypochlorite (NaOCl). There are many reasons behind this approach. The most important reason is that root canal infections are polymicrobial in nature where a variety of microorganisms and/or their interactions are involved directly or indirectly in the pathogenesis and progression of the disease in previously sterile root canals. Systemic antibiotics cannot reach root canals and it has been found that using antibiotics as intracanal medicaments is ineffective. Moreover, these may favour the growth of fungi and cause tooth discolouration (Segura-Egea et al., 2018). Therefore, detection of individual species in the root canal and identification of their antimicrobial susceptibility would be with little benefit in the management of patients with localized endodontic infection. Of note, however, in acute odontogenic infection with systemic involvement, an abscess can be sampled and antimicrobial susceptibility determined, thus allowing an appropriate systemic antibiotic to be employed (Fouad et al., 2020). There is a caveat to the limitations of antibiotic use in endodontic infections, and this is in their use in the form of a triple antibiotic paste in regenerative endodontics where viable stem cells and blood supply is introduced into the root canals to regenerate pulp tissues.

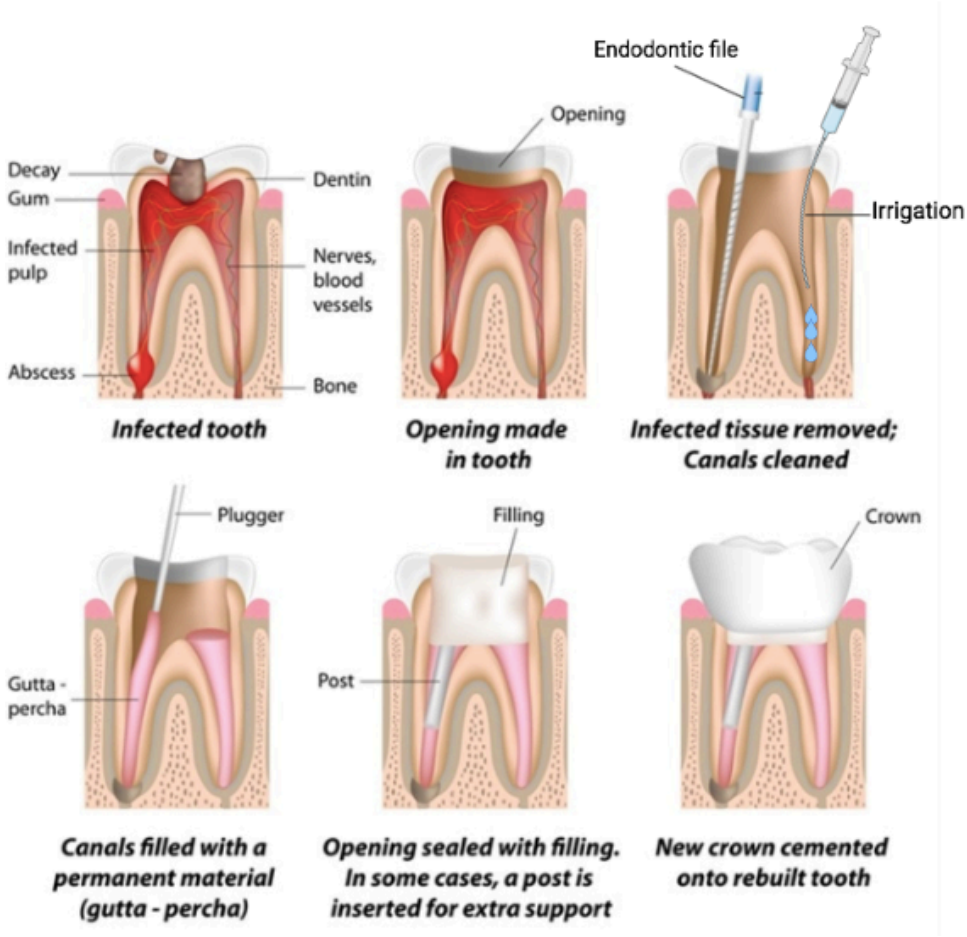


Figure 1.4: Clinical steps of endodontic treatment. Taken from (Shutterstock, 2022).

The use of chemical irrigation is essential for endodontic success. This integral component of endodontic treatments provides killing and removal of microorganisms, necrotic tissues and debris and prevents packing of debris inside the root canal. It also facilitates mechanical cleaning by lubricating instruments and prevents instrument and tooth overheating, especially when using ultrasonic energy (Haapasalo et al., 2014). An irrigant should possess a number of "ideal" characteristics. These include: providing a flushing action, broad spectrum antimicrobial activity, ability to inactivate bacterial endotoxins, ability to dissolve organic tissues, smear layer removal and biocompatibility (Kandaswamy and Venkateshbabu, 2010). However, no single irrigant possesses all of these characteristics. Thus, at present, NaOCl and ethylenediaminetetraacetic acid (EDTA) are the most commonly used irrigants for eradication of the biofilm and disruption of inorganic material resulting from canal instrumentation (Torabinejad, 2011).

Despite NaOCl being the most widely used endodontic irrigant due to its potent and wide spectrum antimicrobial properties and organic tissue dissolving ability, it may be associated with several risks such as tissue toxicity, emphysema, allergy and undesirable taste and smell (Mohammadi, 2008). NaOCl also has a limited ability to dissolve the smear layer which may impede proper disinfection of the dentinal walls and dentinal tubules. For this reason, a chelating (demineralising) agent is required to achieve this goal (Grawehr et al., 2003). Applying chelating agents removes inorganic tissues and debris and exposes dentinal tubules thus providing better penetration and killing by NaOCl (Figure 1.5). Although, the main aim of using a chelating agent is the removal of smear layer, it also possesses an antimicrobial activity, although far inferior to NaOCl. Chelating agents are usually used sequentially following NaOCl irrigation rather than simultaneously. The traditional treatment protocol starts with NaOCl irrigation followed by chelating agent with or without a final flush with NaOCl.

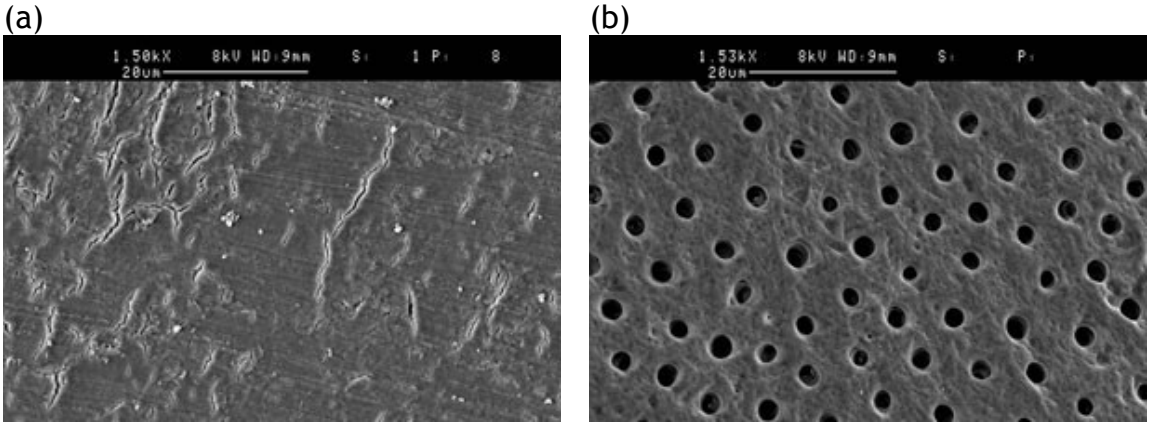


Figure 1.5: SEM images of root canal dentine following chemo-mechanical debridement. (a) The smear layer on the wall of the main root canal after instrumentation. **(b)** Instrumented canal wall after removal of the smear layer by NaOCl and a final rinse by EDTA exposing dentinal tubules. Adapted from (Haapasalo et al., 2014).

Endodontic irrigation is not limited to NaOCl and EDTA only. Various compounds (including novel therapeutics) have been investigated or used to achieve chemical disinfection of the root canal system. Chlorhexidine digluconate (CHX) has good antimicrobial properties and wide applications in the oral cavity such as plaque prevention and as a chemical adjunct in the treatment of periodontitis. It was also widely used as a root canal irrigant. Its major limitation is the lack of organic and inorganic tissue dissolving properties. Moreover, it is less effective against biofilms compared to 5 or 6% NaOCl and kills planktonic cells at a slower rate than NaOCl (Wang et al., 2012). However, it has the advantage of binding to hard dental tissue

which prolongs its antimicrobial effect (substantivity) (Carrilho et al., 2010). Citric acid has also been used for smear layer removal, in a similar fashion to EDTA. However, it is more "aggressive" resulting in greater dentinal erosion (Qian et al., 2011). Recently, 1-hydroxyethane 1,1-diphosphonic acid (HEDP) has been suggested as an alternative chelating agent (Zehnder et al., 2005). HEDP is a mild chelating agent, that unlike others can be used simultaneously with NaOCl. This has led to the development of the concept of 'continuous chelation' (Neelakantan et al., 2012), which is in contrast to the sequential chelation that is required when other chelators such as EDTA are utilized. This single chemotherapeutic strategy has the potential to mitigate any tolerance and persistence observed in conventional approaches. Notably, there have been few studies actively addressing the effectiveness of HEDP, and these have been limited to *E. faecalis* biofilm studies (Zehnder et al., 2005, Arias-Moliz et al., 2014, Morago et al., 2016).

Finally, various natural and synthetic compounds have also been investigated with the aim to replace or synergise with current endodontic irrigants as presented in Table 1.3. In most of the studies, NaOCl, CHX or $Ca(OH)_2$ were used as controls and *E. faecalis* was the most tested endodontic pathogen. However, these studies are limited in number, have high heterogenicity in methodology, they use only single microorganism as a model for what is a complex infection and are *in vitro* in nature. For these novel therapeutics to be considered as real alternatives to conventional antimicrobials, all the drawbacks should be addressed.

Table 1.3: List of experimental novel endodontic therapeutics as compared to traditional antimicrobials. Taken from (Abusrewil et al., 2020a)

Conventional Endodontic antimicrobials	Novel therapeutics	
	Comparable to Conventional antimicrobials	**Inferior to Conventional antimicrobials**
NaOCl EDTA CHX $Ca(OH)_2$	Propolis- Bee glue (Garg et al., 2014, Awawdeh et al., 2018, Tyagi et al., 2013) Nanoparticles (Kishen et al., 2008) Tea tree oil (Kamath et al., 2013) Turmeric (*Curcuma longa*) (Neelakantan et al., 2013) Neem *(Azadirachta Indica)* (Vinothkumar et al., 2013, Rosaline et al., 2013)	Noni plant (*Morinda Citrifolia*) (Prabhakar et al., 2013, Rosaline et al., 2013) Triphala (Prabhakar et al., 2010, Setya et al., 2014, Pujar et al., 2011) German Chamomile (*Marticaria Recutita*) (Sadr Lahijani et al., 2006) Ozone (Hems et al., 2005, Kuştarci et al., 2009) Photodynamic therapy (Meire et al., 2009) Laser irradiation (Meire et al., 2009, Bergmans et al., 2006)

It is important to emphasise the fact that although negative cultures at the time of obturation (filling of root canals following chemo-mechanical disinfection) is associated with increased success rate (Zandi et al., 2019), the presence of bacteria does not always mean treatment failure. It has been found that some periapical lesions can heal despite the presence of bacteria in the root canal at the time of obturation. Sjögren and colleagues (Sjögren et al., 1997) showed that 68% of teeth that yielded a positive culture, at the time of obturation, healed after root canal treatment. To maintain infection and an active immune response, residual bacteria must have virulence attributes, population load, access to periapical tissues, the ability to withstand nutritional deficiency and an altered environment (Sathorn et al., 2007, Siqueira Jr and Rôças, 2008). Causes of persisting periapical infection can be more complex than just persisting bacteria.

1.2 *Candida albicans*

1.2.1 *C. albicans* in endodontic infections

The *Candida* genus comprises chemoorganotroph eukaryotes that reproduce by budding and are found in two forms: a unicellular round or oval form called yeast or multicellular branching tubules called hyphae (Siqueira et al., 2002). The yeast *C. albicans* is a ubiquitous human commensal, but with opportunistic tendencies. Its capacity to morphologically transition from yeast to hyphal cells enables it to react dynamically, both in evasion of host immunity and in its ability to readily form biofilm structures that impact increased antifungal resistance (Ramage et al., 2005). *C. albicans* frequently resides in the oral cavity as a biofilm forming microorganism, interacting with other oral microbiota and the host. Although other *Candida* species were detected in infected root canals, *C. albicans* was the most commonly isolated species (Waltimo et al., 1997, Baumgartner et al., 2000). It has been reported that *C. albicans* has a great affinity for the collagen of dentine compared with non-*albicans Candida* species which may explain the higher prevalence of *C. albicans* in endodontic infection (Siqueira et al., 2002). The occurrence of *C. albicans* in endodontic infection has been reported by culture, molecular methods and electron microscopy. *C. albicans* is more prevalent in secondary root canal infections than in primary infections as reported by various studies (Siqueira Jr et al., 2004, Egan et al., 2002). However, two recent systematic reviews found that *C. albicans* prevalence does not vary significantly between primary and secondary endodontic infections (Mergoni et al., 2018, Persoon et al., 2017b).

Unlike bacteria, which reach the pulp mainly through dentinal tubules, the likelihood of access of yeasts through dentinal tubules to the pulp remains unclear (Waltimo et al., 1997). Previously, it was reported that carious lesions are the main portal of entry of fungi to the root canal system (Najzar-Fleger et al., 1992). The diameter of hyphae, which is 1.9–2.6 μm (Sevilla and Odds, 1986), supports the possibility of dentinal tubule invasion especially in deep carious lesions. However, the higher prevalence of fungi in failed endodontic cases suggests that leaking restorations and the lack of proper isolation of teeth during substandard primary root canal treatment may result in access of yeast to the root canal system (Ashraf et al., 2007). Here, *C. albicans* yeasts are capable of coalescing with one

another in the form of biofilm, which is a multicellular community of yeast and hyphal forms encased in polymeric glue (Ramage et al., 2009). The biofilm lifestyle enhances the ability of the cells to withstand host and antifungal factors, ultimately contributing to persistence within the root canal. This phenotype, along with the complexity of the root canal system, particularly in the apical third of the root, are thought to be the major causes of treatment-resistant apical periodontitis. Furthermore, it has been found that the presence of smear layer enhances the adhesion of *C. albicans* to dentinal tubules (Sen et al., 2003). As described, the smear layer usually forms following the instrumentation of the root canal during endodontic treatment. The presence of this layer increases *C. albicans* resistance to the common endodontic irrigants chlorhexidine and sodium hypochlorite (Şen et al., 1999). *C. albicans* has the ability to penetrate dentinal tubules (Siqueira et al., 2002) and to resist intracanal medicaments such as calcium hydroxide (Waltimo et al., 1999a) which may explain its higher prevalence in persistent root canal infections. *C. albicans* in root canals was shown attached to the dentine surface and invading the dentinal tubules in both yeast and hyphal forms as evident in scanning electron microscope imaging (Figure 1.6).

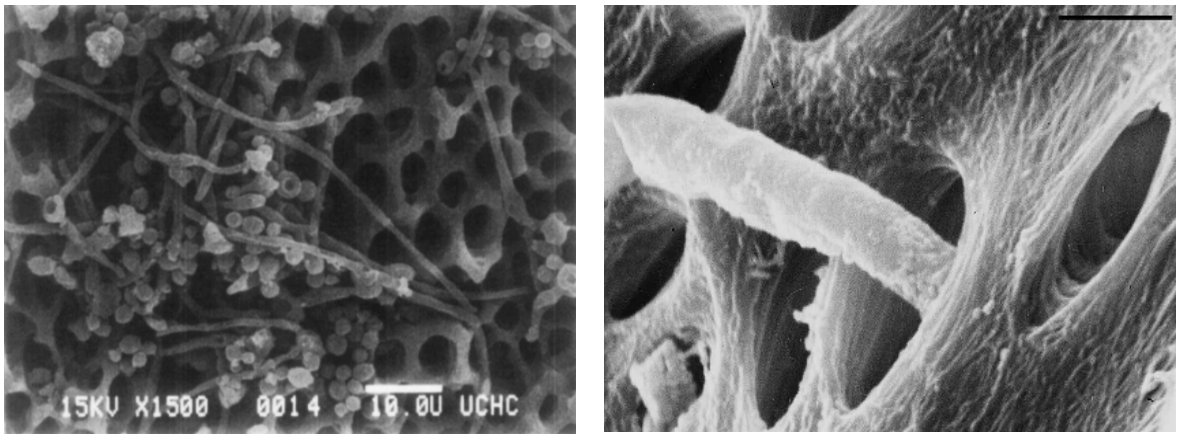


Figure 1.6: Scanning electron microscope visualisation of *C. albicans* formation on radicular dentine and penetration of dentinal tubules. Taken from (Waltimo et al., 2003, Şen et al., 1997).

1.2.2 Mechanisms of *C. albicans* pathogenicity

Several virulence factors have been described for *C. albicans* which may play a role in disease causation (Figure 1.7). These include adhesion to different surfaces, adaptability to various environmental conditions, its ability to produce hydrolytic enzymes, morphogenic changes, hyphal and biofilm formation, host defence and immune modulation, thigmotropism and phenotypic switching

(Waltimo et al., 2003, Siqueira and Sen, 2004). In endodontic infections, *C. albicans* adhesion to and colonisation of dentine are likely to increase its capability to penetrate dentinal tubules and resist antimicrobial procedures and pH changes (Turk et al., 2008, Waltimo et al., 2004). *C. albicans* adhesion is mediated by many surface molecules, including a receptor homologous to the human CR3 integrin. This receptor binds to the RGD motif (arginine-glycine-aspartic acid) on fibrinogen, fibronectin and laminin that binds sugars on epithelial cells and mannose-containing proteins that bind to lectin-like molecules on host cells and tissues (Calderone and Braun, 1991). *C. albicans* binding to collagen type I and IV has been also reported (Chaffin et al., 1998). *C. albicans* adherence is affected by a variety of factors such as cell-surface hydrophobicity, pH and concentration of iron, calcium, zinc and carbon dioxide (Waltimo et al., 2003). Extracellular calcium, which is abundant in dentine, is essential for *C. albicans* adhesion to extracellular matrix protein and type I collagen which may help in explaining the colonisation of radicular dentine by *C. albicans* in endodontic infections (Siqueira et al., 2002).

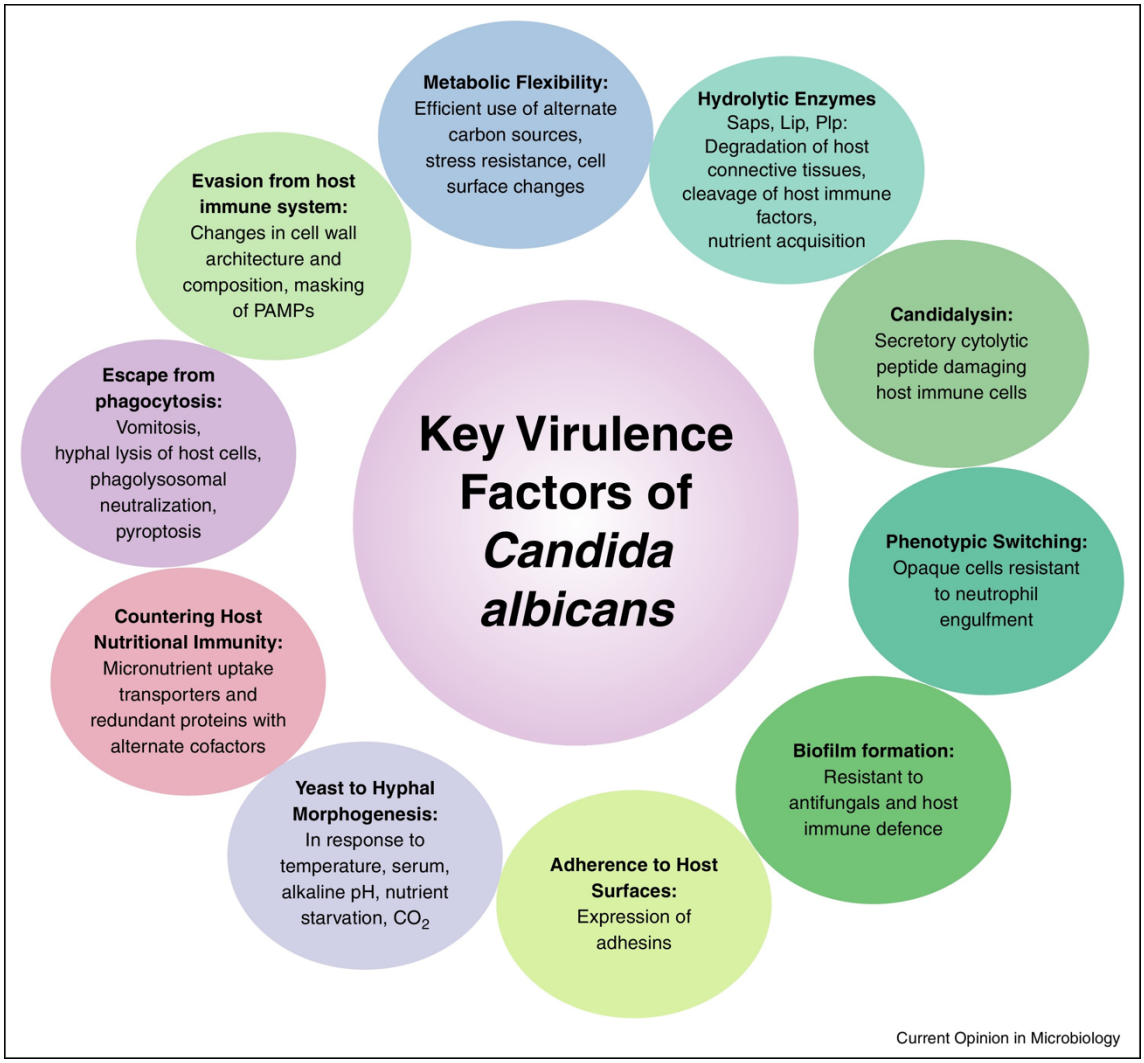


Figure 1.7: Summary of *C. albicans* virulence factors. Taken from (da Silva Dantas et al., 2016).

Candida can adapt to a variety of environmental conditions. For instance, *C. albicans* is capable of adaptation to a range of physiologic extremes such as pH. It can grow in neutral pH found in blood and tissues and in acidic environments such as the vaginal canal (Siqueira and Sen, 2004). The ability of *C. albicans* to adapt to environmental changes is attributable to its tendency to phenotypic alterations and switching of gene expression. Phenotypic switching is a genetically controlled phenomenon which includes changes in colony morphology and protease activity (Waltimo et al., 2003). For instance, *C. albicans* secretion of aspartyl protease and enzymes which degrade dentinal collagen promotes its survival in nutrient limited conditions (Waltimo et al., 2004). These factors may explain the exceptional adaptive ability of *C. albicans* to environmental and nutritional changes during endodontic therapy.

One of the key virulence factors of *C. albicans* is its ability to produce hydrolytic enzymes such as aspartyl proteinase, collagenase and phospholipase. These enzymes are involved in tissue invasion by degrading extracellular matrix proteins (Siqueira and Sen, 2004). It was demonstrated that the amount of protease production of a *C. albicans* strain is directly comparable with its pathogenicity. As a result, the higher rate of protease production by *C. albicans* in comparison with other *Candida* species suggests increased virulence (Waltimo et al., 2003).

C. albicans is a polymorphic microorganism that can exist in a number of growth forms such as yeasts (blastospores), germ tubes, true- and pseudo-hyphae and chlamydospores. With the exception of chlamydospores, the interconversion from one growth pattern to another may occur according to changes in environmental conditions (Brown and Gow, 1999). Although the transition of yeast to hyphal forms has been reported to be associated with increased pathogenicity (Sweet, 1997), hyphal formation is not a prerequisite for infection to occur. Both yeast and hyphal forms were populating most *C. albicans* infections indicating that both forms have a role in disease causation and progression (Siqueira and Sen, 2004). It was suggested that tissue invasion by *C. albicans* is promoted by thigmotropism (contact sensing). This characteristic enables *C. albicans* to grow hyphae to sense changes in topography and composition of surfaces. In other words, *C. albicans* produces hyphae to detect intercellular junctions, tubules, cracks, grooves of surfaces and to follow nutrient sources (Sweet, 1997, Şen et al., 1997). This may be instrumental in *C. albicans* hyphae extension and penetration into dentinal tubules.

C. albicans has the ability to form biofilm on different surfaces which may account for its increased pathogenicity compared with other *Candida* species such as *C. glabrata* and *C. tropicalis* (Haynes, 2001). Biofilms are a community of microorganisms attached irreversibly to a surface and imbedded in a self-produced extracellular matrix (Siqueira and Sen, 2004). Biofilm formation in *C. albicans* has four distinct stages that are governed by complex transcriptional regulations. The first stage (**adhesion**) starts by adherence of planktonic yeast cells to abiotic or biotic surfaces. Initial adhesion to host ligands is mediated by *EAP1* and the ALS-family of genes, mainly *ALS3*. *C. albicans* mutants lacking *ALS1*, *ALS2* or *ALS3* show impaired adhesion (Zhao et al., 2005). Following attachment, yeasts start to proliferate to form microcolonies and switch to form pseudohyphae and true

hyphae (**initiation**). The **maturation** stage is characterised by hyphae elongation, extracellular matrix production and formation of multilayers of 3-dimensional intertwined networks of hyphae infiltrated by yeast cells. In the final phase (**dispersal**), yeast daughter cells detach from the mature biofilm to adhere to a new surface and start the cycle over again. Illustration of the stages of biofilm formation and its genetic control in *C. albicans* is presented in Figure 1.8.

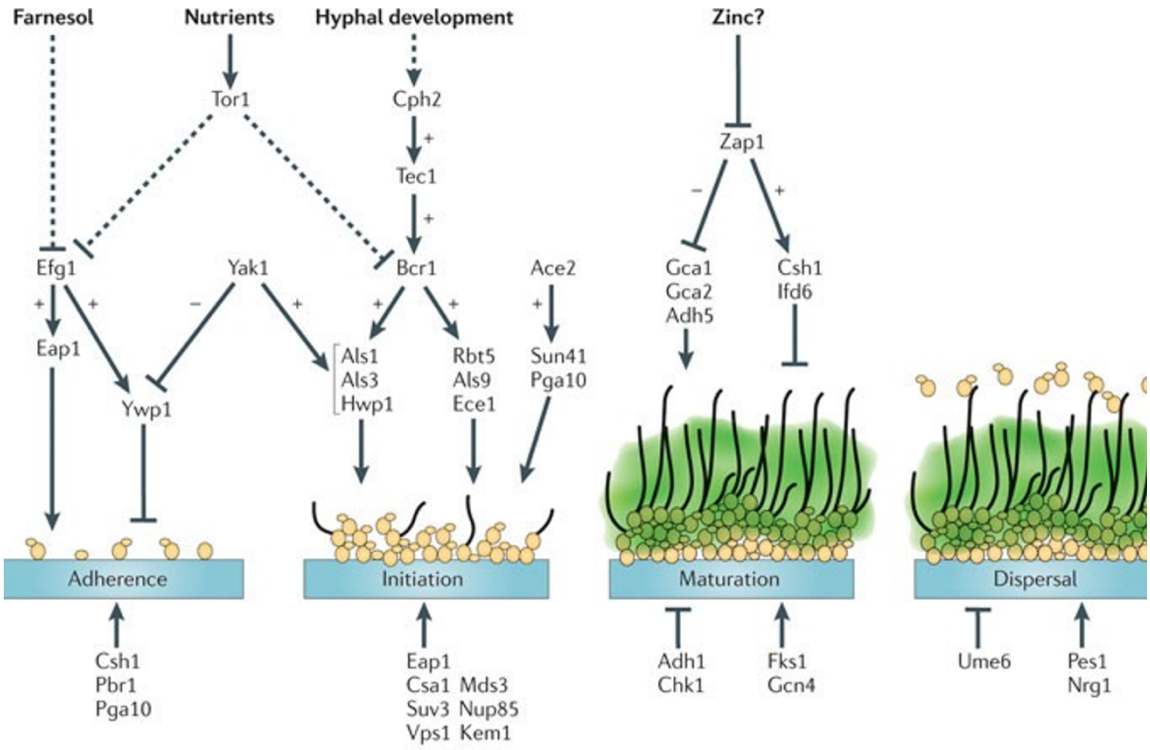


Figure 1.8: Stages of biofilm formation and associated molecular basis. Taken from (Finkel and Mitchell, 2011).

Biofilm formation promotes establishment of *C. albicans* on a given surface and provides protection against potential hazards such as antimicrobial medicaments. It has been reported that *C. albicans* biofilm can be 100 times more tolerant to fluconazole than are planktonic free cells (Chandra et al., 2001). Finally, *C. albicans* possesses a variety of mechanisms to evade host defences. It is capable of blocking polymorphonuclear neutrophils oxygen radical production and degranulation, killing monocytes and degradation of complement factors and immunoglobulins (Danley and Polakoff, 1986, Chaffin et al., 1998). Furthermore, *Candida* can directly or indirectly activate the complement system either through classical, alternative or lectin pathways (Kozel, 1996).

Finally, *C. albicans possesses* several strategies to resist antifungal agents. There are currently three classes of clinically used antifungal drugs such as polyenes, azoles, and echinocandins (Cowen et al., 2015, Gintjee et al., 2020). Polyenes bind to cell membrane ergosterol and cause cell lysis (Ghannoum and Rice, 1999). However, due to increased toxicity, their clinical use is limited (Zotchev, 2003). Azoles act by inhibiting the mechanism of ergosterol biosynthetic pathway, but increased emergence of resistance, particularly the development of point mutations to the target site of azoles or by the expression of efflux pumps in the *C. albicans* hampers its use in treating fungal infections (Sheehan et al., 1999, Perea et al., 2001). Echinocandins, the most recent of the antifungals, acts by inhibiting 1,3 - beta glucans synthase and help in cell wall biosynthesis inhibition, but mutation in the glucan synthase gene *FKS1* has become a crucial problem (Sucher et al., 2009, Wiederhold et al., 2008).

Generally, antifungals resistance mechanisms can involve alterations in drug transport or target, activation of alternative pathways or presence of biofilm structure (Sanglard et al., 2009). At the genetic level, alterations in the genes *ERG11* (encoding target enzyme), *MDR1*, *CDR1* and *CDR2* (efflux pump genes) were linked with azoles resistance (White, 1997, White et al., 2002). Likewise, *GSC1*, *PIL1* and *HSP90* were implicated in echinocandin resistance (Watamoto et al., 2011, Singh et al., 2009). Spettel et al. (2019) found a mutation in the target gene *GSC1* in all echinocandin-resistance *C. albicans* isolates investigated.

1.2.3 Susceptibility of C. albicans to antimicrobial endodontic medicaments

As stated, NaOCl is the most widely used endodontic irrigant due its potent and wide spectrum antimicrobial properties and organic tissue dissolving ability. Various studies have investigated the effect of different NaOCl concentrations on *C. albicans* at a variety of clinically relevant treatment time points. In a series of studies, Waltimo et al. (1999a) tested different disinfectants including NaOCl on seven strains of *C. albicans* isolated from cases of refractory apical periodontitis. They show that NaOCl has the highest efficacy and complete killing of yeast cells was achieved with both 5% and 0.1% NaOCl using filter paper discs immersed in the fungal suspension and colony forming units (CFU) counting. In extracted teeth, 1% NaOCl resulted in 70% killing of *C. albicans*, while 5.25% NaOCl showed higher

antimicrobial activity (Tyagi et al., 2013, Nakamura et al., 2013, Valera et al., 2001). Incomplete killing was attributed to the presence of root canal irregularities.

CHX is another potent antiseptic root canal disinfectant which has the advantage of sustained action after application. However, CHX has a lower antifungal effectiveness compared with NaOCl and EDTA (Sen et al., 2000). Nevertheless, it appears that CHX inhibits the adherence and biofilm formation of yeast due its substantivity. Moreover, a final rinse with CHX following NaOCl application was found to significantly reduce the number of cultivable microorganisms from root canals compared with NaOCl treatment alone (Zamany et al., 2003).

EDTA is commonly used as a part of endodontic irrigation protocol. Its main action is the removal of smear layer to enhance the penetration of other irrigants due to its chelating action (it chelates calcium ions from inorganic tissues). EDTA was reported, by many studies, to have a high antifungal efficacy at concentrations as low as 0.625mg/L (Sen et al., 2000, Ates et al., 2005). They suggest that EDTA antifungal activity is attributed to its ability to decrease the metabolic activity by extracting calcium ions from the cell wall of *C. albicans* and the medium. It may also have anticolonisation action by reducing the adhesion properties of *C. albicans*.

The interim root canal medicament calcium hydroxide ($Ca(OH)_2$) is widely used in endodontic therapy protocol as it has a good antibacterial effect, dissolves necrotic tissues and inactivates endotoxins (Waltimo et al., 2004). The antimicrobial action of calcium hydroxide is assumed to be caused by its high alkalinity (pH, 12.5 – 12.8). However, it is well-documented that calcium hydroxide has little or no effect on *C. albicans* (Mohammadi et al., 2012, Ferguson et al., 2002, Waltimo et al., 1999a). The high tolerance of *C. albicans* to calcium hydroxide can be explained by its ability to survive a wide range of pH values. Furthermore, calcium hydroxide may act as a source for calcium ions required for the growth and morphogenesis of *C. albicans* (Siqueira and Sen, 2004).

In general, many factors may compromise the effectiveness of root canal disinfectants against *C. albicans* including dentine buffering action, smear layer and fungal biofilm. Smear layer favours the growth of *C. albicans* by providing

calcium ions and interferes with the antimicrobial action of endodontic irrigants by providing physical protection. Similarly, biofilm provides protection to *Candida* cells from disinfectants via the extracellular matrix. Previous factors may contribute to the persistence of *C. albicans* following endodontic treatment.

1.3 *Enterococcus faecalis*

1.3.1 The prevalence of *E. faecalis* in endodontic infections

E. faecalis is a Gram positive, lactic acid producing, facultative anaerobic coccus which has been historically associated with endodontic infections. *E. faecalis* are ovoid in shape and can occur singly, paired or as short chains. *E. faecalis* is non-motile and has a diameter of 0.5 - 1μm (Schleifer and Kilpper-Bälz, 1984). In endodontics, *E. faecalis* has been occasionally recovered from primary infections but frequently isolated in persistent infections. Moreover, it has been considered the most often detected organism in post-treatment infections (Sakko et al., 2016) which had led to increased attention of *E. faecalis* in endodontics. In primary infections, *E. faecalis* is more frequently detected in asymptomatic cases than in symptomatic ones (Rôças et al., 2004b). Data of various studies revealed that *E. faecalis* in persistent infections has a prevalence ranging from 29% - 77% (Möller, 1966, Peciuliene et al., 2001, Siqueira Jr and Rôças, 2004) with some studies demonstrating a prevalence as high as 90% (Siqueira JR and Rôças, 2014). Based on this prevalence, *E. faecalis* has been regarded as the main pathogen involved in failed endodontic cases. Nevertheless, the question remains whether this microorganism plays an important role in the pathogenesis of secondary endodontic infections. It is also important to consider the findings of other studies that question the role of *E. faecalis* in endodontic treatment failure. For instance, a study by Rolph et al. (2001) failed to detect *E. faecalis* in persistent endodontic infections. Moreover, it has been reported that *E. faecalis* can be as frequent in lesion-free endodontically treated teeth as it is in those with lesions (Kaufman et al., 2005). It could be equally important to note that failure of detecting *E. faecalis* in some failed cases does not necessarily means its absence. Failure of detection can be a result of limitations in sampling techniques as described above or because of the fact that *E. faecalis* has the ability to penetrate deep into dentinal tubules.

1.3.2 Mechanisms of *E. faecalis* pathogenicity

E. faecalis has some virulence factors which may be involved in disease pathogenesis and enhance its adherence, invasion and resistance through host immunomodulation. These factors include surface adhesins, the toxin cytolysin and lytic enzymes gelatinase and hyaluronidase that are involved in tissue breakdown, Aggregation Substance (AG) which mediates binding to leukocytes and extracellular matrix, lipoteichoic acid (LTA) which promotes adhesion to host surfaces and cytokine production by monocytes, sex pheromones which chemoattract neutrophils as reviewed in Jett et al. (1994). In endodontic infections, surface adhesins and AG may enable *E. faecalis* to bind to dentinal collagen while lipoteichoic acid (LTA) mediates adhesion to dentinal hydroxyapatite (Kayaoglu and Ørstavik, 2004).

Scanning electron microscopic evaluation of *E. faecalis* penetration into dentinal tubules showed that dentinal tubules were heavily infected by *E. faecalis* (Siqueira et al., 1996, Al-Nazhan et al., 2014) (Figure 1.9). In addition, Sedgley et al. (2005a) evaluated the survival of *E. faecalis* in root canals *ex vivo* and found that *E. faecalis* can remain viable for up to 12 months. *E. faecalis* is able to survive environments with scarce nutrients (root filled teeth) and recover from a prolonged starvation state and flourish when nutrient source is re-established (Rôças et al., 2004b). This adaptation to varying environmental conditions is mediated by gene expression regulation in *E. faecalis* (Ruiz-Cruz et al., 2016). Moreover, *E. faecalis* is resistant to calcium hydroxide, the most commonly used endodontic intracanal medicament. This resistance is mediated by proton pump activity which drives protons into the cell to maintain cytoplasmic pH (Evans et al., 2002). The previously described factors may contribute to the high occurrence of *E. faecalis* in persistent endodontic infections.

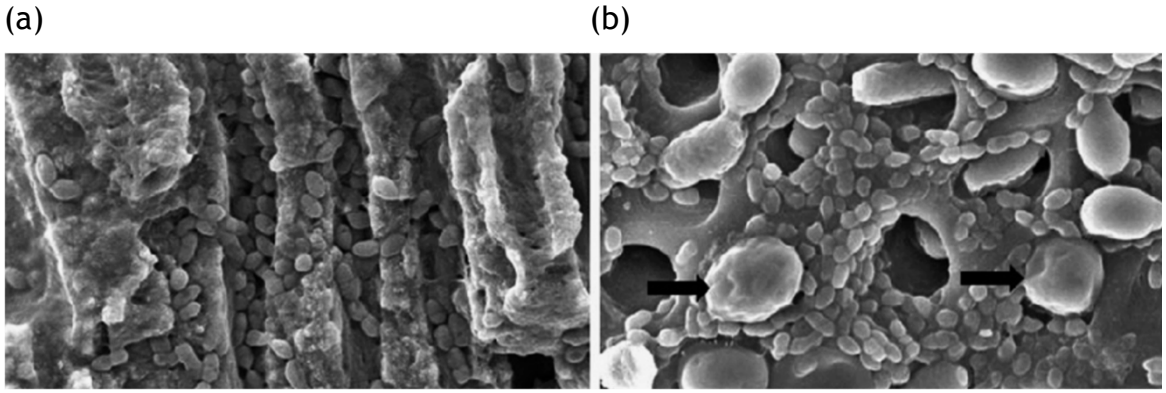


Figure 1.9: Scanning electron microscope image of *E. faecalis* mono- and dual species biofilms on radicular dentine. (a) demonstrates the deep penetration of *E. faecalis* into dentinal tubules. **(b)** *E. faecalis* and *C. albicans* dual species biofilms. Taken from (Al-Nazhan et al., 2014).

1.3.3 Susceptibility of *E. faecalis* to antimicrobial endodontic medicaments

E. faecalis has been used extensively as a model endodontic pathogen for endodontic antimicrobial testing. Radcliffe et al. (2004) reported *E. faecalis* highly resistant to 0.5, 1.0, 2.5 and 5.25% NaOCl. This resistance may be attributed to biofilm formation which is known to affect and limit solute diffusion, the presence of the smear layer and the interaction of NaOCl with tissue fluids, blood, dentine and other organic debris which can reduce its activity. Unlike NaOCl, CHX seems to be more effective against *E. faecalis* (Lima et al., 2001). Similarly, it has been revealed that 2% chlorhexidine solution eliminated *E. faecalis*, when grown in suspension within 15 seconds (Vianna et al., 2004) and 2% concentration can eradicate *E. faecalis* biofilms in 5 minutes (Arias-Moliz et al., 2009). EDTA has no or limited antibacterial activity, which explains why EDTA had no effect on *E. faecalis* at any time or concentration (Arias-Moliz et al., 2008, Arias-Moliz et al., 2009). Lastly, as mentioned previously, *E. faecalis* is also highly resistant to calcium hydroxide (Evans et al., 2002).

1.4 *C. albicans* and *E. faecalis* polymicrobial biofilm

Endodontic infections are polymicrobial, with bacteria being the most frequently isolated microorganism. The microorganisms arrange themselves in complex surface-attached biofilms. The occurrence of biofilms in endodontic infections was reported at 74% and 80% of treated and untreated cases, respectively (Ricucci

and Siqueira, 2010). The microorganisms in the biofilm are embedded in a self-produced extracellular matrix which is composed of polysaccharides, polypeptides and nucleic acids. This extracellular matrix binds microbial cells together, protecting them from environmental stresses and antimicrobials used in endodontic therapy. It was found that microbes in a biofilm can be up to 1000 times more resistant to antimicrobial agents than their planktonic counterparts (Mah and O'Toole, 2001).

C. albicans is a biofilm producing microorganism. *C. albicans* hyphae within a biofilm provide a potential niche for the colonisation of various Gram-positive and Gram-negative bacteria, a phenomenon we term as a mycofilm (Kean et al., 2017). *E. faecalis* and *C. albicans* can be isolated in pure cultures as well as in polymicrobial communities. However, both organisms are frequently isolated in persistent endodontic infections which suggests possibility of an interkingdom interaction between bacteria and fungi in endodontic infections. Some studies revealed that *E. faecalis* could inhibit hyphal morphogenesis and biofilm formation of *C. albicans* (Cruz et al., 2013). The latter effect is mediated by the protein bacteriocin EntV; a protein with demonstrated antihyphal and antivirulence properties without antagonising fungal growth (Graham et al., 2017). Co-infection of these organisms has been shown to attenuate *C. albicans* virulence *in vivo*, particularly with an initial administration of *E. faecalis* (Cruz et al., 2013). This might explain the low fungal prevalence in endodontic infections.

Recent studies suggest that bacteria attaching to *C. albicans* hyphae provides an opportunity to invade and disseminate into the host (Kean et al., 2018b). In endodontic infection, *C. albicans* hyphae may act as a physical carrier for non-motile *E. faecalis* to penetrate into dentinal tubules. *C. albicans* membrane protein Msb2 is able to bind and inactivate host defence proteins and antibiotics (Swidergall et al., 2013). Production of Msb2 could subsequently provide the same protection to *E. faecalis*. Moreover, *C. albicans* has also demonstrated the ability to promote the growth of *E. faecalis* in the gastrointestinal tract (Mason et al., 2012). These same interactions are likely to occur in the root canal, encouraging bacterial growth and preventing resolution of endodontic infection.

The likelihood of fungal and bacterial coaggregation in endodontic infections in the form of complex biofilm communities, creates a potential environment of

mutual protection and tolerance towards antimicrobial agents. *C. albicans* is the most common fungal pathogen frequently co-isolated from polymicrobial biofilm infections and can interact with a number of different bacteria in a variety of ways. Interestingly, *E. faecalis* has been shown to incorporate itself into *C. albicans* biofilms, adhering to both yeast and hyphal forms (Fox et al., 2014). Bacteria preferentially adhere to hyphal as opposed to yeast cells (Ovchinnikova et al., 2012). One potential mode by which this interaction may favour resistance to treatment is through enhancing dentinal tubule penetration. *E. faecalis* is capable of penetrating deep into dentinal tubules (Siqueira et al., 1996, Siqueira Jr and de Uzeda, 1996, Sedgley et al., 2005a, Sedgley et al., 2005b). It may be possible that *E. faecalis* attaches to *C. albicans* hyphae which have themselves been shown to penetrate tubules (Şen et al., 1997), thus providing an opportunity to invade and disseminate into dentine. This model of enhancing the invasive behaviour of bacteria is not without precedent. It has been established that *Staphylococcus aureus* specifically binds to *Candida* hyphae (Kean et al., 2017). Invasive hyphal elements of *C. albicans* thus facilitate the invasion of *S. aureus* across mucosal barriers, leading to systemic dissemination (Schlecht et al., 2015).

Furthermore, various studies have reported the co-occurrence of acidogenic bacteria with fungi in saliva where *C. albicans* load increases with the presence of acidogenic bacteria (O'Donnell et al., 2015). It was also reported in endodontic infections by Persoon et al. (2017a). It was shown that pH below 6 can result in dentine erosion (West et al., 2001). Therefore, it was hypothesised that bacteria can easily invade dentinal tubules, degrade dentine through its acidic activity and create a favourable environment for subsequent fungal colonisation.

It is clear that there is significant microbial complexity associated with endodontic infections. As a biofilm disease, it is not only intrakingdom interactions that are important. Fungi, *C. albicans* in particular, can coexist with multiple bacterial species and are known for their ability to form biofilms with them (Kean et al., 2018a). Synergistic and antagonistic interactions can occur, that may play an important role in endodontic disease development and progression. It is also highly likely that they will create significant challenges for clinical management, and also, for developing strategies to improve management. In exploring *in vitro* strategies, we must be aware of the need for the use of suitable robust model systems. Understanding composition is one thing, but this must be utilized

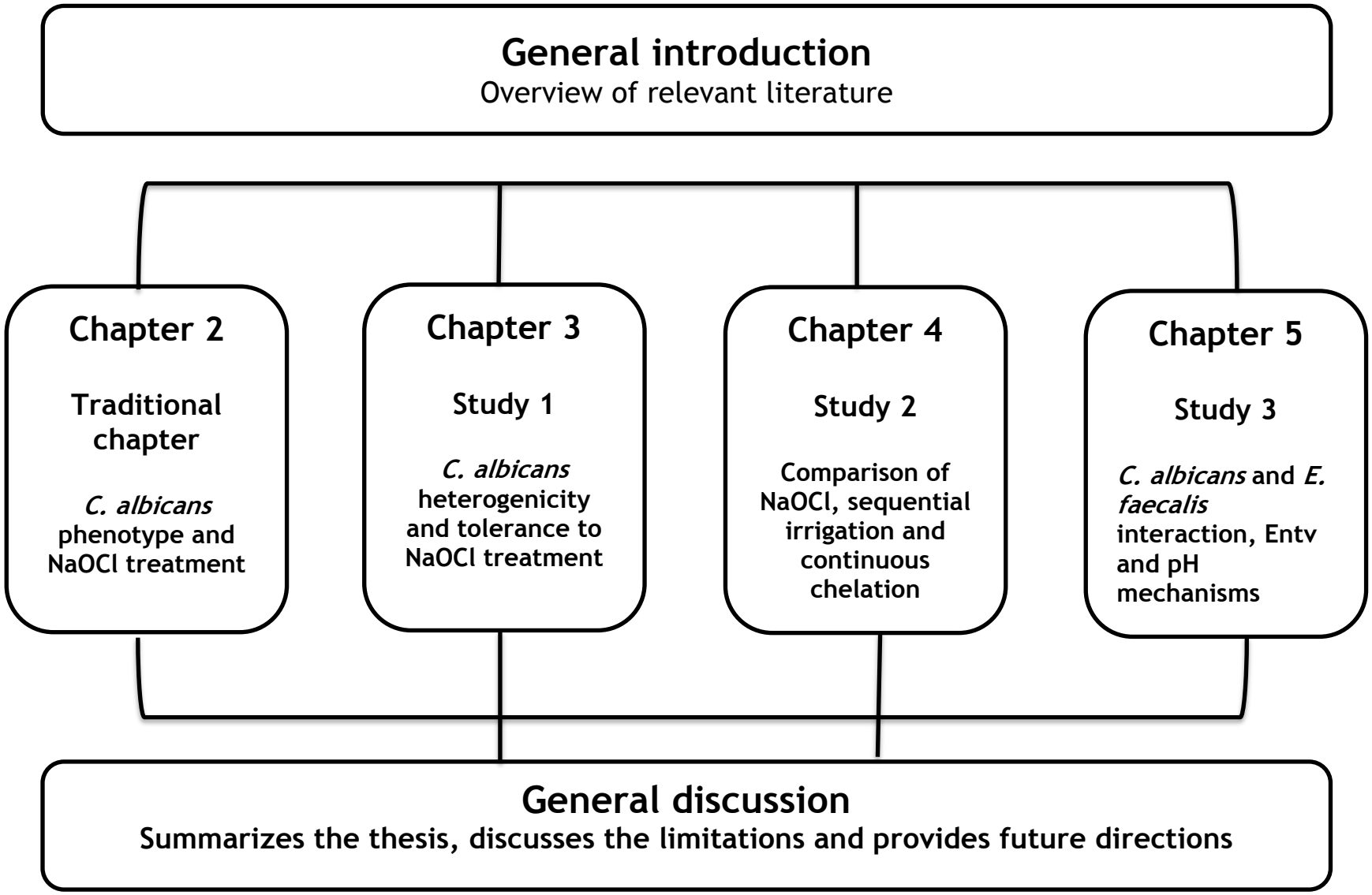
appropriately. It may not immediately have an effect on our management of disease, but it should inform our further exploration of endodontic microbiology. It is clearly not appropriate to use mono-species models to establish the effect of disinfectants or methodologies. Instead, we must build appropriate multispecies models that reflect with at least some degree of accuracy what we find in the clinical situation. However, we must also factor in uninvited microorganisms that exogenously appear.

1.5 Thesis aims and outline

This thesis aims to investigate the response of *C. albicans* to endodontic therapeutics. The unique feature of this work is that it concerned with post-treatment effects and in particular the ability of the tested microorganisms to tolerate endodontic irrigants and regrow following treatment.

Next, it aims to compare the response of *C. albicans* and *E. faecalis* in mono- and dual-species biofilms to sequential treatment with NaOCl and EDTA or continuous chelation using NaOCl+HEDP.

Finally, the thesis explores the molecular basis for the interaction of *C. albicans* and *E. faecalis* and investigates the role of *E. faecalis* acidifying potential as a pH dependent mechanism of action in modulating *Candida* behaviour.


General introduction
Overview of relevant literature
Chapter 2
Traditional chapter
C. albicans phenotype and NaOCl treatment
Chapter 3
Study 1
C. albicans heterogenicity and tolerance to NaOCl treatment
Chapter 4
Study 2
Comparison of NaOCl, sequential irrigation and continuous chelation
Chapter 5
Study 3
C. albicans and *E. faecalis* interaction, Entv and pH mechanisms
General discussion
Summarizes the thesis, discusses the limitations and provides future directions

2 *Candida albicans* biofilm characterisation and its response to sodium hypochlorite treatment

2.1 Introduction

C. albicans is the most commonly isolated fungus from endodontic infections (Siqueira and Sen, 2004). Hyphal morphogenesis and biofilm formation are characteristic features of *C. albicans* and these traits have been linked to virulence and pathogenesis. Furthermore, biofilm formation can result in an increased tolerance to antimicrobials, where higher concentrations of an individual drug are required to kill biofilm enclosed cells compared to equivalent free planktonic cells (Ramage et al., 2012b, Taff et al., 2012). The biofilm phenotype is also associated with enhanced mortality, where it has been shown that this is linked with an increased mortality in candidemia patients (Tumbarello et al. (2007). Moreover, *C. albicans* high biofilm forming isolates (HBFs) have been shown to have higher *in vitro* drug tolerance to antifungals compared to low biofilm forming isolates (LBFs) (Sherry et al., 2014). HBFs have also been shown to be associated with higher mortality using an *in vivo Galleria mellonella* model (Sherry et al., 2014).

Notably, when considering endodontic infections, *C. albicans* in root canals has been shown to be attached to dentine surfaces as biofilms, invading the dentinal tubules in both yeast and hyphal form as evident by scanning electron microscope imaging (Şen et al., 1997). Although it is well established that HBFs are more tolerant to antifungals, less is known about whether the same applies in the context of root canal treatment and how different *C. albicans* phenotypes respond to endodontic irrigants. The effect of endodontic irrigants, including NaOCl on *C. albicans*, has been extensively studied (Valera et al., 2001, Waltimo et al., 1999a). In extracted teeth, 1% NaOCl resulted in 70% killing of *C. albicans*. Incomplete killing was attributed to the presence of root canal irregularities (Valera et al., 2001). Waltimo et al. (1999a) tested different disinfectants including NaOCl on seven strains of *C. albicans* isolated from cases of refractory apical periodontitis. They demonstrated that both 5% and 0.1% NaOCl resulted in complete killing of yeast cells (planktonic cells) in *C. albicans* impregnated filter discs, with all *C. albicans* strains showing similar susceptibility. However, limitations to this and other studies is the failure to fully explore how different biofilm phenotypes respond to standard endodontic treatments.

2.2 Hypothesis and aims

This chapter hypothesised that *C. albicans* clinical isolates show heterogeneity in biofilm formation and HBFs are more tolerant to NaOCl treatment. Specifically, this component of the thesis therefore aims to:

- Characterise *C. albicans* oral isolates based on biofilm formation using standard and transcriptional analysis.

- Establish the susceptibility of these clinical isolates to NaOCl.

- Optimise XTT metabolic assay in order to enhance sensitivity to regrowing populations.
- Undertake transcriptional comparison of LBFs and HBFs for drug resistance genes.

2.3 Materials and methods

2.3.1 Microbial growth conditions and standardisation

C. albicans laboratory strains and clinical isolates used throughout this thesis are listed in Table 2.1.

Table 2.1: *C. albicans* laboratory strains and clinical isolates.

Name	Reference
SC5314	(Gillum et al., 1984)
3153A	(Slutsky et al., 1985)
ATCC10231	(Ghannoum et al., 1986)
ATCC90028	(Espinel-Ingroff et al., 1992)
Day185	(DiRita et al., 2000)
BC009, BC013, BC020, BC023, BC037, BC038, BC039, BC043, BC044, BC045, BC095, BC098, BC099, BC106, BC107, BC108, BC115, BC116, BC117, BC127, BC128, BC129, BC135, BC136, BC137, BC145, BC146, BC159 and BC160	(Coco et al., 2008)

A stock of all used microorganisms were stored in Microbank™ beads (Pro-lab Diagnostics, UK) at -80°C. *C. albicans* clinical isolates were obtained from an oral rinse from patients attending restorative clinics at Glasgow Dental Hospital and School for routine dental care, as previously described (Coco et al., 2008). At the time of experiment, microorganisms were revived and *C. albicans* strains were subcultured on Sabouraud's dextrose agar [SAB (Sigma–Aldrich, Dorset, UK)] and plates maintained aerobically at 30^0C for 48 hours. Afterwards, plates were kept at 4°C for a maximum 2 weeks. Overnight cultures were then prepared in a sterile universal tube (Sterilin® Limited, Cambridge UK) by adding a loopful of yeast colonies in 10 mL of yeast peptone dextrose [1% w/v yeast extract, 2% w/v peptone, 2% w/v dextrose, 1.5% agar [YPD (Sigma-Aldrich, Dorset, UK)] and incubated at 30°C at 120 revolutions per minute (rpm) in an orbital shaker (IKA KS 4000 i control, Berlin, Germany). After 16-18h, the yeast cells were pelleted by centrifugation at 3,000 rpm for 5 minutes, supernatant discarded, pellet washed

twice with sterile phosphate buffered saline (PBS) [10 mM phosphate buffer, 2.7 mM potassium chloride, 137 mM sodium chloride, pH 7.4 (Sigma-Aldrich, UK)] and resuspended in 10 mL of PBS. One hundred times dilution of the cells was then obtained by adding 10 µL cell suspension to 990 µL of PBS in a sterile Eppendorf and counted using a haemocytometer, as outlined in Figure 2.1.

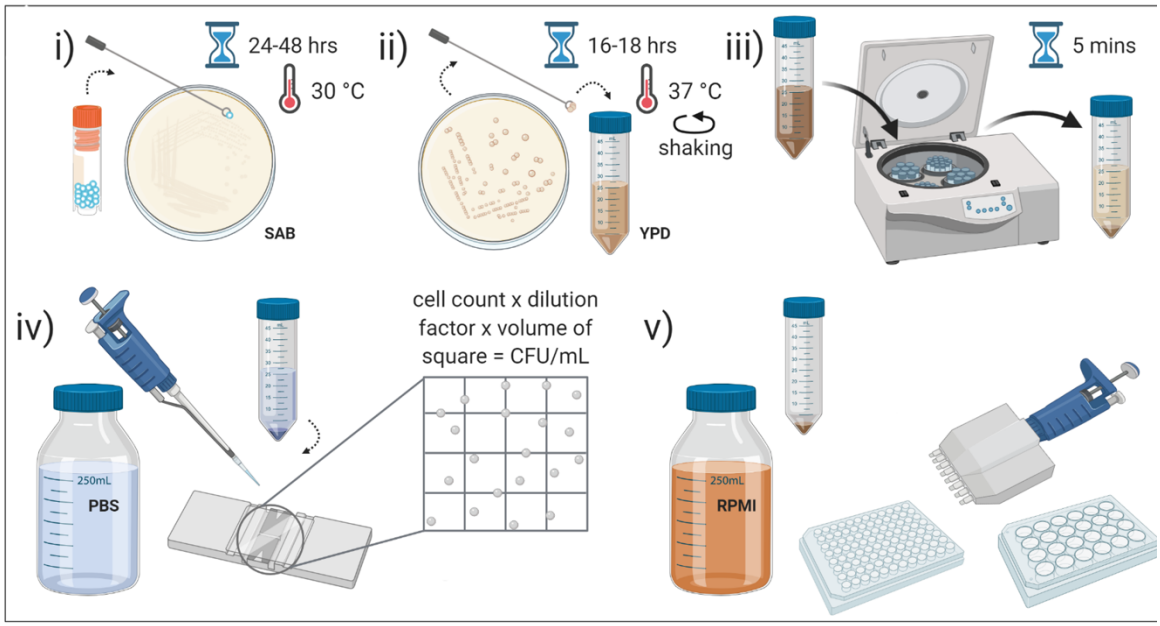


Figure 2.1: Summary of *C. albicans* growth and standardisation. i) Revival of *C. albicans* stock from Microbank beads by plating into SAB agar. **ii)** Preparation of overnight culture by inoculating *C. albicans* colonies into YPD medium. **iii)** *C. albicans* cells were harvested and washed by centrifugation. **iv)** Washed cells suspended in PBS before being counted using the haemocytometer. **v)** *C. albicans* is standardised to the desired cellular density into RPMI medium and dispensed into microtiter well plates. Taken from (Brown et al., 2022)

2.3.2 Biofilm development, metabolic activity and biofilm formation characterisation

C. albicans strains were standardised to the desired cellular density of $1x10^6$ cells/mL into Roswell Park Memorial Institute (RPMI)-1640 medium (Sigma-Aldrich, Dorset, UK) or artificial saliva (AS) [Porcine stomach mucins (0.25% w/v), sodium chloride (0.35% w/v), potassium chloride (0.02% w/v), calcium chloride dihydrate (0.02% w/v), yeast extract (0.2% w/v), lab lemco powder (0.1% w/v), proteose peptone (0.5% w/v)] in deionised distilled water and then supplemented with 40% urea (1.25 µL of urea/mL of AS). Biofilms were formed in pre-sterilised, polystyrene, 96-well flat-bottom microtiter plates (Corning Incorporated, NY, USA) by dispensing 200 µL of *C. albicans* cultures into each well as previously

described (Ramage et al., 2001). Negative control wells containing media only were also included in the same plate. The plates were incubated aerobically at 37ºC for the indicated time. After incubation, the supernatant was discarded and biofilms were washed with PBS to remove the loosely attached cells and the metabolic activity was quantified using (2,3-bis[2-methyloxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide [XTT] [Sigma–Aldrich, Dorset, UK or Fisher Scientific, UK]). XTT action is based on the reduction of the colorless to yellowish tetrazolium salt into an orange formazan dye by the metabolic activity of viable cells that can be then read using a microplate reader, as illustrated in Figure 2.2. However, this reduction may take up to 24h to produce a detectable colour change. Therefore, the electron-coupling agent, menadione, is used to decrease XTT reduction time in a dose dependent manner (Singh et al., 2011). 0.25g/L of XTT salt was dissolved in distilled water and was sterilized by filtration with 0.22-µM-pore size filter tubes and stored in the dark at -80º C. Stock solution of 10 mM of the electron coupler menadione (2-Methyl-1,4-naphthoquinone [Sigma-Aldrich, Dorset, UK]) was also prepared and kept at -20°C. Just before use, XTT was supplemented with menadione 1 µM (1 µL of menadione per 10 mL of XTT) and 100 µL of supplemented XTT was added to each well and incubated aerobically in the dark at 37º C for 2h. Afterwards, 75 µL of the reduced XTT was transferred to a new 96-well flat-bottom microtiter plates and measured spectrophotometrically at 490 nm using a microtiter plate reader (FLUOStar Omega, BMG Labtech, Aylesbury, UK).

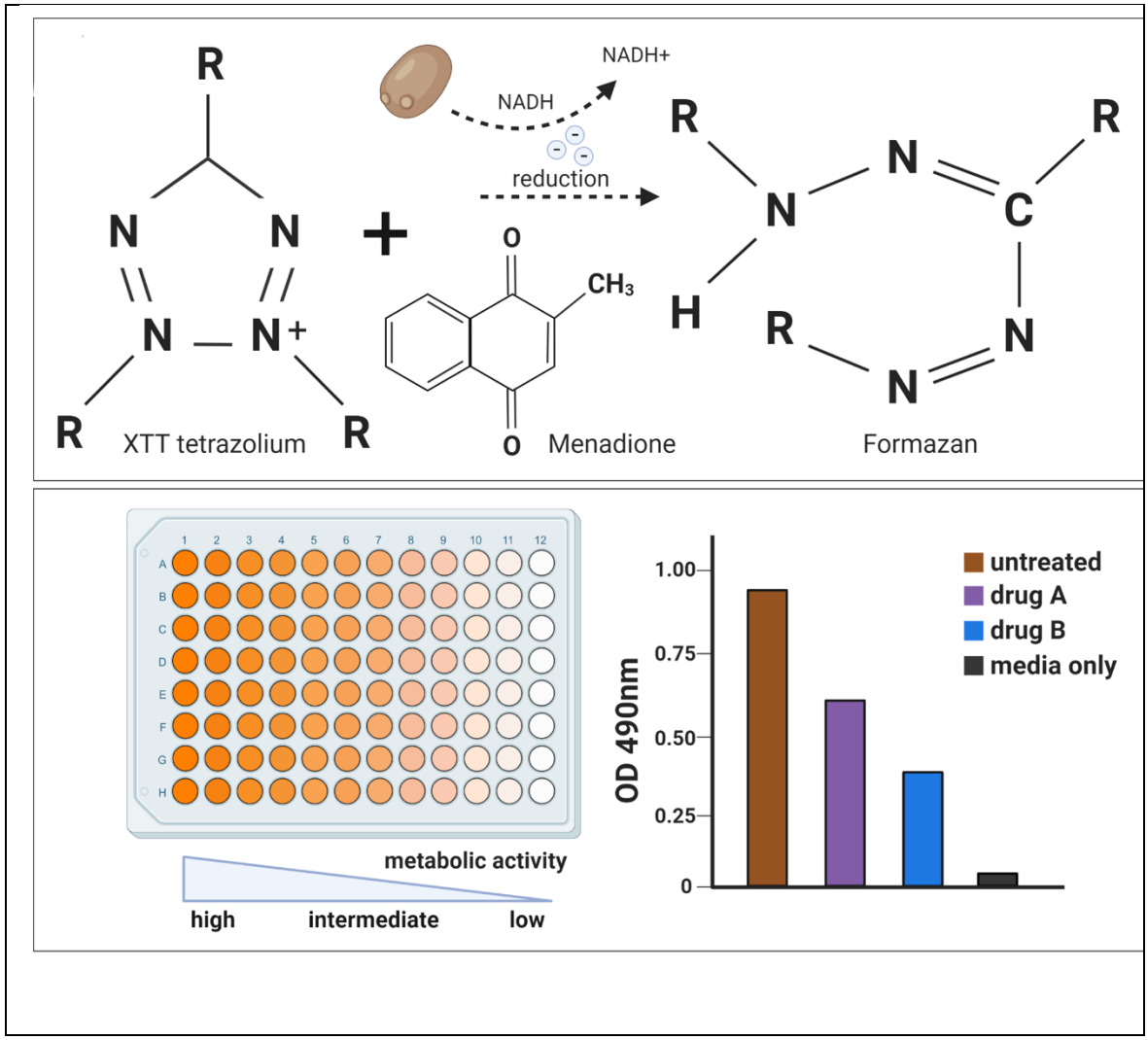


Figure 2.2: XTT mechanism of action and its correlation with cellular metabolic activity. The top panel illustrates the reduction of the colorless XTT tetrazolium into orange formazan in the presence of the electron-coupling agent, menadione. The lower panel illustrates the positive correlation of the color intensity of the reduced XTT and the metabolic activity of the assayed cells. Taken from (Brown et al., 2022).

Biofilm biomass of all *C. albicans* strains was quantified using the crystal violet (CV) assay, as previously described (Jose et al., 2010). In short, the plates were washed with PBS and left to dry overnight at room temperature. Afterwards, 100 µL of 0.05% w/v of CV (Sigma-Aldrich, Dorset, UK) was added to each well. After incubation for 20 minutes at room temperature, unattached dye was washed away gently under tap water and 100 µL of 100% ethanol was added to each well to retrieve bound dye. After agitation with pipetting to get homogenous colour, 75 µL of ethanol was transferred to a new 96-well flat-bottom microtiter plate. The biomass was then quantified spectrophotometrically by reading absorbance at 570 nm using a microtiter plate reader.

Five of the *C. albicans* lowest biofilm formers (LBFs [BC023, BC037, BC038, BC039 and BC044]) and five of the highest biofilm formers (HBFs [BC020, BC117, BC136, BC145 and BC146]) were selected and used in all downstream experiments. Firstly, they were used to test the effect of growth media 80% RPMI with 20% Fetal bovine serum (FBS) (Sigma-Aldrich, Dorset, UK) compared to 80% H_2O with 20% FBS serum on biofilm forming ability. Strains were standardised to the desired cellular density of $1x10^6$ cells/mL into 80% RPMI with 20% FBS or in 80% sterile distilled water with 20% FBS. Biofilms were formed and assessed using XTT and CV assays, as described above.

2.3.3 Transcriptional analysis

2.3.3.1 RNA extraction

The TRIzol method was used for RNA extraction in single species biofilms. *C. albicans* biofilms were developed in 6 well flat-bottom plates for the indicated time, as described above. For assessment of drug resistance genes following fluconazole treatment, the lowest biofilm forming isolate (BC023) and the highest biofilm forming isolate (BC146) were selected. 24h *C. albicans* LBF and HBF biofilms were treated with 64 µg/mL fluconazole for 24h. After incubation, supernatant was discarded, and biofilms washed gently with PBS and 1 mL of TRIzol™ solution (Invitrogen, Paisley, UK) was added to each well. Biofilms were then scraped off using a cell scraper (STARLAB, Milton Keynes, UK) and transferred to an O-ring screw-cap microcentrifuge tube (Stratech, Newmarket, UK) containing about 0.25 mL of 0.5 mm diameter acid washed glass beads (Sigma-Aldrich, Dorset, UK). To mechanically disrupt fungal cells releasing intracellular RNA, a bead beater (Fisher Scientific, Loughborough, UK) was used for 3 cycles of 30 seconds each at 400 rpm. Samples were kept on ice between cycles. One hundred µL of 1-bromo-3- chloropropane (Sigma-Aldrich, Dorset, UK) was then added and samples were vortexed for 30 seconds. Following incubation for 5 minutes at room temperature with occasional inversion, samples were centrifuged at 13,000 rpm for 15 minutes at 4ºC to separate the aqueous layer containing RNA. The upper aqueous layer was then carefully transferred to a fresh microfuge tube and 500 µL of ice-cold isopropanol was added to each sample to precipitate RNA. To allow for maximum RNA precipitation, samples were stored at -20ºC overnight. The following day, samples were centrifuged at 10,000 rpm for 10 minutes at 4ºC

before discarding the supernatant. The remaining pellet of each sample was washed with 500 µL of ice-cold 70% ethanol. Samples were then inverted 3-4 times and centrifuged at 10,000 rpm for 5 minutes at 4ºC. Ethanol was discarded, and the pellet left to dry for 30 minutes in a laminar flow cabinet before being suspended in 25 µL RNase free water and incubated at 65ºC for 5 minutes.

2.3.3.2 DNase digestion and RNA clean up

Following TRIzol RNA extraction, DNase digestion kit (Qiagen, Crawley, UK) was used as per manufacturer's instructions to remove any DNA contamination from RNA samples. A mixture of 10 µL DNase,10 µL of RDD buffer and 55 µL Rnase free water was added to each sample giving a total volume of 100 µL and incubated at room temperature for 20 minutes. For RNA clean up, RNeasy MiniElute clean up kit (Qiagen, Crawley, UK) was used as per manufacturer's instructions. Briefly, 350 µL of RLT buffer and 250 µL of 100% ethanol were added to the extracted RNA samples and mixed by pipetting. Samples were then transferred to RNeasy MiniElute spin columns contained in a 2 mL collection tube, centrifuged at 10,000 rpm for 15 seconds and the flow-through was discarded. Afterwards, spin column membranes were washed with 500 µL of RPE buffer and centrifuged at 10,000 rpm for 15 seconds. The column binding the RNA was transferred into a new 1.5 mL microfuge tube and 50 µL of RNase free water was added directly to the centre of the column membrane and centrifuged at 10,000 rpm for 1 minute to elute RNA. The released RNA was then re-eluted by passing through the column membrane a second time and centrifuged for an additional minute. RNA quality and quantity were checked using a NanoDrop spectrophotometer (ND-1000, Thermo Scientific, Loughborough, UK).

2.3.3.3 cDNA synthesis for real time PCR

For this purpose, a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Paisley, UK) was used. Ten µL of High-Capacity RNA-to-cDNA reverse transcription mastermix was added to 10 µL of RNA in 0.2 mL dome-capped PCR microtubes. No Reverse transcription (RT) controls were also included by adding a mastermix lacking reverse transcriptase enzyme to RNA samples. Afterwards, samples were transferred to a MWG-Biotech Primus 96 plus thermal cycler and the applied thermal cycles were: 25º C for 10 minutes, 37º C for 120 minutes, 85º C for

5 minutes and final hold step at 4° C. cDNA was then stored at -20° C until use for PCR.

2.3.3.4 Gene expression analysis using real time PCR

A mastermix containing 10 µL Fast SYBR GreenER™ (Thermo Fisher Scientific, Paisley, UK), 1 µL of 10 µM forward/reverse primers (Table 2.2), and 7 µL UV treated RNase-free water was prepared and added to 1 µL of cDNA. The used thermal cycles were a holding stage of 50°C for 2 minutes, 95°C for 2 minutes, 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds using Step-One plus real time PCR machine and StepOne software V2.3 (Life Technologies, Paisley, UK). cDNA of no RT controls was also included. ΔΔCT method was used to analyse gene expression for *C. albicans* in relation to untreated controls for fluconazole treatment experiment after normalising cycle threshold (Ct) values to ACT1 housekeeping gene. The expression of biofilm related genes in LBF and HBF was calculated as a percentage of expression in relation to ΔΔCT of the housekeeping gene.

Table 2.2: *C. albicans* primers used for quantitative and real time qPCR

Primer	Gene name	Sequence (5′– 3′)	Function
	ACT1	F - AAGAATTGATTTGGCTGGTAGAGA	Housekeeping
		R - TGGCAGAAGATTGAGAAGAAGTTT	
	HWP1	F- GCTCAACTTATTGCTATCGCTTATTACA	Hyphal wall protein
		R - GACCGTCTACCTGTGGGACAGT	
	ALS3	F - CAACTTGGGTTATTGAAACAAAAACA	Adhesion
		R - AGAAACAGAAACCCAAGAACAACCT	
	ECE1	F - GCTGGTATCATTGCTGATAT	Hyphae specific protein
		R - TTCGATGGATTGTTGAACAC	
	EFG1	F - CCAGTGGTGGCAGTAATGTG	Adhesion and virulence
		R - CAGTGGCAGCCTTGGTATTT	
	HSP90	F - GGTTGCTGATCACGTCCAAGTT	Heat shock protein
		R - AACTTACCACCAGCGTTAGATTCC	
	CDR1	F - GTACTATCCATCAACCATCAGCACTT	Efflux pump
		R - GCCGTTCTTCCACCTTTTTGTA	
	MDR1	F - TCAGTCCGATGTCAGAAAATGC	Efflux pump
		R - GCAGTGGGAATTTGTAGTATGACAA	
	GSC1	F - GAACCAGTGCCTGAAGGTGA	ß-1,3-glucan synthase subunit
		R - AGCCCATCTTGACGAAGCAA	
	ERG11	F - GGTGGTGATTTGAATGATTTGACTTAT	Ergosterol biosynthesis
		R - GGCATATGCATTCTAAGAGTTTCCT	
	PIL1	F - TAAGCAATTGAGTGCTTGGG	Eisosome component has a role in endocytosis
		R - GGTTGGACAGAACCTTCGAT	

2.3.4 *C. albicans* biofilm treatment with NaOCl and regrowth assessment

NaOCl is used in endodontics in concentrations ranging from 0.5% - 5.25%, where higher concentrations showed faster and increased antimicrobial and tissue dissolving effects but were associated with higher cytotoxity (Jefferson et al., 2012). Three percent NaOCl (Parcan; Septodont, Saint-Maur-des-Fosses, France) was chosen in the downstream analysis as this concentration is the most commonly used concentration in endodontic irrigation and showed a deeper penetration into dentinal tubules compared to 0.5% (Wong and Cheung, 2014). Biofilms of four LBFs (BC023, BC037, BC038 and BC039) and five HBFs (BC020, BC117, BC136, BC145 and BC146) were grown in RPMI for 24h in 96-well flat-bottom microtiter plates. After incubation, supernatant was discarded, and biofilms were washed with PBS and treated with 200 µl of 3% NaOCl for 5 or 30 minutes. Untreated controls were also

included for comparison. The effect of NaOCl was deactivated with 5% sodium thiosulfate for 10 minutes (Fisher Chemicals, UK). Sodium thiosulfate was also applied to untreated controls to normalise results. The viability and biofilm biomass of the treated biofilms was quantified immediately after treatment using XTT assay supplemented with 1 μM menadione and CV assay as described above. To further explore the post-treatment effect, the treated biofilms were washed with PBS to remove any remnants of treatment or neutralizer and fresh RPMI medium was replenished to allow remaining *C. albicans* cells to grow. The plates were then re-incubated at 37ºC for another 24h, 48h and 72h and XTT viability and CV assessment were performed. Of note, one of the selected five LBFs isolates (BC044) showed an odd response to NaOCl and was shown to be a non *albicans candida* isolate based on chromogenic *candida* agar analysis and therefore was excluded.

In order to confirm that *C. albicans* regrowth observed with NaOCl was actually arising from cells persisting after treatment rather than a possible contamination from untreated control wells within the same plate, an extra *C. albicans* plate was used. All the wells of this plate containing 24h *C. albicans* biofilm were treated with NaOCl for 5 minutes and deactivated with sodium thiosulfate. After re-incubation with fresh RPMI for 72 h, *C. albicans* regrowth was confirmed microscopically.

2.3.4.1 Assessment of media replenishment on *C. albicans* regrowth

To further investigate the persistence of *C. albicans* following NaOCl treatment, continuous re-incubation with fresh RPMI growth media was compared with replenishment re-incubation. In continuous re-incubation, 24h *C. albicans* biofilms (four LBF and five HBF) were treated with 3% NaOCl for 5 minutes and re-incubated with fresh RPMI for another 24h, 48h and 72h as described above. In replenishment re-incubation, 24h *C. albicans* biofilms were treated and re-incubated with fresh RPMI similar to continuous re-incubation. However, at 24h and 48h post re-incubation, RPMI was removed and fresh RPMI added. *C. albicans* persistence following both types of re-incubation was assessed immediately after treatment (0h), at 24h, 48h and 72h using XTT and CV assays.

2.3.5 Metabolic activity XTT assay optimisation

2.3.5.1 Determining the sensitivity of XTT with 1 µM menadione

The four LBFs and the five HBFs *C. albicans* isolates were standardised in PBS to a cell density ranging from $1x10^8$ to $1x10^3$ cells/mL. One mL of each cellular density was centrifuged in 1.5 mL Eppendorf tubes at 13,000 rpm for 10 min to pellet cells. Supernatant was discarded and 100 µL of XTT solution containing 1 µM menadione was added to each Eppendorf. After 2h of incubation in the dark on a shaking incubator at 37°C, 75 µL of XTT was transferred to a new 96-well flat-bottom microtiter plates and optical density was assessed using a microtiter plate reader at wavelength 490 nm.

2.3.5.2 Determining the sensitivity of XTT with different menadione concentrations and reading wavelengths

In an attempt to enhance the sensitivity of the XTT assay to detect cell concentrations below $1x10^6$ cells/mL, the effect of gradual increase in menadione concentration was investigated. For this experiment, LBF and HBF were used. One mL of a cellular density ranging from $1x10^6$ to $1x10^3$ cells/mL of each isolate was centrifuged in an Eppendorf tube to pellet cells as described above. After discarding supernatant, 100 µL of XTT solution containing the desired menadione concentration (1 µM, 2 µM, 4 µM, 8 µM or 10 µM) was added and the optical densities were measured as above. To test the difference in reading XTT at two different wavelengths, cellular densities ranging from $1x10^8$ to $1x10^3$ cells/mL were prepared and assayed with XTT containing 1 µM or 8 µM as described above. Absorbance was then measured using a plate reader at 2 different wavelengths (460 nm or 490 nm).

2.3.5.3 Comparison of alamar blue with XTT assay in detecting *C. albicans* regrowth

C. albicans biofilms (four LBFs and five HBFs) were treated and re-incubated with fresh RPMI as previously described. At 0h, 24h, 48h and 72h, metabolic activity of the biofilms was assessed using XTT or alamar blue. Alamar Blue™ (Thermo Fisher Scientific, USA) was diluted by 1:10 in RPMI media. One hundred µL of alamar blue were then dispensed into each well and incubated aerobically in the dark at 37°C for 1h. After incubation, 75 µL of XTT and alamar blue were transferred to a new

96 well flat-bottom plate and absorbance was measured at 490 nm for XTT and 544/590 (excitation/emission) for alamar blue using the microtiter plate reader.

2.3.6 Statistical analysis

Statistical analysis was performed, and figures were created using GraphPad Prism (version 7.0 d, GraphPad, La Jolla, CA, USA). To compare data sets and calculate significance, one-way ANOVA non-parametric Kruskal–Wallis test with Dunn's multiple comparison test was used and statistical significance was considered if $P < 0.05$.

2.4 Results

2.4.1 Oral *C. albicans* clinical isolates exhibit variation in metabolic activity and biofilm forming ability

The first aim of this investigation was the characterisation of 36 *C. albicans* laboratory strains and clinical oral isolates based on their biofilm forming ability. This characterisation would aid in understanding the response of different *C. albicans* phenotypes to the classical endodontic irrigant, NaOCl. In order to choose the appropriate time and growth media for this evaluation, *C. albicans* was grown in either RPMI or AS for 4h and 24h. Following incubation, metabolic activity and biofilm biomass were assessed. The biofilm forming strain SC5314 was used as a reference. As shown in Figure 2.3, there was a wide variation in the metabolic activity (Figure 2.3a) and the biomass (Figure 2.3b) of the screened *C. albicans* isolates, which was more evident with RPMI media compared with AS and at 24h compared to 4h. AS was less supportive for *C. albicans* growth, as indicated by metabolic activity. There was a 4.4-fold reduction in metabolic activity and 1.3-fold reduction in biofilm mass of *C. albicans* SC5314 grown in RPMI and AS at 24h. As a result, biofilm biomass data obtained in RPMI media at 24h was chosen for subsequent isolate categorisation. *C. albicans* isolates were then arranged based on biomass readings and five isolates of the lowest (LBFs) and five isolates of the highest (HBFs) biofilm forming ability were defined (Figure 2.4). Representative microscopic images of isolates of high, moderate and low biofilm forming ability are presented in Figure 2.5.

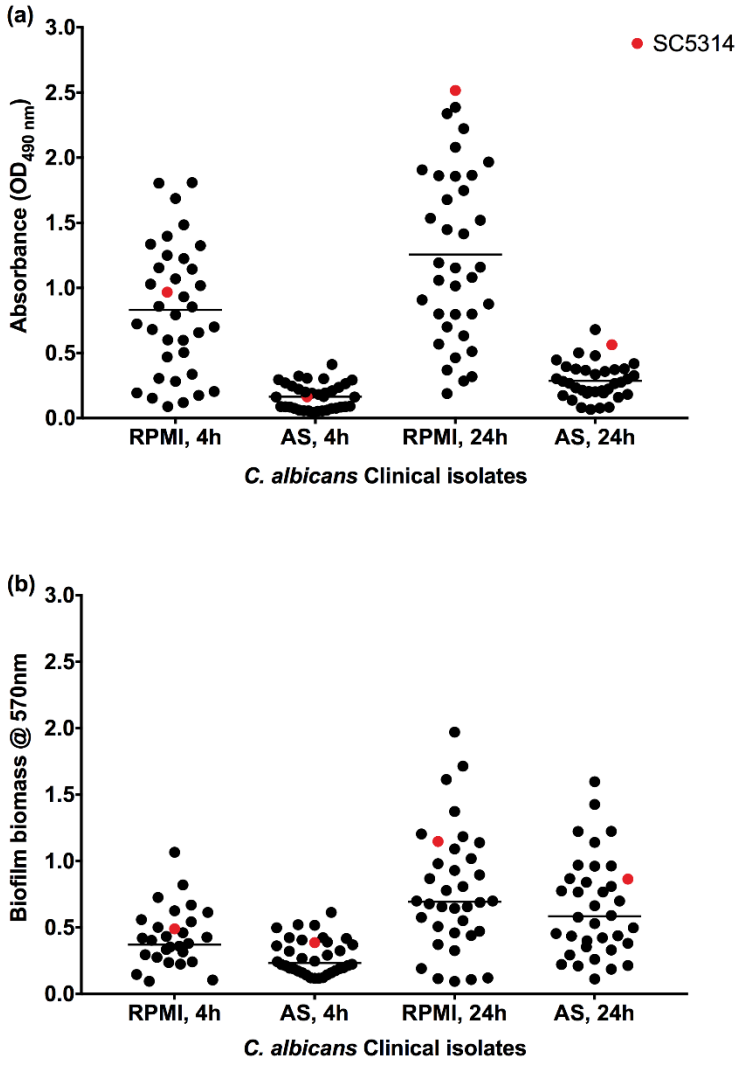


Figure 2.3: Biofilm characterisation of *C. albicans*. *C. albicans* clinical isolates (n=29) and laboratory strains (n=5) were grown in RPMI or AS to select the optimum media to characterise *C. albicans* isolates based on their biofilm forming ability. Metabolic activity **(a)** and biofilm biomass **(b)** assessed at 4h and 24h of incubation. Each dot represents the average of data obtained from triplicates of three independent experiments for each strain/isolate. The horizontal line represents the grand mean of each condition/time point. Laboratory strain SC5314 (red colour) used as a reference.

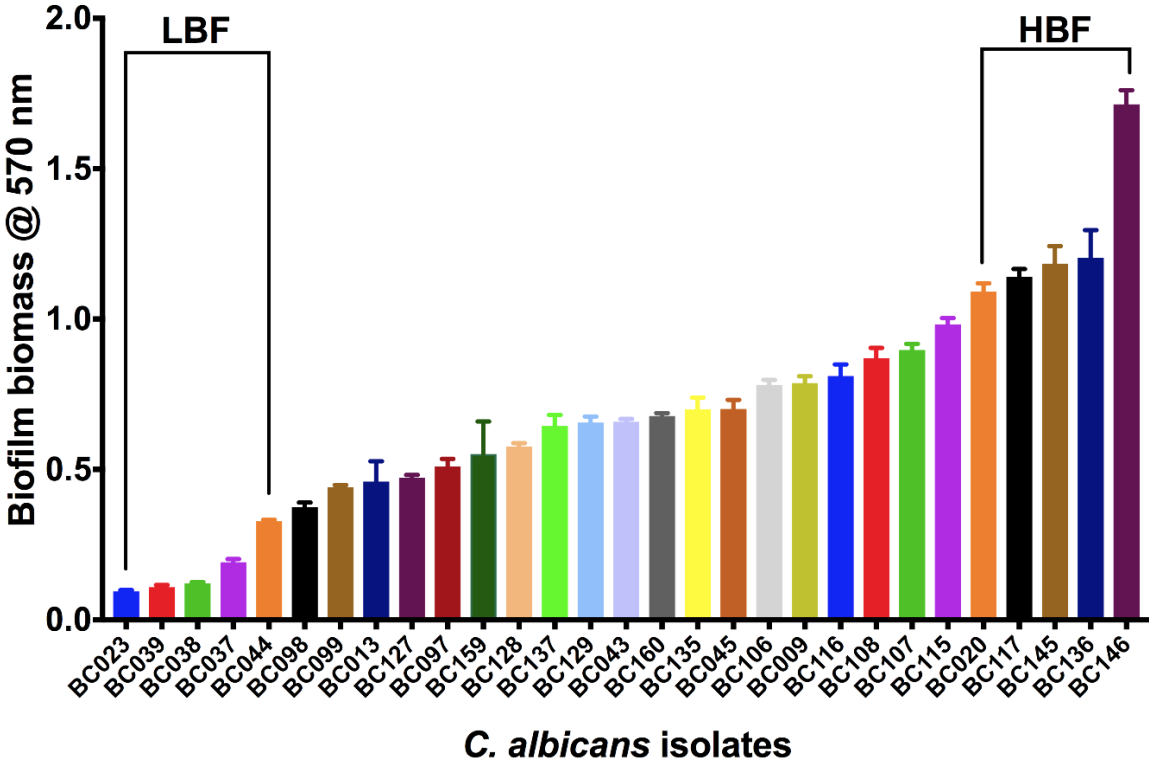


Figure 2.4: Selected five LBFs and five HBFs from *C. albicans* clinical isolates. LBFs and HBFs were selected based on their biofilm biomass as assessed with CV assay following incubation in RPMI for 24h. Each bar represents the average of data obtained from triplicates of three independent experiments for each isolate. Error bars represent the standard error of the mean.

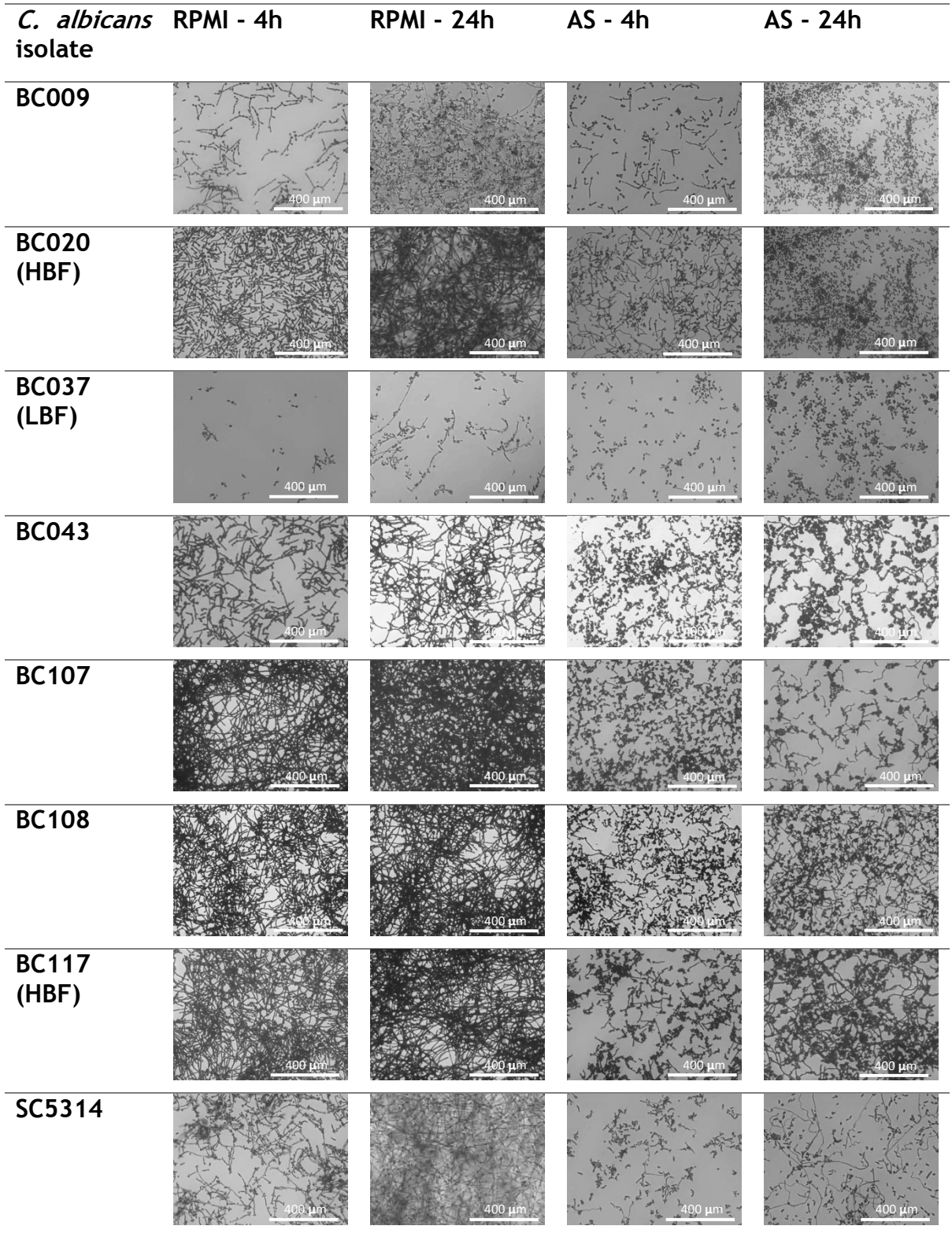


Figure 2.5: Light microscopic images of a number of *C. albicans* clinical isolates and laboratory strain. Isolates were grown in RPMI or AS for 4h and 24h. Images represent examples of isolates of high, moderate and low biofilm forming ability. Scale bar is 400 µm.

Serum is a potent inducer of hyphal morphogenesis in *C. albicans* and impacts biofilm formation (Pendrak and Roberts, 2007). We tested whether fetal bovine serum (FBS) alone, or combined with RPMI, would alter our previous selection of

LBFs and HBFs. As expected, the biofilm biomass of all *C. albicans* isolates increased when FBS was used. The highest biomass was observed when FBS was combined with RPMI at 24h (Figure 2.6). Nevertheless, FBS induced enhancement of biofilm formation was consistent across all 10 isolates, and therefore did not impact our previous categorisation of the isolates into LBF and HBF isolates. Furthermore, serum was shown to inactivate NaOCl (Pappen et al., 2010) and therefore we refrained from using FBS for NaOCl treatment assessment in the subsequent studies. Collectively, the previous data led to the identification of RPMI as an appropriate growth medium for the subsequent NaOCl treatment assessment.

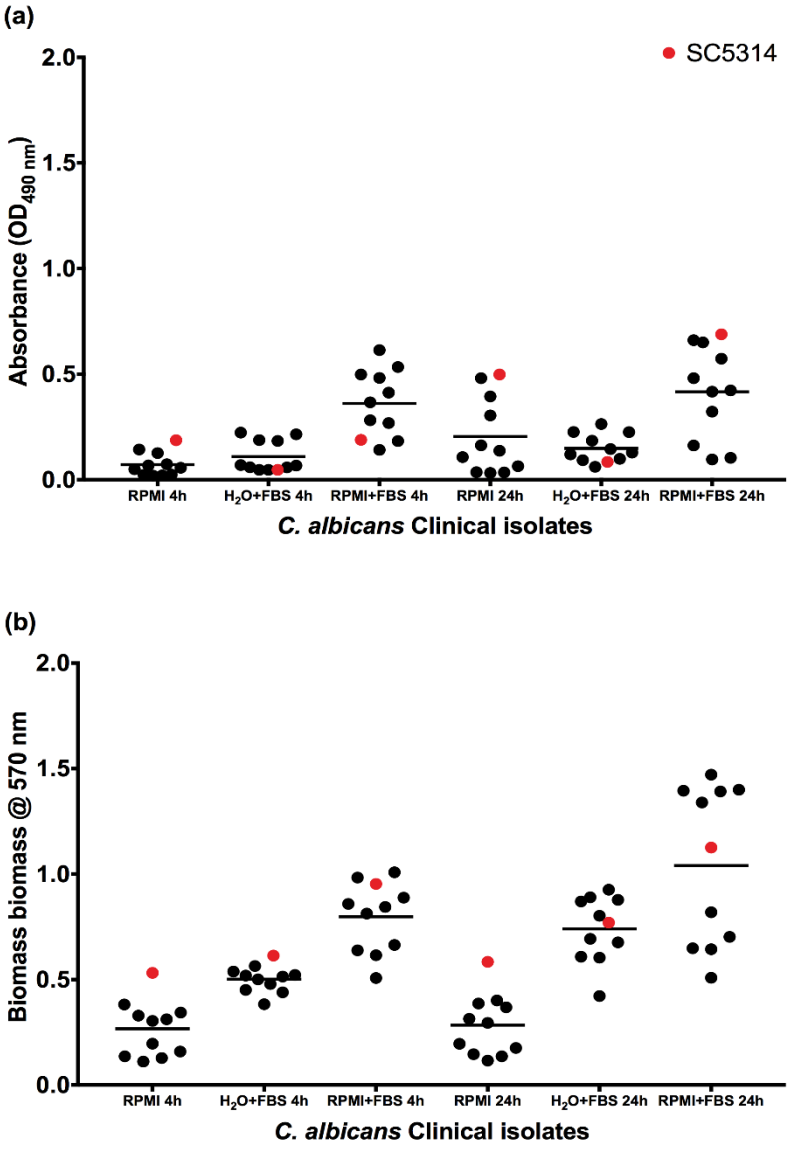


Figure 2.6: The effect of FBS on *C. albicans* biofilm forming ability. *C. albicans* LBFs (n=5) and HBFs (n=5) grown in RPMI, FBS or RPMI+FBS. Metabolic activity **(a)** and biofilm biomass **(b)** assessed at 4h and 24h of incubation. Each dot represents the average of data obtained from triplicates of three independent experiments for each strain/isolate. The horizontal line represents the grand mean of each condition/time point. Laboratory strain SC5314 (red colour) used as a reference.

Following biofilm biomass characterisation, the lowest biofilm former isolate (BC023=LBF) and the highest biofilm former isolate (BC146=HBF) were selected for transcriptional assessment for key biofilm related gene expression at 4h and 24h. Percentage of expression relative to the *ACT1* housekeeping gene is illustrated in Figure 2.7. The expression of *ACT1* was shown to be stable across all conditions. Overall analysis shows that the expression of the two closely associated genes, *HWP1* (filamentation) and *ALS3* (adhesion) was higher in the HBF compared to LBF at 4h and 24h. While the expression of *HWP1* and *ALS3* in LBF remained relatively stable between 4h and 24h, their expression increased in HBF at 24h. Therefore, the difference in *HWP1* and *ALS3* expression between LBF and HBF becomes more evident at 24h with a 6.3 and 3.8-fold difference, respectively. This transcriptional analysis further confirms our biomass-based initial categorisation of *C. albicans* isolates into LBF and HBF. On the other hand, the expression of *ECE1* at 4h was comparable in LBF and HBF and becomes higher in LBF at 24h. The expression of *EFG1* was slightly higher in LBF at 4h and becomes comparable for both isolates at 24h.

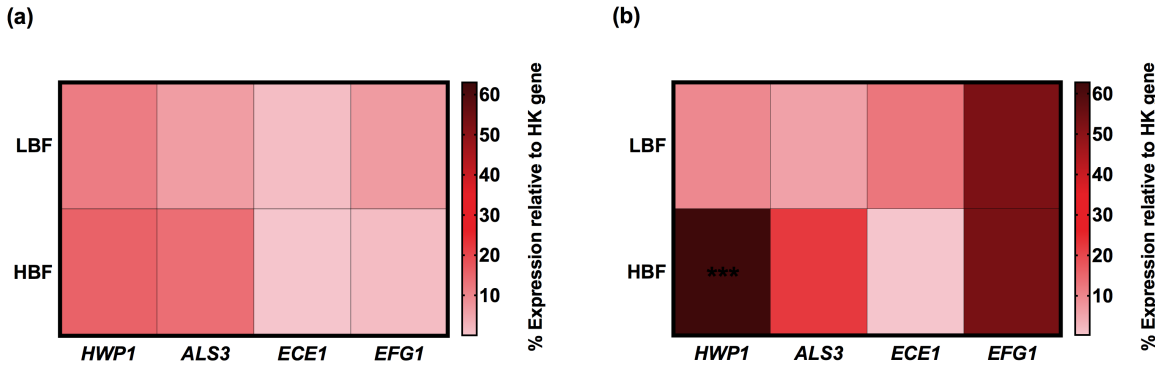


Figure 2.7: Heatmap of *C. albicans* LBF and HBF key biofilm genes. *C. albicans* LBF (BC023) and HBF (BC146) were grown in RPMI for 4h **(a)** and 24h **(b)**. Gene expression was assessed using real time qPCR and percentage expression was normalised to *ACT1* housekeeping gene. Data obtained from triplicates of three independent experiments. Statistical significance in the expression of each gene between LBF and HBF was calculated using Kruskal-Wallis test and presented as *** $p < 0.001$.

2.4.2 *C. albicans* biofilm treatment with Sodium hypochlorite

2.4.2.1 Comparison of 5 minutes versus 30 minutes *C. albicans* biofilm treatment with NaOCl

Next, we evaluated how different *C. albicans* isolates respond to the classical and the most widely used endodontic irrigant, NaOCl. The first step of this analysis was to choose the appropriate treatment time. For this purpose, the 2 clinically relevant time points (5 and 30 minutes) were compared. 24h biofilms of LBFs and HBFs were treated with NaOCl for 5 or 30 minutes. The treatment outcome, determined by the metabolic activity and the biofilm biomass, was assessed immediately (Figure 2.8) or following 24h of re-incubation with fresh RPMI media (Figure 2.9). The overall analysis shows that there was not a significant difference in the metabolic activity nor the biofilm biomass between the two time points either at the immediate or re-incubation level. Thirty minutes treatment time was not superior to 5 minutes. Indeed, there was a trend of increased viability of the treated biofilms at 30 minutes for some LBFs and HBFs. Based on this data, 5 minutes of treatment was selected for all downstream NaOCl experiments. Of note, during this analysis, *C. albicans* LBF (BC044) showed an odd response to NaOCl compared with other *C. albicans* isolates. Further analysis revealed that this isolate was not a *C. albicans* isolate and was therefore excluded.

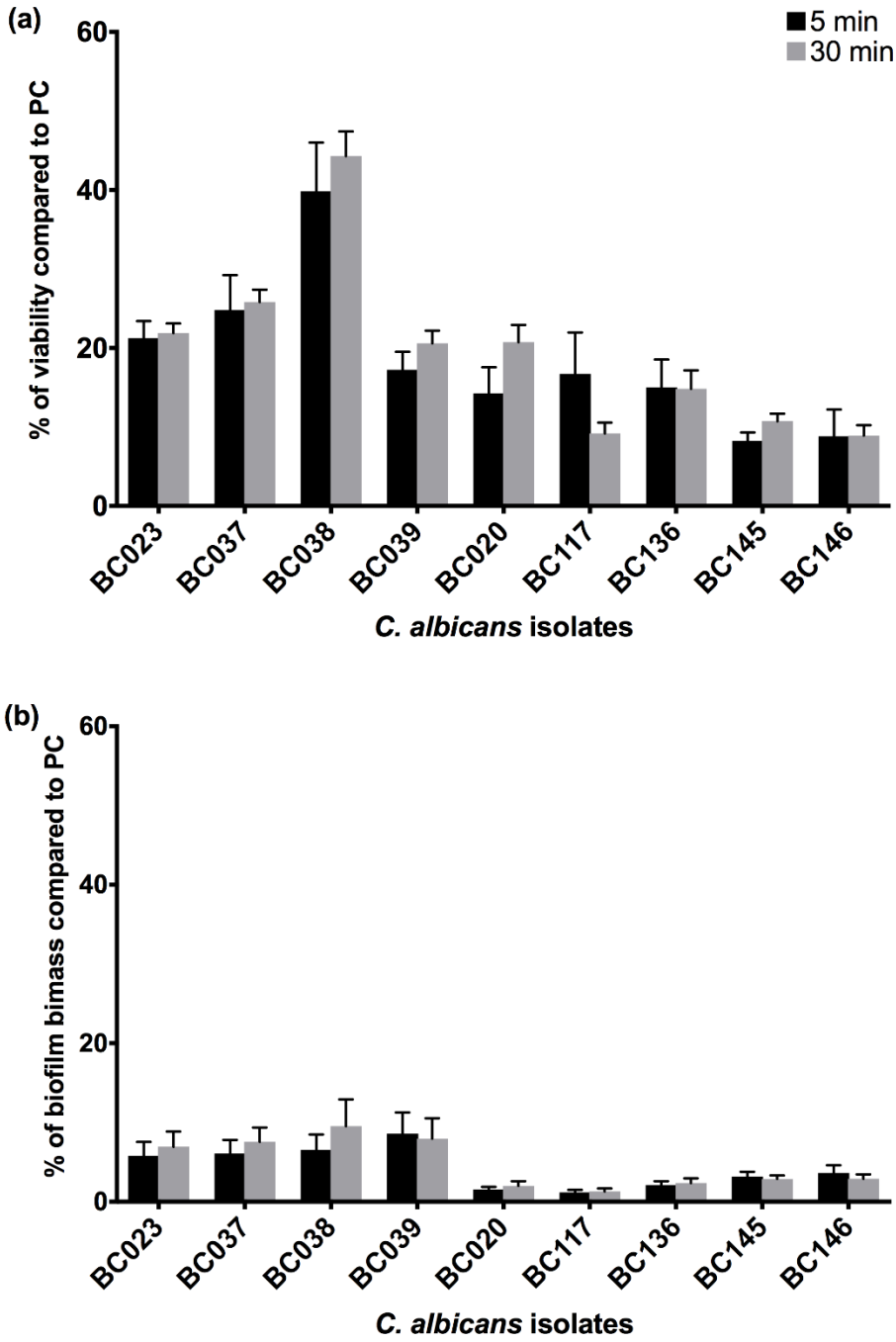


Figure 2.8: Comparison of the immediate effect of 5 minutes and 30 minutes treatment with NaOCl on the viability and biofilm biomass of the LBFs and HBFs. (a) Viability percentage of treated biofilms in relation to untreated controls as assessed by XTT assay immediately following the treatment. **(b)** Biofilm biomass percentage of treated biofilms in relation to untreated controls as assessed by CV assay. Each bar represents the average of data obtained from triplicates of three independent experiments for each isolate. Error bars represent the standard error of the mean. PC = positive untreated control. There was no statistical significance between 5 minutes and 30 minutes treatment for each isolate.

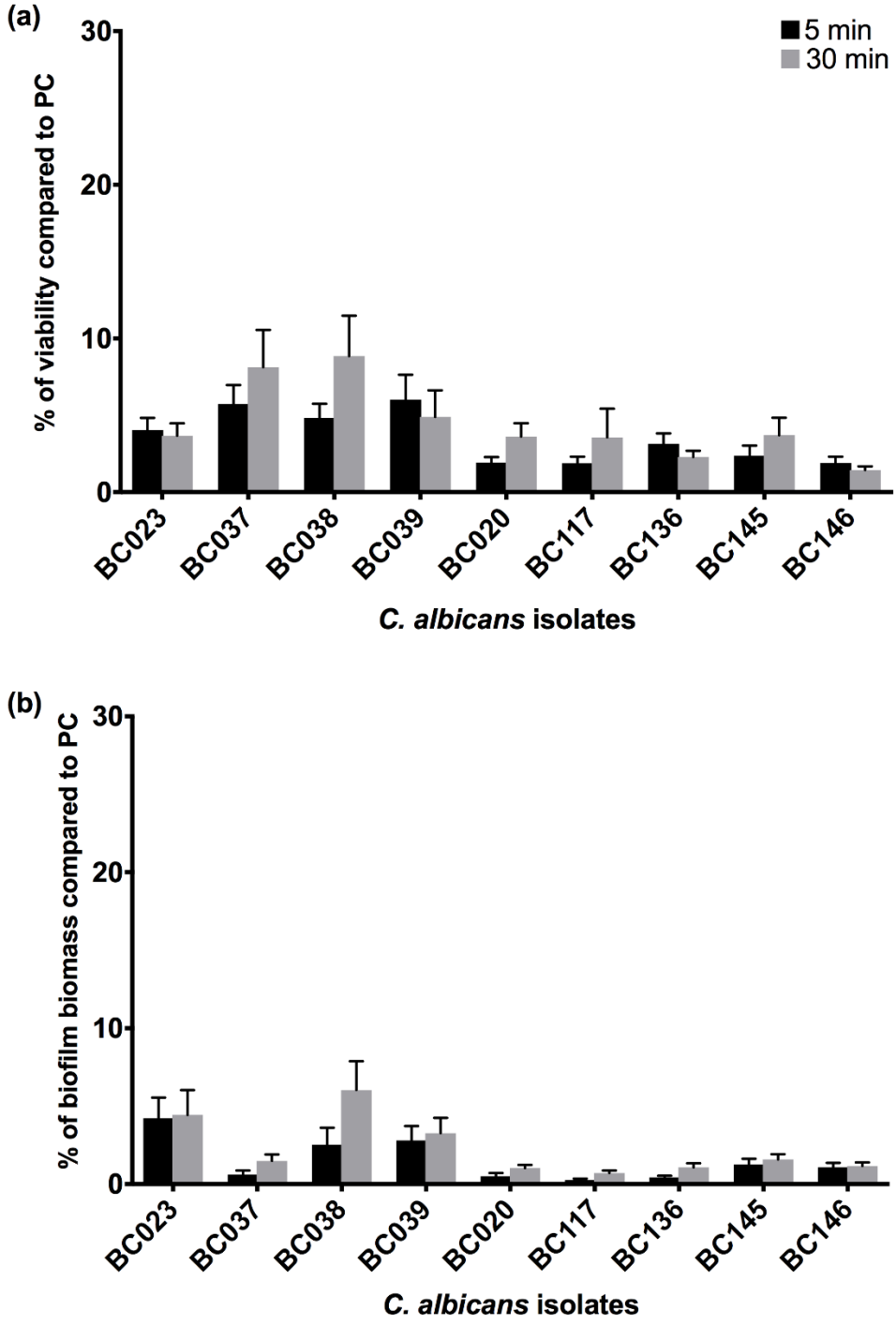


Figure 2.9: Comparison of the effect of 5 minutes and 30 minutes treatment with NaOCl on the viability and biofilm biomass of the LBFs and HBFs after 24h of re-incubation. 24h biofilms were treated for 5 minutes or 10 minutes with NaOCl and re-incubated with fresh RPMI for another 24h**. (a)** Viability percentage of treated biofilms in relation to untreated controls as assessed by XTT assay. **(b)** Biofilm biomass percentage of treated biofilms in relation to untreated controls as assessed by CV assay. Each bar represents the average of data obtained from triplicates of three independent experiments for each isolate. Error bars represent the standard error of the mean. PC = positive untreated control. There was no statistical significance between 5 minutes and 30 minutes treatment for each isolate.

2.4.2.2 *Candida albicans* persists following NaOCl treatment

As mentioned earlier in section 1.1.1, the regrowth of treated populations has significant implications for recurrent and secondary endodontic infections. Thus, treated biofilms were assessed not only immediately, but also after re-incubation with fresh media at 24h, 48h and 72h. *C. albicans* LBFs and HBFs 24h biofilms were treated for 5 minutes with 3% NaOCl. Viability of the treated biofilms were expressed as percentages relative to untreated controls. As illustrated in Figure 2.10a, b, both LBFs and HBFs were able to regrow over time to reach maximum viability at 48h and decrease slightly at 72h. Interestingly, LBFs showed higher viability immediately after treatment with ranges between 17.8 - 29.5% compared to 2.8 - 7.1% for HBFs. This was consistent among all LBF and HBF isolates.

However, biofilm biomass measurements did not follow the same trend. There was an obvious decrease in biomass at 24h and then remained relatively stable at 48h and 72h for all LBFs and HBFs (Figure 2.10c, d). Microscopic examination showed some persistent populations of *C. albicans* LBF and HBF immediately after treatment with NaOCl. These populations then continued to grow over the re-incubation period to reach a density comparable to that of untreated controls. It was also evident that most regrowing cells for both LBF and HBF are in yeast form (Figure 2.11). This may explain why there was not an increase in biomass in parallel with increased viability over time of re-incubation. *C. albicans* mono-species response to NaOCl treatment and regrowth is further discussed in chapter 3.

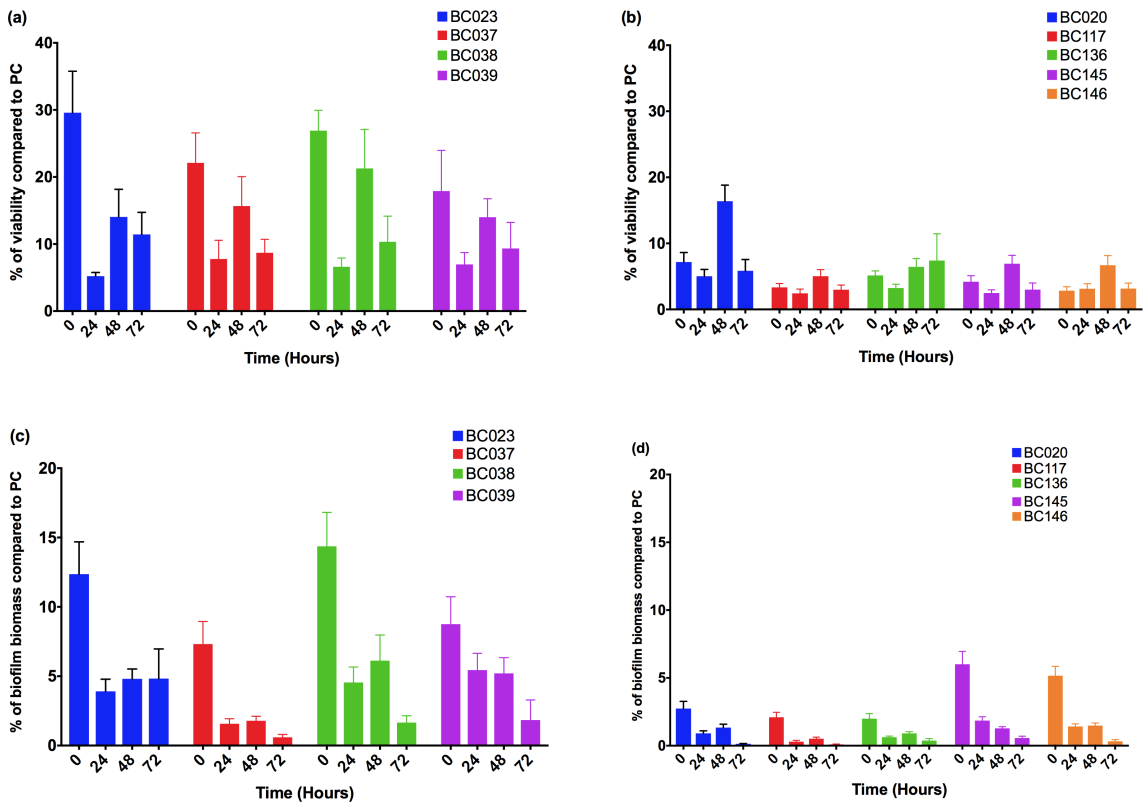


Figure 2.10: Percentage of biofilms viability and biofilm biomass following 5 minutes NaOCl treatment and re-incubation with fresh RPMI. Viability and biomass percentage of treated biofilms in relation to untreated controls was assessed by XTT and CV assay immediately post-treatment (0h), 24h, 48h and 72h. **(a)** Viability of LBFs **(b)** Viability of HBFs **(c)** Biomass of LBFs **(d)** Biomass of HBFs. Each bar represents the average of data obtained from triplicates of three independent experiments for each isolate. Error bars represent the standard error of the mean. PC = positive untreated control.

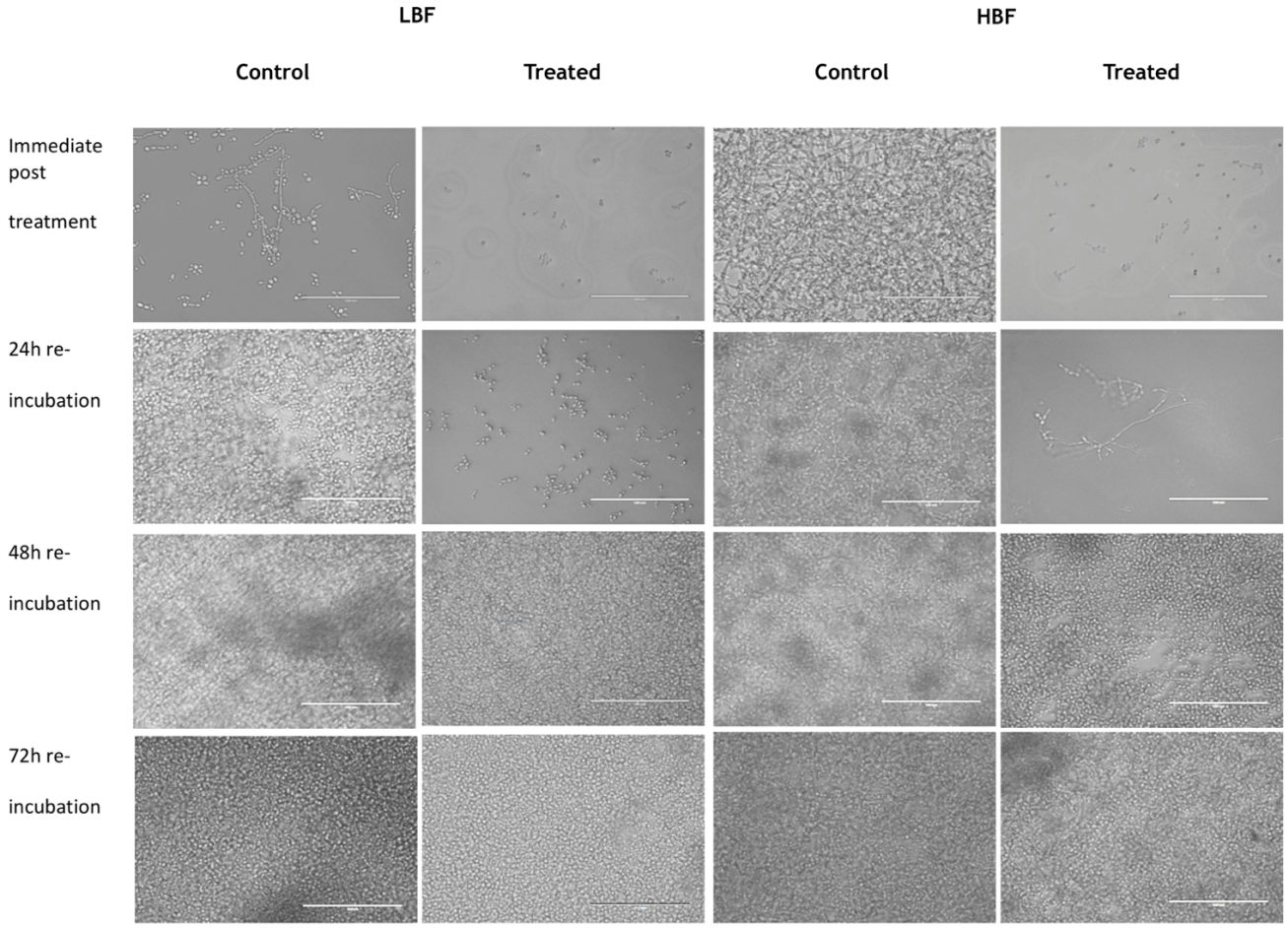


Figure 2.11: Light microscopic images of LBF (BC023) and HBF (BC146) following NaOCl treatment. Biofilms were treated and re-incubated with fresh media for 24h, 48h, and 72h and images were taken at the indicated time points. Scale bar is 100 µm.

It was evident that LBFs showed a higher percentage of viability following NaOCl treatment in comparison to HBFs. Therefore, it was hypothesised that the LBF phenotype may intrinsically pose higher resistance to drugs. Thus, the final step of this part of the analysis was to compare the expression of drug resistance genes in LBF and HBF when treated with the traditional antifungal drug, fluconazole. In contrast to NaOCl, fluconazole treated fungal cells can be harvested for RNA extraction and gene expression analysis. 24h biofilms of LBF and HBF were treated with fluconazole for 24h. After treatment, RNA was extracted and the expression of drug resistance genes, *CDR1*, *MDR1*, *GSC1*, *ERG11*, *PIL1* and *HSP90* was measured. With the exception of MDR1, there was no statistically significant difference in the expression of the above-mentioned genes between LBF and HBF. *MDR1* was the only gene that showed significantly higher expression in HBF (Figure 2.12). Although, *ERG11* and *CDR1* expression was higher in LBF, this difference was statistically insignificant. Based on this gene expression profile, it can be concluded that LBFs isolates are not intrinsically more drug resistant than HBF to fluconazole. However, it is essential to note that NaOCl has a completely different mechanism of action and therefore, it is expected that it would result in different expression profiles distinct from those associated with fluconazole.

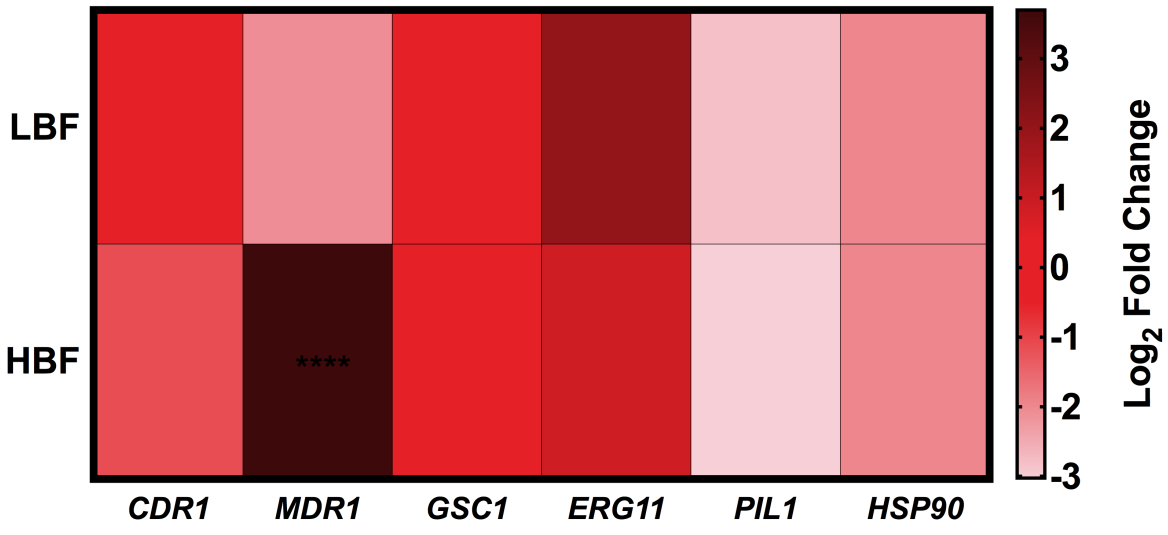


Figure 2.12: Heatmap of LBF and HBF genes associated with drug resistance. The expression of these genes was measured in LBF (BC023) and HBF (BC146) untreated and fluconazole treated biofilms at 24h. The expression of each gene was first normalised to *ACT1* housekeeping gene and the mean log fold change of the expression in treated biofilm was calculated in relation to untreated biofilms for each gene. Data obtained from triplicates of three independent experiments. Statistical significance in the expression of each gene between LBF and HBF was calculated using Kruskal-Wallis test and presented as **** $p < 0.0001$.

2.4.2.3 *C. albicans* persistence was not impacted by replenishment of RPMI medium

C. albicans cells remaining after NaOCl treatment require fresh media to be able to regrow. In this analysis, we evaluated whether replenishment of media at 24h and 48h would enhance *C. albicans* regrowth. LBFs and HBFs biofilms were treated as described above and re-incubated with fresh RPMI media. At 24h and 48h, fresh RPMI was added in the replenishment group. No fresh media was added to the continuous re-incubation group. Regrowth was then assessed at 48h and 72h as a percentage of viability using the XTT assay. There was no significant difference in viability between the two tested groups at both 48h and 72h for all LBFs and HBFs (Figure 2.13, Figure 2.14). Media replenishment may have resulted in loss of planktonic unattached cells in the supernatant, therefore, did not offer increased viability over continuous incubation. Again, LBFs show higher regrowth potential compared to HBFs.

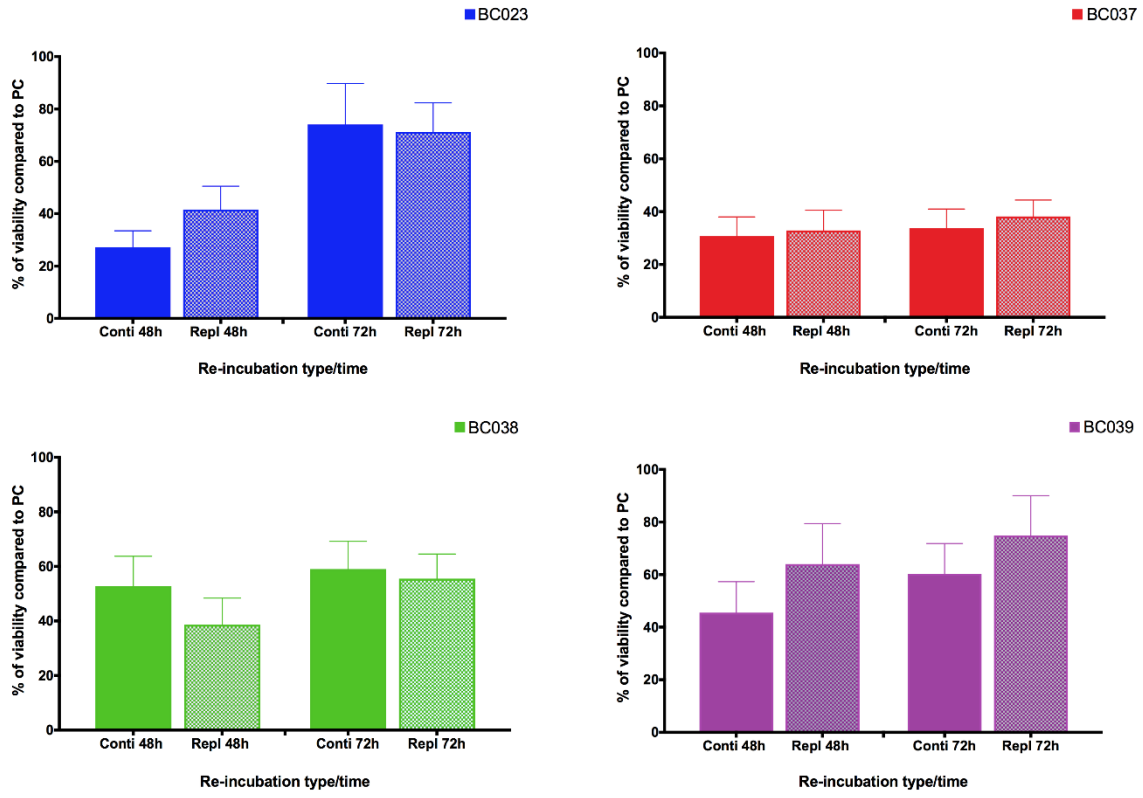


Figure 2.13: Percentage of biofilms viability following 5 minutes NaOCl treatment and continuous or replenishment re-incubation of LBFs. Biofilm were treated and re-incubated with fresh RPMI and assessed with XTT assay at 48h and 72h. RPMI was replenished at 24h and 48h in replenishment (Repl) re-incubation but not in the continuous re-incubation (Conti). Each bar represents the average of data obtained from triplicates of three independent experiments for each isolate. Error bars represent the standard error of the mean. PC = positive untreated control.

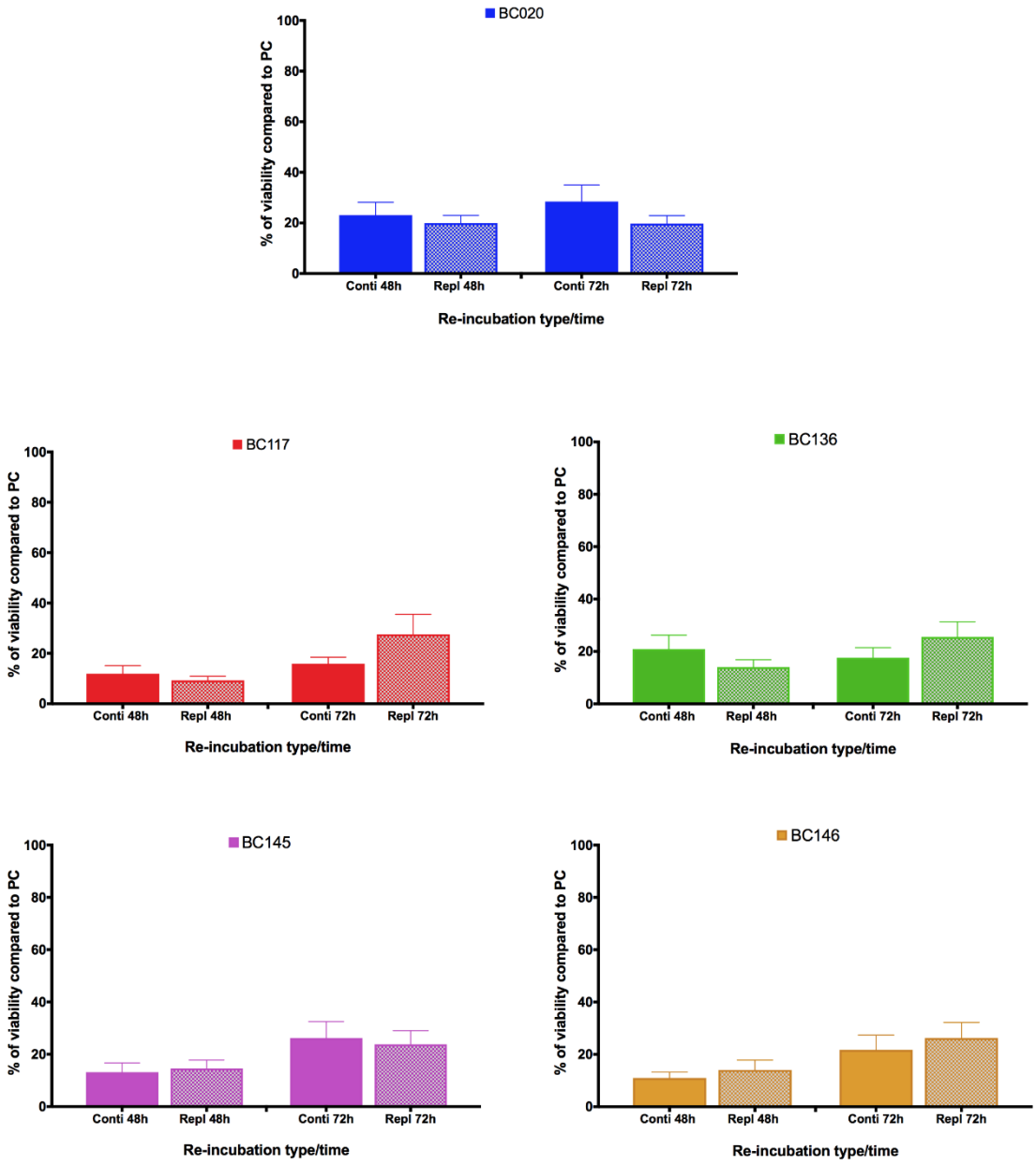


Figure 2.14: Percentage of biofilm viability following 5 minutes NaOCl treatment and continuous or replenishment re-incubation of HBFs. Biofilms were treated and re-incubated with fresh RPMI and assessed with XTT assay at 48h and 72h. RPMI was replenished at 24h and 48h in replenishment (Repl) re-incubation but not in the continuous re-incubation (Conti). Each bar represents the average of data obtained from triplicates of three independent experiments for each isolate. Error bars represent the standard error of the mean. PC = positive untreated control.

2.4.3 Metabolic activity XTT assay optimisation

The XTT metabolic assay is widely used to assess viability of various cell types, including *C. albicans* as shown above. It is a rapid and inexpensive method that is commonly used to assess the effectiveness of different treatments. It was evident from the previous microscopic images that there was a small number of cells

remaining immediately following NaOCl treatment. Also, it was surprising to observe higher viability percentage with LBFs compared to HBFs. HBFs were previously shown to be more resistant to antifungal treatment (Sherry et al., 2014). Therefore, we aimed to validate the XTT results and test whether XTT was sensitive enough to detect these persistent populations. Also, the sensitivity of XTT to different cellular density and how it can be enhanced was evaluated.

2.4.3.1 XTT sensitivity is limited at cellular density below 1x10^6 cells/mL

We tested the sensitivity of XTT at different cellular densities ranging from 1x10^3 - 1x10^8 cells/mL. *C. albicans* LBFs and HBFs were adjusted to the indicated cellular density in PBS. XTT was then added and incubated in the dark for 2h after which, absorbance was measured using a microplate reader. The results show that while there was a clear difference in absorbance between cellular density of 1x10^8, 1x10^7 and 1x10^6 cells/mL, XTT failed to detect differences in cellular density below 1x10^6 cells/mL (Figure 2.15, Figure 2.16). This was consistent for all LBFs and HBFs tested. Interestingly, absorbance of LBFs was generally lower than HBFs of the same cellular density. For instance, LBFs absorbance at 1x10^8 cells/mL ranges between 0.45 – 0.6 and for HBFs was between 0.5 – 1.2.

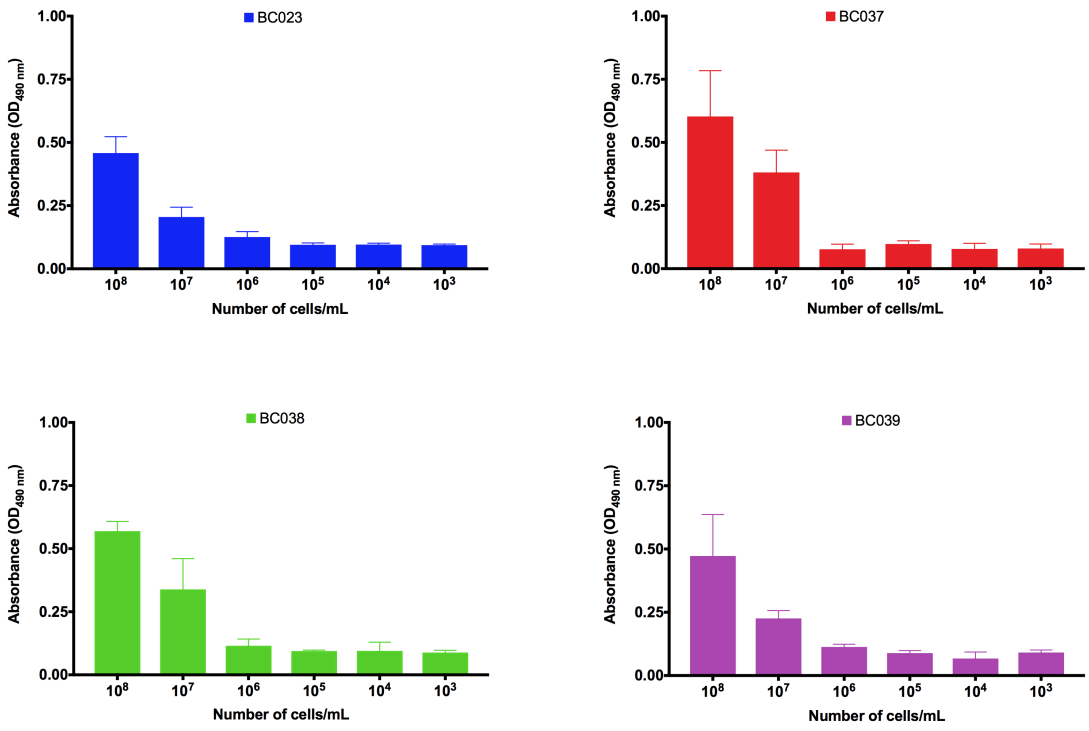


Figure 2.15: Sensitivity of XTT metabolic assay with 1µM menadione in detecting cellular density of 1x10^8 to 1x10^3 cells/mL in LBFs. Each bar represents the average of data obtained from triplicates of three independent experiments for each isolate. Error bars represent the standard error of the mean.

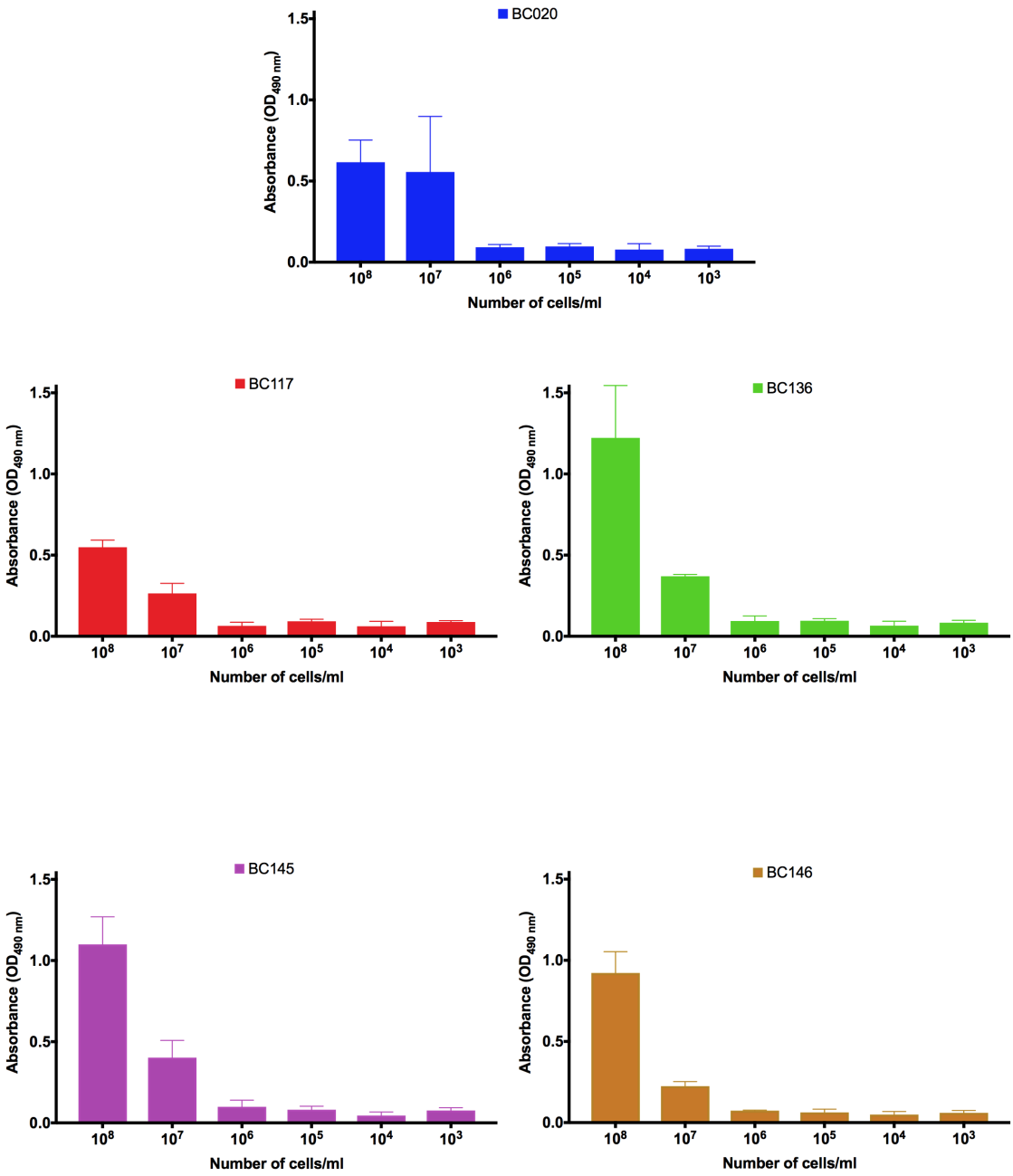


Figure 2.16: Sensitivity of XTT metabolic assay with 1µM menadione in detecting cellular density of 1x10^8 to 1x10^3 cells/mL in HBFs. Each bar represents the average of data obtained from triplicates of three independent experiments for each isolate. Error bars represent the standard error of the mean.

In an attempt to enhance XTT sensitivity, menadione was added in different concentrations to test if XTT sensitivity can be enhanced at differentiating cellular density below 1x10^6 cells/mL. XTT with menadione concentrations of 1µM, 2 µM, 4 µM, 8 µM or 10 µM were added to *C. albicans* LBF and HBF as described above. In LBF, 8 µM showed the best sensitivity to differentiate cellular density 1x10^6-1x10^4 cells/mL (Figure 2.17a). XTT was not sufficiently sensitive to detect cellular

density below $1x10^4$ cells/mL regardless of menadione concentration. All other tested menadione concentrations did not enhance XTT sensitivity below $1x10^6$ cells/mL where the difference in absorbance between10^6-10^3 cells/mL was negligible. In HBF, with the exception of 4 µM, all menadione concentrations showed high difference in absorbance between $1x10^6$ and $1x10^5$ cells/mL. This difference becomes less evident between $1x10^5$ and $1x10^4$ cells/mL and negligible below $1x10^4$ cells/ mL (Figure 2.17b). Secondly, we tested whether reading XTT absorbance at different wavelengths would enhance sensitivity. XTT manufacture recommend measuring XTT absorbance at wavelengths between 450-500 nm. Therefore, the absorbance of the same sample was measured at 460 nm and 490 nm. As illustrated in Figure 2.18a and b, there was no significant difference between the two wavelength readings for LBFs and HBFs. Again, 8 µM was more sensitive than 1 µM in differentiating cells of $1x10^8$ and $1x10^6$ cells/mL. Overall, when considering both LBFs and HBFs, it seems that increasing menadione concentration above 8 µM does not provide extra XTT sensitivity which remains predominantly limited in differentiating cellular density below $1x10^5$ cells/mL. Nevertheless, 8 µM could be the appropriate menadione concentration that provides better sensitivity to differentiate $1x10^6$ and $1x10^5$ cells/mL populations.

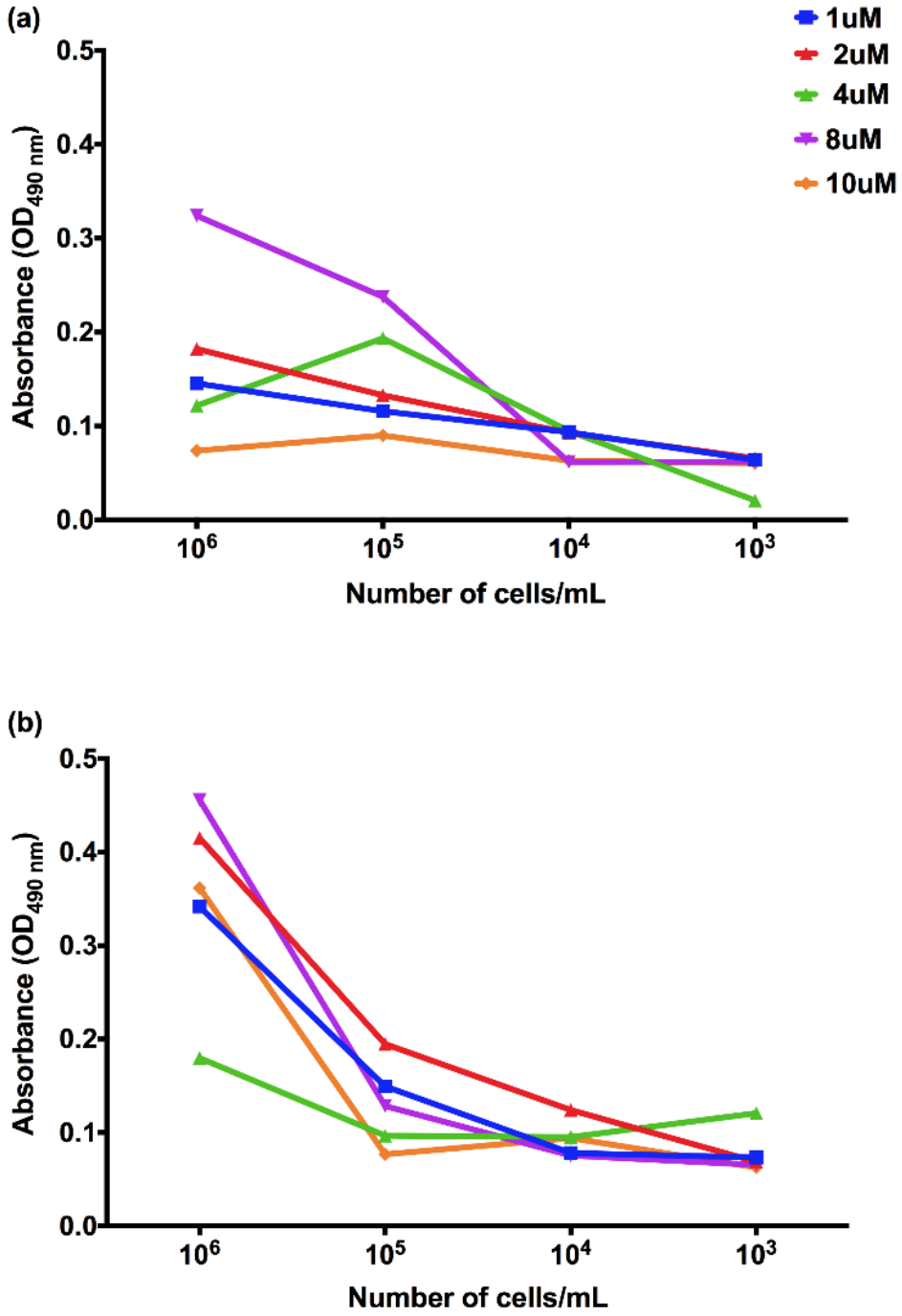


Figure 2.17: Sensitivity of XTT with 1µM, 2 µM, 4 µM, 8 µM or 10 µM menadione in detecting cellular density of 1x10^6 to 1x10^3 cells/mL. (a) LBF (BC023) **(b)** HBF (BC146). Data obtained from triplicates of three independent experiments for each isolate.

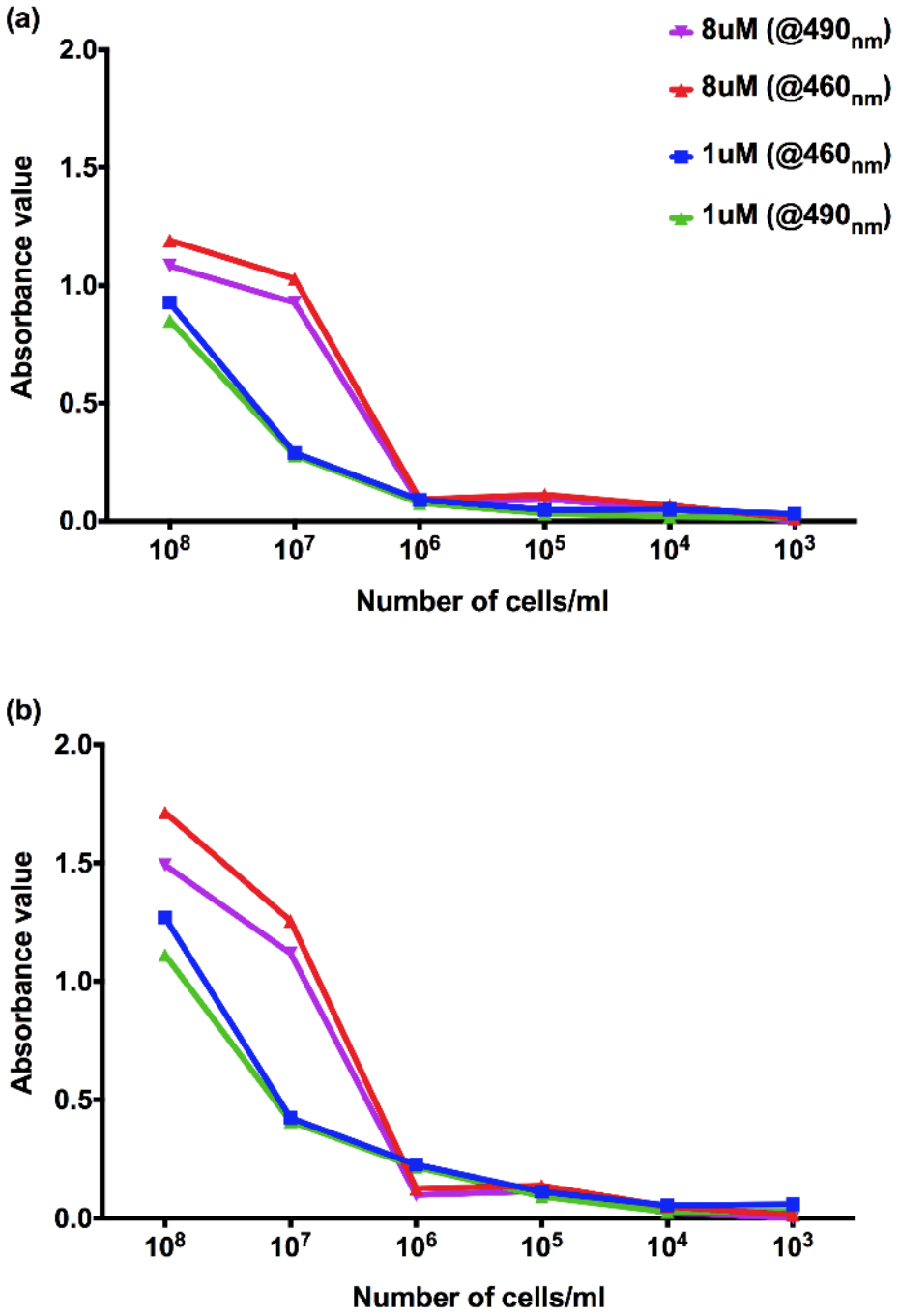


Figure 2.18: Sensitivity of XTT with 1µM and 8 µM when reading at wavelengths 460 or 490 nm in detecting cellular density of $1x10^8$ to $1x10^3$ cells/mL. (a) LBF (BC023) **(b)** HBF (BC146). Data obtained from triplicates of three independent experiments for each isolate.

2.4.3.2 Alamar blue metabolic activity assay is not superior to XTT in detecting *C. albicans* persistence

Alamar blue is another colorimetric metabolic activity-based assay which has similar applications as XTT. Here, the sensitivity of alamar blue was compared to that of XTT in LBFs and HBFs immediately following treatment, at 24h, 48h and

72h. To be able to compare the sensitivity of the two assays, the percentage of viability of the treated biofilms was calculated relative to untreated controls of the corresponding time points. The results presented in Figure 2.19 and Figure 2.20 suggest that XTT has better detection rate at 0h (immediately after treatment) in all LBFs and HBFs although statistical significance was only observed in the LBF BC038 isolate. Data of XTT and alamar blue for other time points were inconclusive, none of them were consistently superior in detecting regrowth across all the isolates or time points.

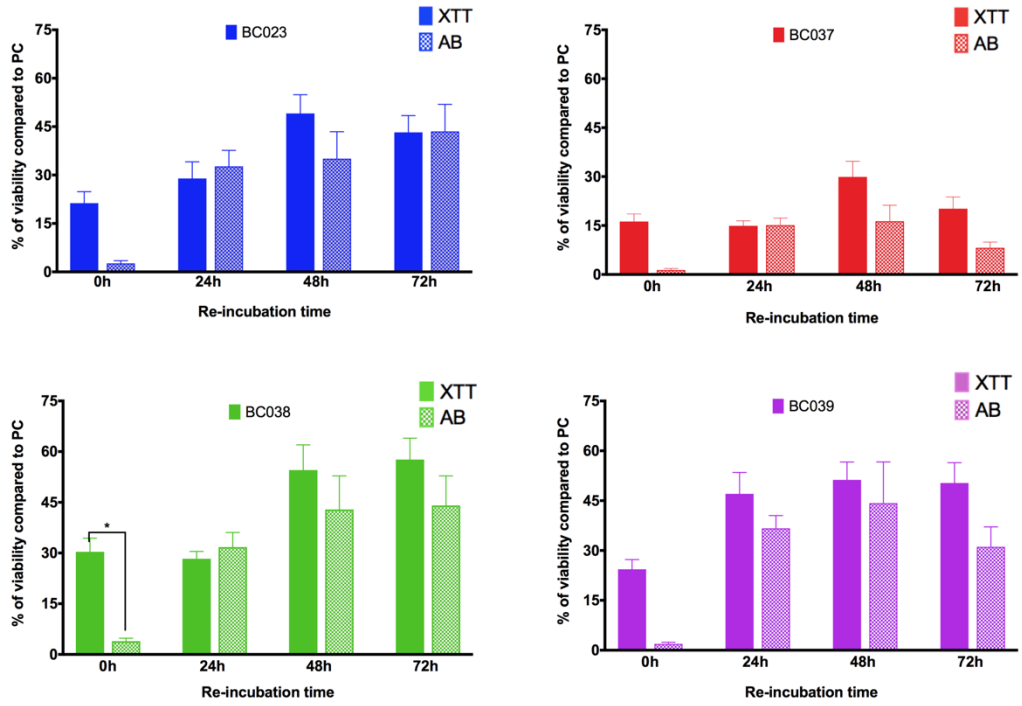


Figure 2.19: Comparison between XXT and alamar blue metabolic assay in LBFs. Viability percentage of *C. albicans* biofilms in relation to untreated controls after 5 min treatment with NaOCl and re-incubation with fresh RPMI. Metabolic activity was assessed using XTT or alamar blue assay immediately post-treatment (0h), 24h, 48h and 72h. Each bar represents the average of data obtained from triplicates of three independent experiments for each isolate. Error bars represent the standard error of the mean. PC = positive untreated control. Statistical significance was calculated using Kruskal-Wallis test and presented as * $p < 0.05$.

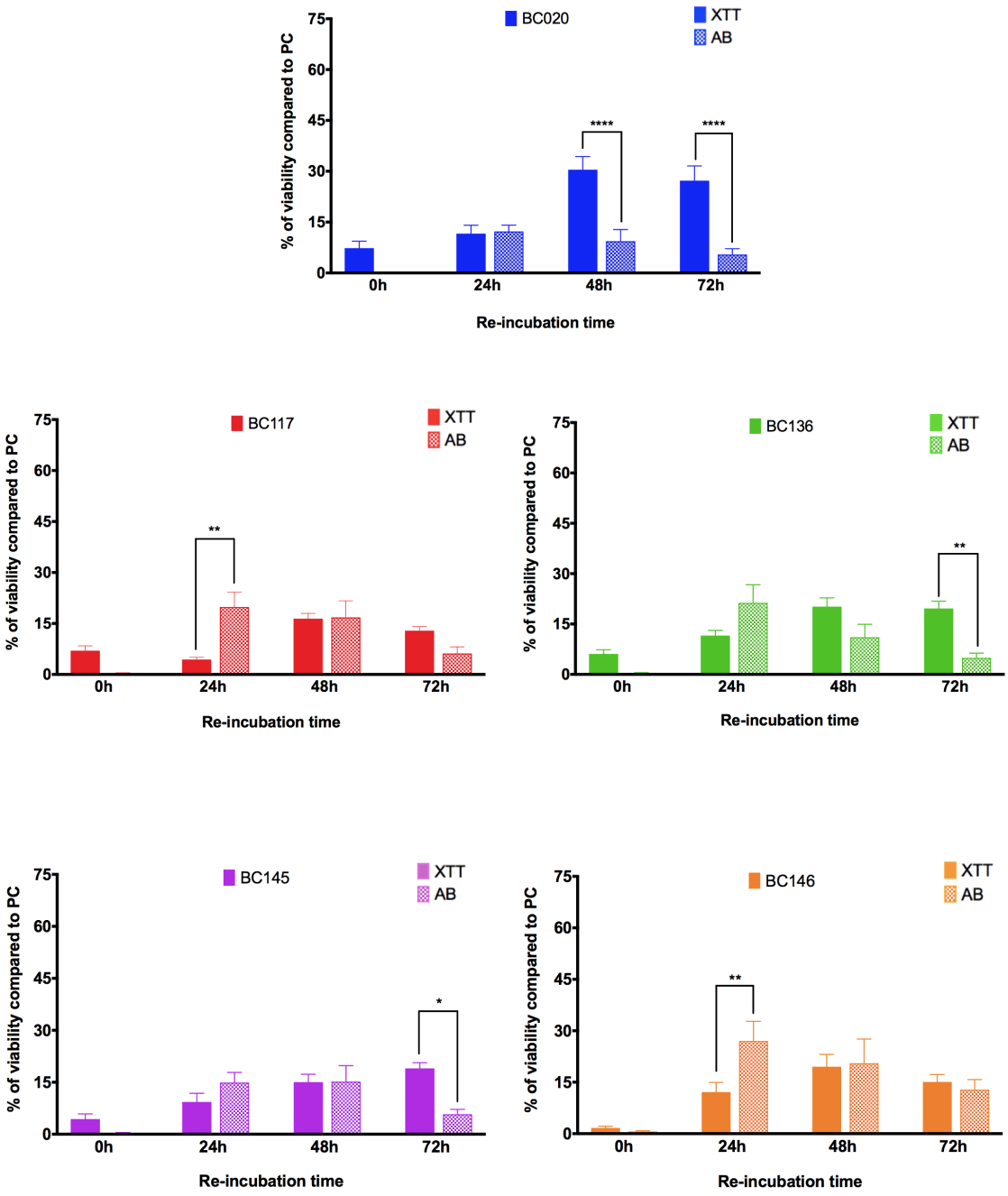


Figure 2.20: Comparison between XXT and alamar blue metabolic assay in HBFs. Viability percentage of *C. albicans* biofilms in relation to untreated controls after 5 min treatment with NaOCl and re-incubation with fresh RPMI. Metabolic activity was assessed using XTT or alamar blue assay immediately post-treatment (0h), 24h, 48h and 72h. Each bar represents the average of data obtained from triplicates of three independent experiments for each isolate. Error bars represent the standard error of the mean. PC = positive untreated control. Statistical significance was calculated using Kruskal-Wallis test and presented as * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

Overall, it can be concluded that XTT has a limited sensitivity to differentiate between cellular densities below $1x10^6$ cells/mL. XTT sensitivity can be possibly enhanced by adding 8μM menadione though not significantly. The number of cells remaining immediately after NaOCl treatment or those regrowing at 24h, 48h, and 72h was only quantified by XTT on this occasion. However, as indicated from the

microscopic examination in Figure 2.11, there are few cells remaining following treatment, afterwards the cellular density increases overtime. It can be inferred that the cell number at 0h or 24h may be less than the detection limit of XXT. However, at 48h and 72h, the cells appear to cover the majority of the well surface, and therefore the XTT is more likely to be sensitive enough to detect cellular density in these time points. Furthermore, viability results following NaOCl treatment were expressed as a percentage relative to untreated controls rather than expressing the absolute absorbance values. Given the fact that the viability of untreated controls increases over time, or at least reaches a growth plateau, higher viability percentages at 48h and 72h are more likely indicative of increased regrowing populations and not due to limitation in XTT assay itself. Finally, XTT results of NaOCl treatment were further validated using a molecular method (qPCR) in chapter 4.

2.5 Discussion

C. albicans was isolated from the root canals of teeth with endodontic infections (Siqueira Jr and Rôças, 2004). However, the specific role of *C. albicans* in these infections is yet to be determined. *C. albicans* is a polymorphic fungus with a distinct ability to change morphology between yeast, pseudohyphae and hyphae (Villar et al., 2004). Yeast to hyphae switch and biofilm formation are major virulence attributes of *C. albicans* and associated with increased pathogenicity (Ponde et al., 2021). Several studies reported that bloodstream *C. albicans* isolates with higher biofilm forming ability are shown to be more pathogenic and may be associated with increased mortality (Tumbarello et al., 2012, Sherry et al., 2014, Hasan et al., 2009). The endodontic micro-environment is different from other infections sites with respect to the lack of blood supply, restricted nutrients and anaerobic atmosphere. Therefore, it can be assumed that, in this unique environment, the *C. albicans* phenotype and virulence attributes may be impacted in a way that is different from other infection sites. Scanning electron microscope examination showed *C. albicans* on the radicular dentine surface and penetrating the dentinal tubules in both yeast and hyphal form (Şen et al., 1997). However, there is lack of evidence whether yeast or hyphal form are more virulent and more resistant to treatment in the context of endodontic infections. In this analysis, we firstly categorised *C. albicans* oral isolates based on biofilm forming ability and further confirmed this classification with the analysis of gene expression of biofilm

related genes at early 4h and 24h. *C. albicans* isolates used in this analysis are isolated from the oral cavity rather than from endodontic infections. However, it can be argued that *C. albicans* in endodontic infections are originally oral isolates that gained entry to the originally sterile root canal system due to breach of the surrounding protective dental tissues.

Following characterisation, we showed that *C. albicans* LBFs have a higher percentage of viability following NaOCl compared to HBFs isolates. Previous studies of our group on *C. albicans* blood isolates (Sherry et al., 2014) and on denture isolates (O'Donnell et al., 2017) also categorised *C. albicans* into LBFs and HBFs and investigated their response to antifungals and denture hygiene products respectively. In contrast to our findings, HBFs demonstrated higher resistance to amphotericin B, but not to voriconazole (Sherry et al., 2014). Also, O'Donnell et al. (2017) were unable to demonstrate a positive correlation of biofilm heterogeneity and patient outcomes in denture stomatitis. There was a positive corelation of the disease with the absolute number of candidal cells rather than *C. albicans* phenotype. Increased resistance to amphotericin B of HBFs reported by Sherry et al. (2014), can be explained by the fact that HBFs biofilms are more complex with predominant hyphae and extracellular matrix that cannot be readily penetrated by the antifungal drug. As a result, higher viability is seen with HBFs following treatment as not all the cells were challenged. In contrast, LBF biofilms, as evident from the above microscopic examination, are composed mainly of one layer of yeast cells with occasional hyphae and therefore, more cells are exposed and affected by the same dose of antifungal.

NaOCl is a potent bleaching agent with its mechanism of action different from that of antifungals. NaOCl has the ability to dissolve organic tissue and degrade lipid and fatty acid. Its antimicrobial action is exerted by either the high pH (pH>11) or by the release of free chlorine. NaOCl solution contains hypochlorous acid, that when it comes in contact with organic tissue, releases chlorine, which in turn, combines with amino acids leading to amino acid degradation (Estrela et al., 2002). With this high organic tissue dissolving ability, it can be inferred that even HBFs biofilms cannot withstand the antimicrobial effect of NaOCl and the majority of biofilms cells are now exposed to the bleaching agent. Nevertheless, this does not explain the higher tolerance observed in LBFs to NaOCl.

While there is no specific explanation of why LBFs are more tolerant to NaOCl, many possibilities exist. One is the presence of persister cells within the treated biofilms, a phenomenon that is well reported for *C. albicans* biofilms (LaFleur et al., 2006, Galdiero et al., 2020, Wuyts et al., 2018). This unique population of *C. albicans* cells are phenotypic variants of yeast cells present only in biofilms and are highly tolerant to antimicrobials. *C. albicans* persisters were first reported with amphotericin B treatment and their presence indicated by the characteristic biphasic killing curve where most (but not all) fungal populations are killed leaving a subpopulation that survived the lethal dose of antifungal (LaFleur et al., 2006). Considering the fact that the majority of *C. albicans* cells in LBFs biofilms are in the yeast form, it may naturally harbour more persister cells compared to HBFs which accounts for the observed increased tolerance to NaOCl. Future work looking into the percentage of persisters in LBFs and HBFs would be of interest and can form the basis for understanding persistence mechanisms and therefore, may impact upon clinical diagnosis and treatment prognosis.

Endodontic treatment is predictable with a high success rate ranging from 74% to 92% (Friedman et al., 2003). However, treatment failures still occur even in cases where the treatment was carried out to the highest standard (Siqueira Jr, 2001). There is a well-established belief that treatment failure and persistence of infections is attributable to the inability to completely disinfect all the anatomical complexities within the root canal system. It was shown that regardless of the technique or instruments used, some areas remain untouched and therefore, harbour viable microorganisms that may give rise to recurrent infections (Şen et al., 1997). However, persistence of endodontic pathogens that were undoubtedly exposed to and survived the treatment is less commonly considered as a cause of failure. Furthermore, the results in the majority of endodontic studies investigating the effectiveness of antimicrobials or techniques against endodontic microorganisms are limited to the immediate post treatment outcomes (Mohammadi, 2008). Only a few studies have explored further the regrowth potential of tolerant populations and the implications it may have in the recurrence of infections (Dametto et al., 2005, Byström and Sunvqvist, 1985, Sjögren and Sundqvist, 1987). In agreement with our findings, these studies showed the persistence of bacteria after NaOCl treatment at 2-7 days following the initial treatment. These studies evaluated persistence in natural human teeth

(either extracted or in clinical sessions) which are more relevant to clinical scenarios than our *in vitro* investigation. However, even in these studies, it was not possible to differentiate whether the persistence arises from bacterial populations that were treated and survived the treatment or those that remained untouched due to root canal complexity. Therefore, the possibility that these persistent populations evaded rather than tolerated the treatment remains valid.

In vitro investigation has its own limitations, such as the use of a single microorganism and a non-dental substrate. Root canal infections are polymicrobial in nature with microorganisms colonising the dentinal walls of the root canals. Therefore, it would be more relevant to use multispecies endodontic models on a dentine substrate for *in vitro* investigation. Nevertheless, the present study clearly shows that tolerance of the treated microorganism can contribute to infection persistence. We were able to show that despite the absence of anatomical complexity in our *in vitro* investigation, *C. albicans* cells that were totally exposed to treatment were able to tolerate NaOCl and regrow overtime. Contradictory results were seen in an early *in vitro* analysis by Waltimo et al, (Waltimo et al., 1999a) who showed that NaOCl resulted in complete killing of *C. albicans* cells. They used filter discs impregnated by *C. albicans* suspension that were subsequently immersed in the NaOCl. Therefore, similar to our model, *C. albicans* cells were completely exposed to treatment. However, glass beads with vigorous shaking were employed to retrieve *C. albicans* cells from filter discs for CFU counting. This may have resulted in disruption of the fragile remaining persistent *C. albicans* cells and may well explain the difference in results.

XTT is a quantitative colorimetric measure of metabolic activity of cells that is usually used as an indicator of cell viability. It has been used in fungal susceptibility testing, biofilm formation and drug resistance (Kuhn et al., 2003). XTT is colourless or has a light yellowish colour that is reduced to an orange colour through the formation of formazan in the presence of metabolically active cells. It has been shown that there is a direct relation between the intensity of the colorimetric signal produced by XTT and the number of cells (Hawser, 1996). However, Kuhn et al (Kuhn et al., 2003) demonstrated that while XTT can be useful in comparing parameters of the same strain, different *Candida* isolates and species may metabolise XTT differently. Therefore, the assumption that there is a linear relationship between the cellular number of different isolates and colorimetric

signal may not be necessarily true. This limitation in XTT applies to the different XTT readings we noticed with LBFs and HBFs of the same cell number (Figure 2.15). The absorbance of LBFs was lower than their HBFs counterparts of the same cell number. As a result, it cannot be claimed that LBFs and HBFs have similar capacity to metabolise XTT. To overcome this, we expressed viability post treatment as a percentage of untreated controls rather than absolute absorbance values to make a valid comparison between the different isolates. Furthermore, we aimed to enhance the sensitivity of XTT to detect the small number of cells remaining immediately post treatment. Despite our efforts, XTT sensitivity remained limited to cellular density below $1x10^6$ cells/mL. Likewise, sensitivity was not enhanced with the use of the alamar blue metabolic assay. Therefore, in subsequent analyses of dual-species biofilms (Chapter 4) we used quantitative PCR for outcome assessment.

Hyphal growth and biofilm formation are a characteristic feature of *C. albicans* that is regulated via different pathways (Finkel and Mitchell, 2011). Our transcriptional characterisation of *C. albicans* LBF and HBF isolates demonstrated increased expression of the key *C. albicans* biofilm genes, *HWP1* and *ALS3* in HBFs, a finding previously reported for HBF by Sherry et al. (2014). However, the expression of another biofilm related gene *ECE1* was higher in LBF at 24h. The expression of *EFG1* was higher in LBF at 4h and demonstrated no difference between LBF and HBF at 24h. While *EFG1* was shown to be involved in the adherence stage where yeast cells adhere to a substrate (Finkel and Mitchell, 2011), its higher expression in LBF at early stages of biofilm formation may be indicative of enhanced adherence of LBF. *ECE1*, on the other hand, is a hyphae specific gene that is involved in the initiation step in which the cells proliferate to form microcolonies and form germ tubes (Finkel and Mitchell, 2011). Early characterisation of *ECE1* demonstrated that *ECE1* expression is strictly associated with hyphae formation and no expression is detected when *C. albicans* grows as yeast cells. However, it is not essential for biofilm formation as an *ECE1* null mutant showed no morphological alteration from the wild type (Birse et al., 1993). Furthermore, *ECE1*, *HWP1* and *ALS3*, are regulated by the transcriptional factor Bcr1, therefore, increased expression of *ECE1* in LBF was surprising. This reflects the complexity of gene expression profiles of *C. albicans* during the different stages of biofilm development that involves different intersecting pathways.

Finally, the enhanced tolerance of LBFs to NaOCl lead to the hypothesis that LBFs may show enhanced expression of drug resistance genes compared to HBFs. Fluconazole was chosen for this purpose because, in contrast to NaOCl, fluconazole treated cells can be harvested and RNA can be extracted for gene expression analysis. The expression patterns of LBF and HBF show that there was not a significant difference in the expression of the investigated genes set except for *MDR1* whose expression was significantly higher in HBF.

2.6 Highlights

- *C. albicans* biofilm heterogeneity of oral clinical isolates impacts the response to the commonly used endodontic irrigant NaOCl.

- *C. albicans* biofilms are tolerant to NaOCl and have the potential to regrow following incubation with fresh media.

- XTT metabolic assay is limited in detecting low cellular density populations.

- Expression of drug resistance genes is not different between LBF and HBF except for *MDR1*.

3 *Candida albicans* biofilm heterogeneity and tolerance of clinical isolates: implications for secondary endodontic infections

3.1 Preface

It was shown in the previous chapter that *C. albicans* isolates persist following NaOCl treatment. In this chapter, we further explore regrowth and employ the xCELLigence system in an attempt to enhance detection of the regrowing populations. Additionally, we show that *C. albicans* heterogeneity impacts tolerance and sequential treatment with NaOCl followed by EDTA significantly inhibits *C. albicans* regrowth compared with NaOCl only.

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3.2 Author declaration and contribution

Conceptualisation: The work presented in this publication was initially started by Mrs Suror Shaban as part of her dissertation for her MSc Endodontics at the University of Glasgow in 2018. She started testing NaOCl against a *C. albicans* reference strain. My contribution was to develop the idea of *C. albicans* heterogeneity with regards to tolerance to endodontic irrigants by using clinical isolates of different phenotypes. I also developed the concept of regrowth following NaOCl treatment and testing sequential treatment of *C. albicans* biofilms.

Data Curation: I had the complete responsibility of data curation

Formal Analysis: I completed all the formal analysis presented in this publication.

Investigation: I conducted 90% of all the laboratory experiments presented in this publication. Mrs Suror Shaban contributed by supporting the experiments conducted to generate data in Figure 3.2 and Figure 3.4 by helping in operating the xCELLigence system.

Methodology: I designed all the experiments presented in this publication.

Project Administration: Prof Gordon Ramage, Dr William McLean and Dr Christopher J Nile were responsible for project Administration.

Visualisation: I was responsible for all the preparation and presentation of data presented in this publication.

Writing – original draft: I wrote the complete original draft for this publication.

Writing – review and editing: I completed all the editing requested by the journal. Prof Gordon Ramage, Dr William McLean and Dr Christopher J Nile provided review and feedback for the original draft.

3.3 *Candida albicans* biofilm heterogeneity and tolerance of clinical isolates: implications for secondary endodontic infections

Om-Alkhir Alshanta[Ψ], Suror Shaban[Ψ], Christopher J Nile, William McLean and Gordon Ramage*

Glasgow Dental School, School of Medicine, Dentistry and Nursing, College of Medical, Veterinary and Life Sciences, Glasgow, United Kingdom.

[Ψ]Both authors contributed equally to this work

Running title: *Candida albicans* biofilm tolerance

*Corresponding Author: Gordon Ramage, Oral Sciences Research Group, Glasgow Dental School, School of Medicine, Dentistry and Nursing, College of Medical, Veterinary and Life Sciences, University of Glasgow, 378 Sauchiehall Street, Glasgow, G2 3JZ, UK. Phone: +44(0)141 211 9752. e-mail: gordon.ramage@glasgow.ac.uk

3.4 Abstract

Aim: Endodontic infections are caused by the invasion of various microorganisms into the root canal system. *Candida albicans* is a biofilm forming yeast and the most prevalent eukaryotic microorganism in endodontic infections. In this study we investigated the ability of *C. albicans* to tolerate treatment with standard endodontic irrigants NaOCl (sodium hypochlorite), ethylenediaminetetraacetic acid EDTA and a combination thereof. We hypothesized that biofilm formed from a panel of clinical isolates differentially tolerate disinfectant regimens, and this may have implications for secondary endodontic infections. **Methodology:** Mature *C. albicans* biofilms were formed from 30 laboratory and oral clinical isolates and treated with either 3% NaOCl, 17% EDTA or a sequential treatment of 3% NaOCl followed by 17% EDTA for 5 min. Biofilms were then washed, media replenished and cells reincubated for an additional 24, 48 and 72h at 37^{o}C. Regrowth was quantified using metabolic reduction, electrical impedance, biofilm biomass, and microscopy at 0, 24, 48 and 72h. **Results:** Microscopic analysis and viability readings revealed a significant initial killing effect by NaOCl, followed by a time dependent significant regrowth of *C. albicans*, but with inter-strain variability. In contrast to NaOCl, there was a continuous reduction in viability after EDTA treatment. Moreover, EDTA significantly inhibited regrowth after NaOCl treatment, though viable cells were still observed. **Conclusions**: Our results indicate that different *C. albicans* biofilm phenotypes grown in a non-complex surface topography have the potential to differentially tolerate standard endodontic irrigation protocols. This is the first study to report a strain dependent impact on efficacy of endodontic irrigants. It is suggested that within the complex topography of the root canal, a more difficult antimicrobial challenge, existing

endodontic irrigant regimens permit cells to regrow and drive secondary infections.

Keywords: biofilm; *Candida albicans*; EDTA; NaOCl; tolerance

3.5 Introduction

Fungal infections are generally perceived as being relatively uncommon, yet are reported to affect more than a billion people (Gow and Netea, 2016). This is exacerbated when we consider the alarming global burden of antifungal resistance that we now experience (Fisher et al., 2018). Many infections we believe to be of bacterial origin are in fact fungal. Indeed, the yeast *Candida albicans* is a ubiquitous human commensal, but with opportunistic tendencies. Its capacity to morphologically transition from yeast to hyphal cells enables it to react dynamically, both in evasion of host immunity and in its ability to readily form biofilm structures that impact increased antifungal resistance (Ramage et al., 2005). *C. albicans* frequently resides in the oral cavity as a biofilm forming microorganism, interacting with other oral microbiota and the host. When we consider root canal infections, pathogenic yeasts have also been isolated from teeth associated with primary apical periodontitis and post-treatment disease (Siqueira Jr and Rôças, 2004).

Dental pulp is generally a sterile tissue containing nerves and vascular tissues that are connected to the surrounding periradicular tissues, though recent studies appear to suggest the presence of bacterial DNA in the pulp of pristine healthy teeth (Widmer et al., 2018). The pulp is protected from the oral microbiome by layers of mineralised tissues (dentin, enamel and cementum). Breach to these tissues, as a result of dental caries, tooth fractures or cracks, can expose the dentin and provide routes for oral microbiota to ingress towards the pulp (Yu and Abbott, 2007). Another route of pulp infection is the direct invasion of microorganisms to the pulp during root canal treatment during substandard clinical procedures. *C. albicans* is the most frequently isolated fungal species in

endodontic infections (Mergoni et al., 2018, Persoon et al., 2017b). The prevalence of yeast in persistent infections is higher than that in primary infections (Siqueira Jr and Rôças, 2004). However, two recent systematic reviews found that *C. albicans* prevalence does vary significantly between primary and secondary endodontic infections (Mergoni et al., 2018, Persoon et al., 2017b). In this environment *C. albicans* can colonise dentinal walls and penetrate into dentinal tubules (Siqueira et al., 2002, Şen et al., 1997). The route of fungal invasion to the root canals is likely to be through cracks or via leaking restorations, though the diameter of hyphae, which is 1.9–2.6 μm (Sevilla and Odds, 1986), cannot exclude the possibility of dentinal tubules invasion especially in deep caries lesions. Here, candida yeasts are capable of coalescing with one another in the form of biofilm, which is a multicellular community of yeast and hyphal forms encased in polymeric glue. The biofilm lifestyle enhances the ability of the cells to withstand host and antifungal factors, ultimately contributing to its persistence within the root canal. This phenotype, along with the complexity of root canal system, particularly in the apical third of root canals, are thought to be the major causes of treatment-resistant apical periodontitis.

Root canal treatment (RCT) is the treatment of choice for these endodontic infections. RCT aims to: (1) eliminate microorganisms from the root canal system to a level that promotes the healing of periradicular tissues, and (2) provide a three-dimensional hermetic seal to prevent reinfection. Different hand and rotary instruments are used during RCT in order to mechanically debride the biofilm on the root canal walls. Nevertheless, the cross-sectional root canal configuration can pose a challenge to adequate debridement, as these instruments tend to leave untouched recesses in oval canals (Taha et al., 2010, Weiger et al., 2002). This instrumentation can however create a smear layer that covers the root canal walls,

which consists of organic (pulp tissue remnants) and inorganic (dentin chips) tissues (Gwinnett, 1984). This layer might act as a protective barrier to encase biofilms formed on root canal walls (Paque et al., 2009) and might also encourage the adherence of microorganisms, such as *C. albicans* (Sen et al., 2003). Furthermore, it may compromise the quality of root canal sealing (Kokkas et al., 2004). Therefore, the use of chemical irrigating solutions to maximise root canal debridement and removal of the smear layer is vital for successful RCT, which includes ethylenediaminetetraacetic acid (EDTA), though not universally (Willershausen et al., 2015). EDTA acts mainly as an adjunct irrigant to remove smear layer, though an alternative effect is the inhibition of filamentation through chelation of necessary divalent cations in the pathogenic yeast *C. albicans* (Ramage et al., 2007), a structural element strongly associated with robust biofilm formation.

Recent work from our group has highlighted the clinical relevance of biofilm heterogeneity between isolates of *C. albicans* from the oral cavity and other clinical sites (Kean et al., 2018a). The overriding message from this review is that with respect to particular groups of microorganisms they cannot all be considered as a single entity and "one size does not fit all" when it comes to treatment regimens. We have shown that a greater capacity to form biofilm is linked to enhanced virulence and increased resistance to antifungals (Sherry et al., 2014). Further studies of this yeast, alongside the emerging yeast pathogen *C. auris*, have demonstrated that biofilm-mediated patterns of resistance also exist for both antiseptics and disinfectants, including sodium hypochlorite (Kean et al., 2018b). Given that sodium hypochlorite (NaOCl) and (EDTA) are principal components of the management of endodontic infections (Zehnder, 2006), we hypothesised that root canal treatment failure involving *C. albicans* may be driven by biofilm

heterogeneity resulting in an enhanced ability to withstand treatment and regrow within root canals. We therefore aimed to assess how different biofilm phenotypes responded *in vitro* to NaOCl and EDTA treatments.

3.6 Material and Methods

3.6.1 Microbial growth conditions and standardisation

C. albicans laboratory strains SC5314 (Fonzi and Irwin, 1993) and 3153A (Slutsky et al., 1985), and 28 clinical isolates obtained from an oral rinse from patients attending restorative clinics at Glasgow Dental Hospital and School for routine dental care, as previously described (Coco et al., 2008). Antifungal susceptibility profiles and proteolytic activity of these strains have been described previously (Ramage et al., 2012a, Ramage et al., 2011). Notably, these studies showed that these isolates (saliva derived) were highly sensitive planktonically to azoles, polyenes and echinocandins, whereas in sessile mode they were resistant to only azoles (Ramage et al., 2011). Moreover, proteolytic assessment of these isolates showed that those of a higher biofilm forming capacity were more proteolytic (Ramage et al., 2012a). The strains were subcultured on Sabouraud's dextrose agar [SAB (Sigma–Aldrich, Dorset, UK)] plates and maintained at 30^0 C for 48 hours. An overnight culture was prepared in yeast peptone dextrose [YPD (Sigma-Aldrich, Dorset, UK)] and incubated at 30^{o}C at 120 rpm in an orbital shaker (IKA KS 4000 i control, Berlin, Germany). After 18h, the yeast cells were pelleted by centrifugation, washed twice with phosphate buffered saline [PBS (Sigma–Aldrich, Dorset, UK)] and counted using a haemocytometer.

3.6.2 Biofilm development and biofilm forming ability characterisation

C. albicans strains were standardised to the desired cellular density of 1×10^6 cells/mL into Roswell Park Memorial Institute (RPMI)-1640 (Sigma–Aldrich, Dorset, UK) medium and the biofilms formed onto pre-sterilised, polystyrene, 96-well flat-bottom microtiter plates (Corning Incorporated, Corning, NY, USA) as previously described (Ramage et al., 2001). The plates were incubated at 37ºC for 24h. After incubation, the biofilms were washed with PBS to remove the loosely attached cells and the biofilm biomass of all strains was quantified using the crystal violet (CV) assay, as previously described (Jose et al., 2010). The biomass was then quantified spectrophotometrically by reading absorbance at 570 nm using a microtiter plate reader (FLUOStar Omega, BMG Labtech, Aylesbury, UK).

The growth profiles of the selected test strains were also assessed using an xCELLigence system (ACEA, Biosciences Inc., San Diego, California, USA). Briefly, this system is a real-time cell analysis instrument that uses patented microtitre plates (E-plates®) that contain gold biosensors embedded in the bottom of each well. Quantifiable data was expressed as cell index, which represents the cell impedance when the cells adhere, or associated matrix, to the electrodes (Junka et al., 2012). These biosensors continuously and non-invasively monitor changes in cell number, cell size, cell-substrate attachment quality, and extrapolymeric substance (EPS) production. *C. albicans* (1×10^6 cells/mL) in RPMI-1640 was seeded into 96-well E-plates (ACEA, Biosciences Inc.) and the plates incubated overnight at 37ºC with readings taken every 30 min.

3.6.3 Planktonic minimum inhibitory concentration (MIC)

MIC was determined using a broth microdilution method according to the M27-A3 standard for fungi (Wayne, 2002) (CLSI). Briefly, cells were adjusted to the desired density of 2×10^4 cells/mL in Roswell Park Memorial Institute (RPMI) media (Sigma-Aldrich, Dorset, UK). A series of two-fold dilutions of 3% (30000 ppm) NaOCl (Parcan; Septodont, Saint-Maur-des-Fosses, France) and 17% (EDTA (ENDO-SOLution, Stalowa Wola, Poland) were performed using 96-well round-bottom microtiter plates (Corning Incorporated, Corning, NY, USA). The plates were incubated at 37^{o}C. After 24h, the MIC concentration was determined as the lowest concentration that prevents visible growth.

3.6.4 Biofilm treatment and regrowth assessment

After biofilm growth, they were washed with PBS and treated with 3% NaOCl, 17% EDTA for 5 min, or sequentially treated with NaOCl followed by EDTA for 5 min each. Untreated controls were used for comparison. The effect of NaOCl was deactivated with 5% sodium thiosulfate for 10 min (Fisher Chemicals, London, UK) and EDTA was deactivated using Dey Engley Neutralising broth (Sigma-Aldrich, London, UK) for 15 min. Sodium thiosulfate and Dey Engley Neutralising broth were also applied to untreated controls to normalise results. The viability of the biofilms was quantified immediately after treatment using XTT metabolic activity assay (Sigma-Aldrich, UK) and CV as described in Chapter 2. The absorbance was measured spectrofluorometrically using a plate reader (FLUOStar Omega, BMG Labtech, Aylesbury, UK) at a wavelength of 490 nm for XTT and 570 nm for CV according to the manufacturer's recommendation. To further explore the effect of this treatment, post-treatment, the microtitre plates were washed with PBS and fresh RPMI-1640 media replenished. The plates were re-incubated at 37^{o}C for 24,

48 and 72h and XTT viability and CV assessment performed, as described above. To validate this approach, we mirrored this experimental procedure using the xCELLigence system. Instead of static endpoint measurements, we were able to monitor biofilm regrowth in real-time. Briefly, following the initial treatment, deactivation and washing steps, the wells were replaced with RPMI and the entire plate sonicated for 5 min to remove retained adherent biofilm cells. The sonicated cells were then transferred into 96-well plastic E-plates, and the plates re-incubated at 37^{o}C for 72h, with cell impedance quantified every 30 min. In parallel, plates were imaged using EVOS FL Cell Imaging system (Thermo Fisher Scientific, Waltham, MA, USA).

3.6.5 Statistical analysis:

Viability and biomass data of *C. albicans* BC023 and BC146 were chosen to represent the low biofilm forming (LBF) and high biofilm forming (HBF) strains respectively. GraphPad Prism (version 7.0d) was used for creating graphs and statistical analysis. Data was tested for normal distribution using D'Agostino-pearson omnibus normality test and were plotted as log where required. Student *t*-test was used to compare LBH and HBF viability. One-way ANOVA with Tukey's multiple comparison test was used to statistically compare biofilm viability after NaOCl treatment for each isolate over time while a Kruskal–Wallis test with Dunn's multiple comparison test was used to compare viability following the three treatment conditions.

3.7 Results

3.7.1 Oral *Candida albicans* differentially form biofilms and are NaOCl sensitive

Initially, biofilm biomass was assessed for the 28 *C. albicans* clinical isolates (Figure 3.1). Isolates showed different biofilm forming abilities with the biofilm biomass for 18 isolates ranging between 0.4- 1.0 mean optical density. The five lowest biofilm forming isolates (LBFs) and the five highest biofilm formers (HBFs) were subsequently selected to test their susceptibility to NaOCl and EDTA treatment. In terms of growth profiles, BC023 (the lowest biofilm forming clinical isolate), BC146 (the highest biofilm forming clinical isolate), 3153A (low biofilm forming laboratory strain) and SC5314 (high biofilm forming laboratory strain) were used as controls. Growth profiles of BC023 and 3153A (Figure 3.2a) were clearly different from those of BC146 and SC5314 (Figure 3.2b). LBFs showed less irregularities before both reach saturation phase when the E-plate electrodes become entirely covered with *Candida* cells. This difference suggests the potential of xCELLigence system to differentiate LBF and HBF isolates based on their growth profiles. NaOCl MIC for all LBFs and HBFs was 0.093% (930 ppm) and 0.13% for EDTA.

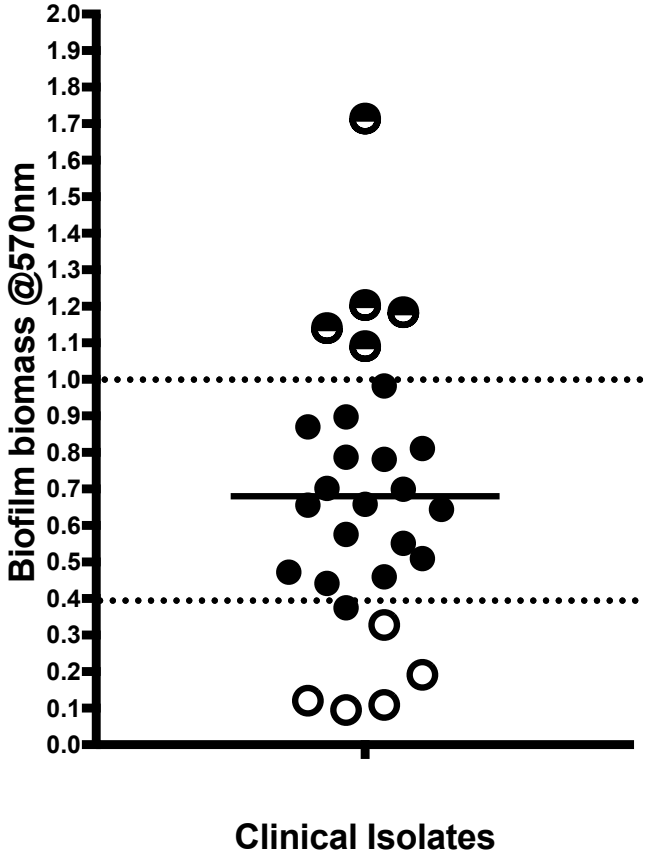


Figure 3.1: Quantification of biofilm biomass from clinical isolates. LBF and HBF identified by horizontal lower and higher dotted lines. Those isolates with an open circle or half dark/white circle represent the five LBF and HBF isolates selected for downstream analysis, respectively.

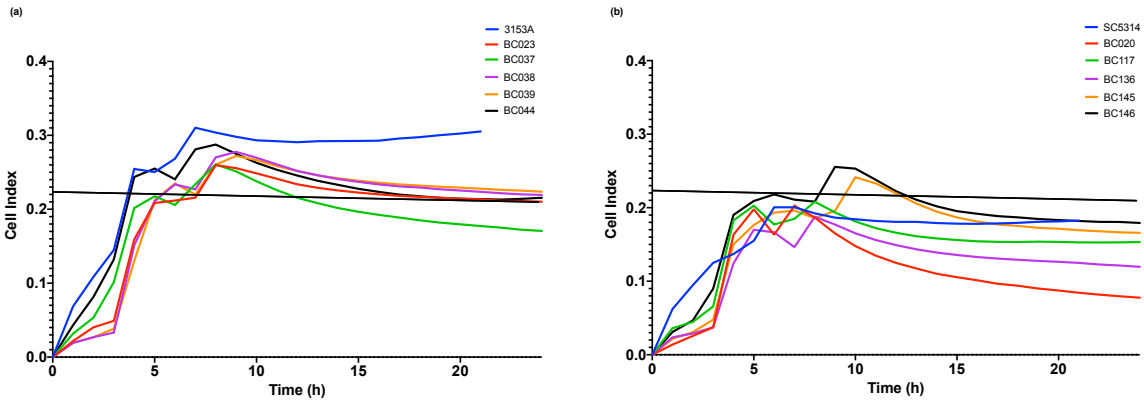


Figure 3.2: Growth profile as shown by xCELLigence system. Data represent the mean of normalized cell index values taken every 30 min over 24h of **(a)** *C. albicans* 3153A and five LBFs **(b)** *C. albicans* SC5314 and five HBFs. Mrs Suror Shaban supported data generation in this figure by helping in operating the xCELLigence system.

3.7.2 Oral *Candida albicans* persists following NaOCl treatment

Following NaOCl application, cell viability expressed in percentage in relation to untreated controls was assessed (Figure 3.3). In spite of the initial killing effect of NaOCl (78.7%, 98.3% reduction in viability for LBF and HBF, respectively), both isolates were able to regrow over time. LBF and HBF showed comparable regrowth profiles. However, LBF revealed higher immediate post-treatment viability compared to HBF ($p<0.05$). In terms of post-treatment regrowth, the viability increased significantly between 0 and 72h for both LBF ($p<0.0001$) and HBF ($P<0.01$) with maximum viability at 48h ($p<0.0001$). No significant difference was observed between viability at 48 and 72h for both isolates. Data from xCELLigence system confirm the above-mentioned results for HBF (Figure 3.4b), but not for LBF (Figure 3.4a).

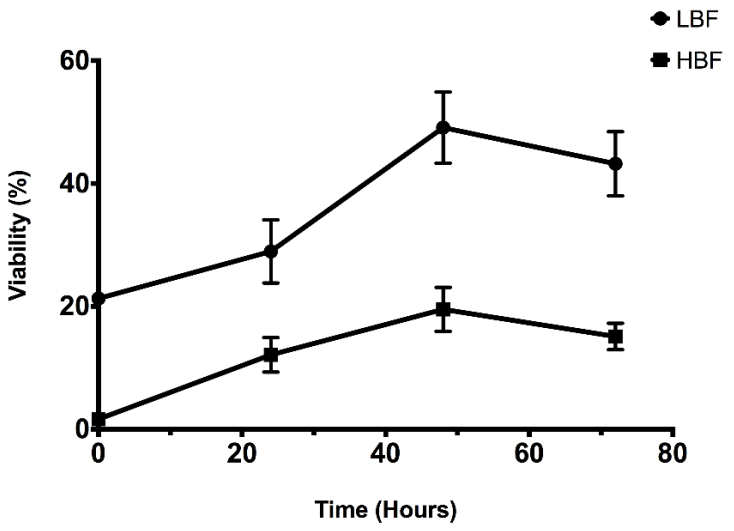


Figure 3.3: Viability percentage of biofilms in relation to untreated biofilms after treatment with NaOCl and reincubation with fresh RPMI media. Immediate post-treatment (0), 24, 48 and 72h for LBF (upper line) and HBF (lower line). Values represent the mean with SEM (standard error of the mean) from three independent experiments.

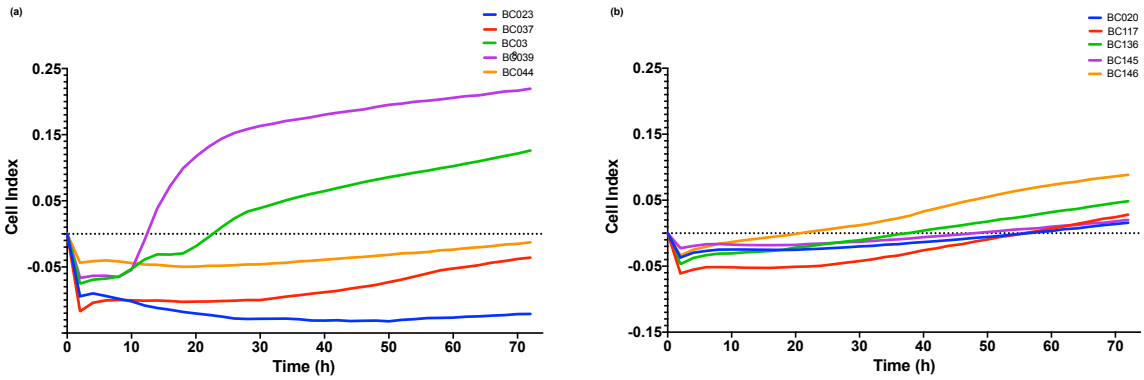


Figure 3.4: Regrowth from 0 to 72h as detected by xCELLigence system and presented as normalized cell index for (a) five LBF (b) five HBF. Mrs Suror Shaban supported data generation in this figure by helping in operating the xCELLigence system.

3.7.3 Oral *Candida albicans* regrowth after NaOCl treatment is significantly inhibited by EDTA.

As shown in the previous data, *C. albicans* persists following NaOCl treatment and has the potential to regrow. In terms of EDTA treatment, the metabolic activity of LBF (Figure 3.5a) and HBF (Figure 3.5b) was initially decreased by 82%, 97%, respectively, when compared to untreated biofilms. Again, LBF showed significantly higher immediate post-treatment tolerance to EDTA than HBF ($p<0.001$). In contrast to NaOCl, the metabolic activity following EDTA treatment showed a continued reduction between 0 and 72h for both isolates. Similar trends were observed for NaOCl + EDTA treatment of LBF, but not for HBF. The metabolic activity is significantly lower in EDTA and NaOCl + EDTA in comparison to NaOCl alone for both isolates at 48 ($p<0.0001$) and 72h ($p<0.05$, $P<0.01$ for LBF and $p<0.0001$ for HBF). Finally, there was not a significant difference between EDTA and NaOCl + EDTA at all time points.

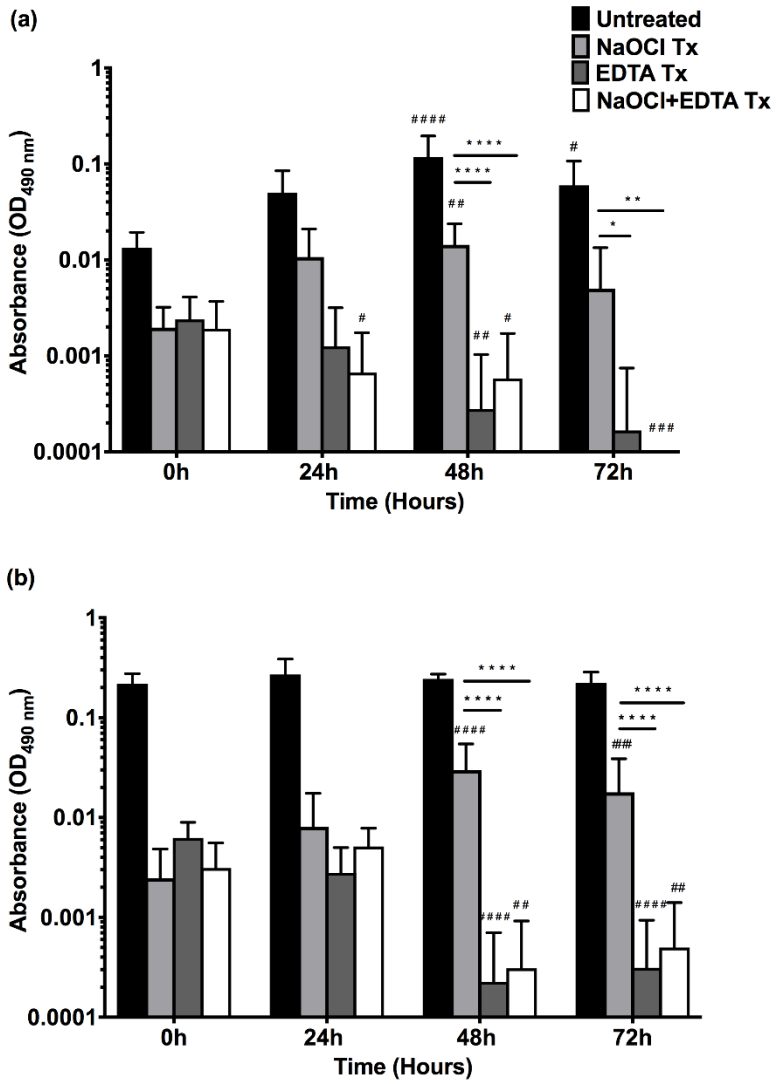


Figure 3.5:The effect of the three treatment conditions NaOCl, EDTA and NaOCl + EDTA on pre-formed 24h biofilms viability in relation to untreated biofilms. Biofilms were treated for 5 min and re-incubated with fresh RPMI. XTT readings were taken at 0, 24, 48 72h. Values were plotted as $\log_{10}$ on the Y axis. **(a)** absorbance values for LBF **(b)** absorbance values for HBF. Statistical significance between the three treatment conditions at 48, and 72h was presented as *p<0.05, **p<0.01 and ****p<0.0001. Whereas the significant difference in relation to untreated biofilms of the same treatment condition over time was presented as $^{\#}$ p<0.05, $^{\#\#}$ p<0.01, $^{\#\#\#}$ p<0.001 and $^{\#\#\#\#}$ p<0.0001.

Increased viability over time with NaOCl treated biofilms was not matched with an increase in biofilm biomass for both isolates. Furthermore, EDTA treated HBF shows significantly higher biomass in comparison to NaOCl and NaOCl + EDTA at all

time points (Figure 3.6b). At 72h, NaOCl + EDTA is not superior in decreasing biofilm biomass compared to NaOCl alone for HBF, but it is more effective in decreasing biomass compared with NaOCl treatment in LBF (P<0.001) (Figure 3.6a).

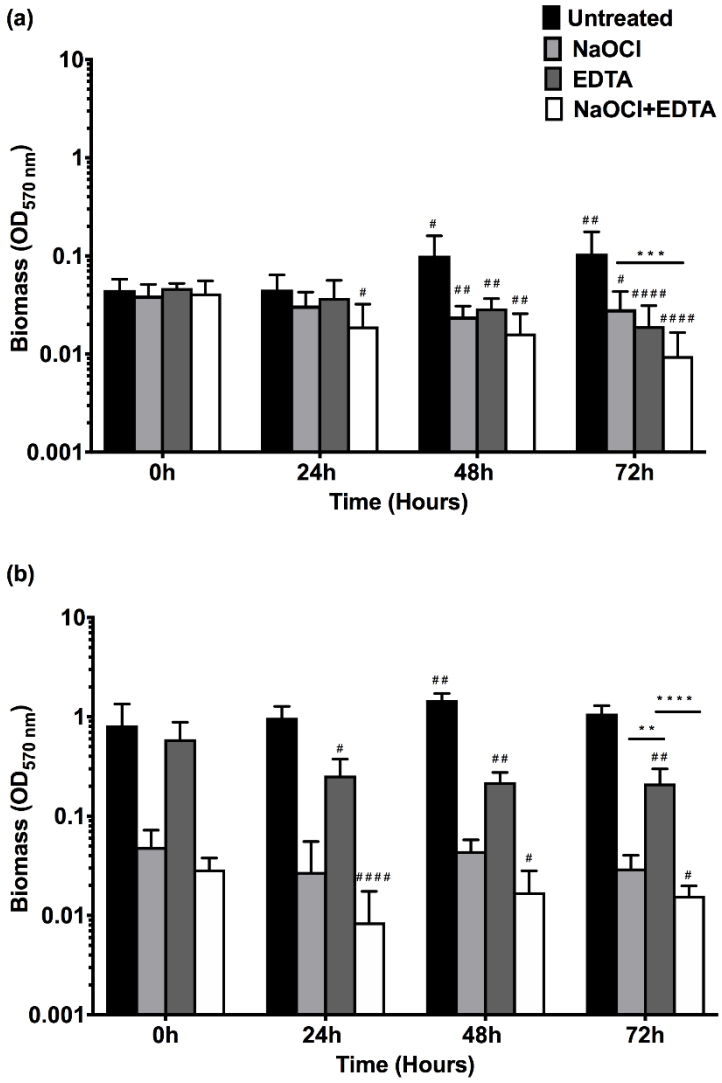
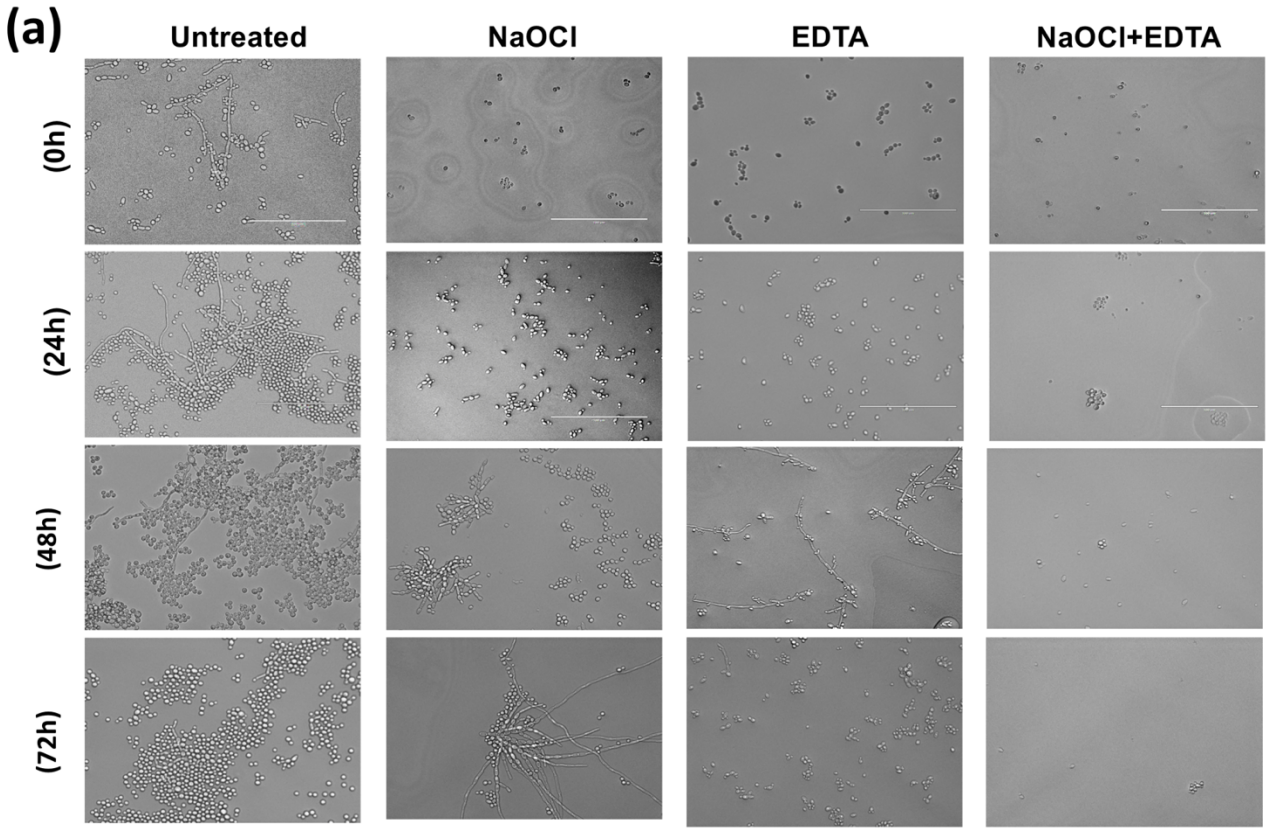


Figure 3.6: The effect of the three treatment conditions NaOCl, EDTA and NaOCl + EDTA on pre-formed 24 h biofilms biomass in relation to untreated biofilms. CV readings were taken at 0, 24, 48 72 h. Values were plotted as $\log_{10}$ on the Y axis. (a) Biofilm biomass for LBF (b) Biofilm biomass for HBF. Statistical significance between the three treatment conditions at 72 h was presented as **p<0.01, ***p<0.001 and ****p<0.0001. Whereas the significant difference in relation to untreated biofilms of the same treatment condition over time was presented as $^{\#}$ p<0.05, $^{\#\#}$ p<0.01 and $^{\#\#\#\#}$ p<0.0001.

Microscopic examination is consistent with these data. There are visibly evident cells for both isolates at 0h in all treatment conditions (Figure 3.7a and b). However, the visible quantity of cells increased significantly over time in NaOCl treated biofilms for both isolates. In EDTA treatment, the number of cells remained relatively stable over time in both isolates, with maintaining biofilm architecture in HBF. Finally, NaOCl + EDTA showed significantly less cells at 24, 48 and 72h compared with NaOCl alone and EDTA alone.


(a)
Untreated
NaOCl
EDTA
NaOCl+EDTA
(0h)
(24h)
(48h)
(72h)

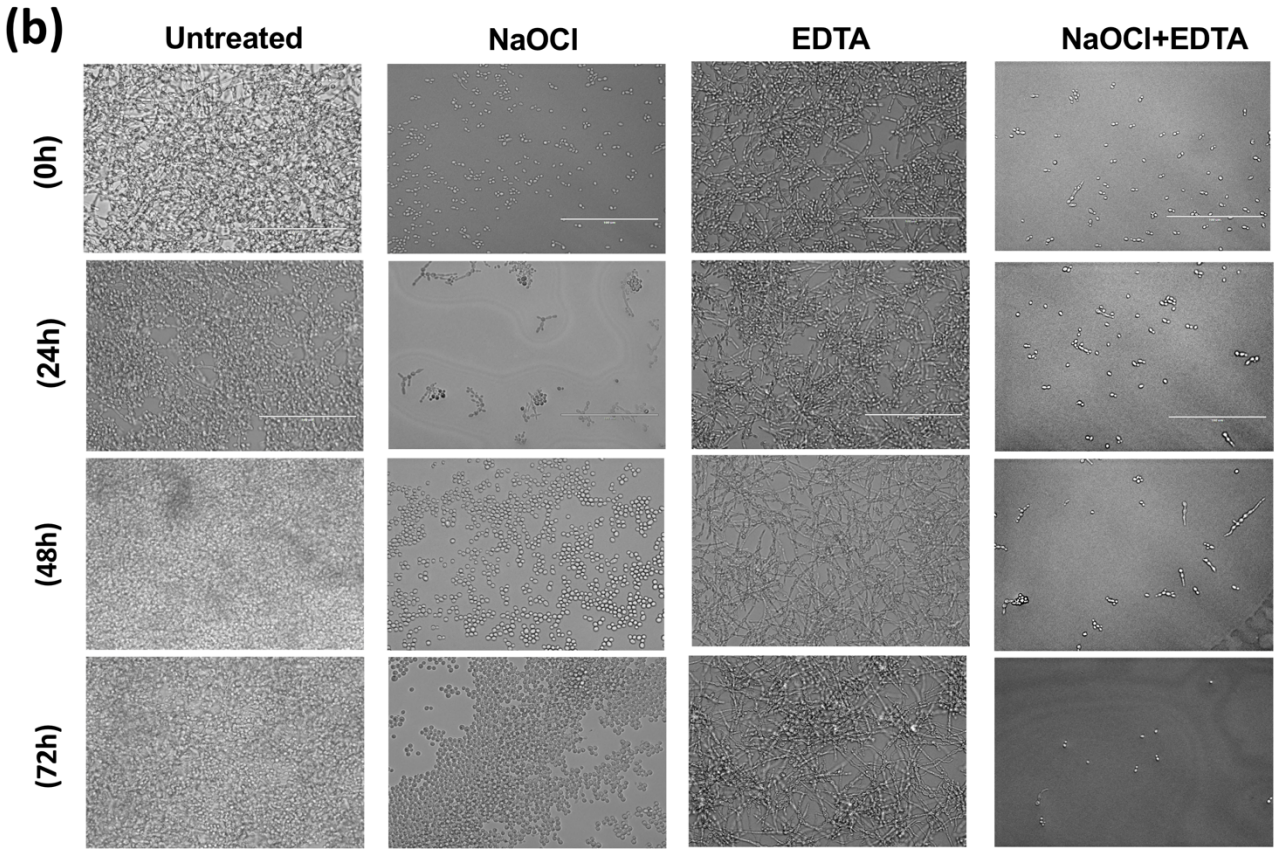


Figure 3.7: Microscopic examination of biofilms at four conditions: untreated, NaOCl, EDTA and NaOCl + EDTA treated biofilms. It demonstrates the biofilms at 4 time points, 0, 24, 48 and 72h. (a) LBF (b) HBF. Bar is 40 μm for all panels.

3.8 Discussion

C. albicans biofilm infections are heterogeneous, and it has been shown that depending on the phenotype of a specific clinical isolate then this can have a profound impact on patient outcomes (Rajendran et al., 2016). Therefore, we sought to test whether these characteristics played a role in a dentally relevant scenario. Root canal treatment is the treatment of choice for endodontic infections. The use of chemical irrigating solutions to disinfect the root canal and to remove the smear layer is vital for success. An ideal irrigant should provide a flushing action, have a broad spectrum antimicrobial activity, be able to inactivate bacterial endotoxins, have the ability to dissolve the necrotic organic tissues, remove the smear layer, have no adverse effect on dentin and be biocompatible with the periradicular tissues (Kandaswamy and Venkateshbabu, 2010). However, no single irrigant possesses all of these characteristics. Here we modelled the *in vitro* ability of standard endodontic irrigants to manage *C. albicans* biofilms, demonstrating a flaw in the capacity of NaOCl and EDTA to effectively eradicate the biofilm.

The present study has demonstrated significant variation between candidal phenotypes, with LBF (mainly yeasts) and HBF (hyphal forms) differing in their susceptibility to NaOCl and EDTA. Although HBF would be expected to show higher resistance to these irrigants attributed to its higher biofilm forming ability, LBF showed greater tolerance in comparison to untreated control than HBF, possibly due to the capacity of budding yeast cells to disperse. Radcliffe et al. (2004) previously examined the resistance of two strains of *C. albicans* to NaOCl of concentrations ranging from 0.5% to 5.25% and found that NaOCl as low as 0.5% lowered the colony forming units (CFU) to below the detection limit for the tested strains. No difference was noted between the response of the tested strains. However, this study examined treatment of planktonic cells rather than biofilm. As highlighted previously, it is becoming clear that the heterogeneity of microbial isolates requires attention when considering treatment strategies; it is no longer appropriate to assume that because a protocol is effective for one clinical isolate that it will be equally effective for another. We are now in the era of personalised antimicrobial therapy, although this may not be directly relevant in endodontic

treatment it does highlight the potential need for fine tuning of irrigation protocols that can take into account differential sensitivities.

In an attempt to enhance the sensitivity of the analysis, we employed an xCELLigence system that relies on electric impedance. Not previously used in either fungal or endodontic studies, the system provided the potential to detect early phases of regrowth. It allowed determination of biofilm growth kinetics in untreated samples, however, was less informative following NaOCl treatment. Due to the degradation of the gold electrodes used in the E-plates, it was necessary to grow and treat biofilms in conventional polystyrene, 96-well flat- bottom microtiter plates then sonicate and transfer the persistent cells to xCELLigence system E-plates. There is a possibility that cells were lost during sonication or transfer which may account for the poorly detectable regrowth in some LBF strains. Nonetheless, the novelty of this real-time biofilm monitoring system enabled us to assess biofilm regrowth in real-time for several of these treated isolates.

NaOCl is the most widely used endodontic irrigant due to its potent and wide spectrum antimicrobial properties and organic tissue dissolving ability although it may be associated with several risks such as tissue toxicity, emphysema, allergy and undesirable taste and smell (Mohammadi, 2008). Various studies investigated the effect of different NaOCl concentrations on *C. albicans* at a variety of clinically relevant treatment time points and concentrations. Previously, it was shown that NaOCl has the highest efficacy against *C. albicans* and NaOCl (0.5% and 5%) completely killed all *C. albicans* cells in 30 sec, with all the tested *C. albicans* strains showing similar susceptibility (Waltimo et al., 1999a). However, our results show that with 3% NaOCl application for 5 min on biofilms, some *C. albicans* cells were still viable and are able to regrow. Waltimo *et al* (1999) tested the NaOCl against planktonic *Candida* cells, whereas 24h biofilm was used in the present study which may explain the different results (Waltimo et al., 1999a). This is particularly true when considering that biofilms are believed to be significantly more tolerant to antimicrobials than planktonic free cells (Kumamoto, 2002, Mah and O'Toole, 2001). Given the nature of endodontic infections that develop over a period of time, and the fact that *C. albicans* has a great affinity for the collagen of the dentine which promotes yeast adhesion (Siqueira et al., 2002), it is more

likely that *C. albicans* will occur in a biofilm rather than planktonic cells in endodontic infections.

Recently, similar findings to our results were reported, where viable candidal cells were retrieved from root canals following 3% treatment and incubation with growth media for 72h (Roy and Bhaumik, 2017). However, they tested the effect of NaOCl on *C. albicans* grown in root canals of previously healthy teeth, therefore, it is more likely that viable cells would be retrieved due to the complexity and topography of root canals and the presence of smear layer which may impede the action of NaOCl. Whereas, in the present study biofilms were grown and treated on an uncomplicated 96-well flat-bottom microtitre plates. It is widely believed that persistence of endodontic infections is mainly due to complexity of root canal anatomy which impedes the disinfection of inaccessible areas (Taha et al., 2010, Weiger et al., 2002). In addition, the buffering action of dentine and the small volume of irrigant within root canals are believed to decrease the killing efficiency of NaOCl (Karale et al., 2016, Siqueira Jr et al., 2000). However, our results challenge this concept. We were able to demonstrate that even though *C. albicans* biofilms were entirely exposed to a large volume of NaOCl in the absence of dentine buffering effect, eradication of cells was unachievable.

EDTA is recommended as a part of endodontic irrigation protocols. Its main action is to remove the smear layer to enhance the penetration of other irrigants due to its chelating action (it chelates calcium ions from inorganic tissues). EDTA was reported, by many studies, to have a high antifungal efficacy at a concentration as low as 0.625mg/L (Sen et al., 2000). They suggest that EDTA antifungal activity is attributed to its ability to decrease the metabolic activity by extracting calcium ions from *C. albicans* cell wall and the medium and its anti-colonisation action by reducing adhesion properties of *C. albicans*. Calcium ions have a crucial role on the morphogenesis and pathogenesis of *C. albicans* by affecting adherence and growth (Ramage et al., 2007). Interestingly, the metabolic activity of EDTA treated HBF was significantly lower while the biofilm biomass of the same biofilms was significantly higher in comparison to untreated biofilms. Furthermore, biofilm architecture of the same biofilms was maintained when viewed microscopically. EDTA inhibits the metabolic activity of *Candida* cells, which may explain the low

XTT readings while maintaining high biomass. Previous research reported that EDTA inhibits biofilm formation of *C. albicans*, but minimally affects pre-formed biofilm (Ramage et al., 2007). It was also reported that growth and biofilm formation can be restored if EDTA is removed and fresh media was added. However, our present findings showed a significant reduction in viability of EDTA treated pre-formed biofilms. Furthermore, there was a continuous reduction in viability over time rather than regrowth. This variation can be explained by the significant difference in EDTA concentration. A clinically relevant EDTA concentration of 17% (equivalent to 581 mM) was used in this study, whereas the highest EDTA concentration used by Ramage and colleagues was 250 mM (Ramage et al., 2007).

Contrary to the results at our baseline time point, Sen and colleagues (2000) showed that EDTA was superior to NaOCl in its antifungal effect (Sen et al., 2000). In our study, there was no difference between the two disinfectants on LBF, but there was a higher antifungal effect observed with NaOCl on HBF. This group also used agar diffusion tests to compare the two, whilst we used biofilms and the XTT reduction assay to assess antifungal activity which may account for the difference in the results (Şen et al., 1997). Our findings highlight the importance of including EDTA in irrigation protocols not only for smear layer removal, but also for its antifungal properties and its ability to inhibit candidal regrowth after treatment with NaOCl. It is important to note that our studies did not explore the effect of irrigant activation, which is an integral part of endodontic disinfection protocols and could potentially, in combination with NaOCl and EDTA, eliminate *C. albicans* and prevent its regrowth. Furthermore, *C. albicans* coexist with multiple species and are known for their ability to form biofilms with and without other bacterial species (mono-microbial and poly-microbial biofilms), such as *Enterococcus faecalis* and *Staphylococcus aureus* (Delaney et al., 2018). As a result, future investigation should explore whether interkingdom interactions of *C. albicans* with bacteria could complicate root canal treatment and provide protection to one another ultimately leading to persistence.

In conclusion, *C. albicans* clinical isolates have variable biofilm forming abilities, which differentially tolerate endodontic irrigants. These isolates were able to persist after 3% NaOCl treatment and regrow to levels that are comparable with

untreated biofilms, with more regrowth potential observed for yeast cell phenotypes. EDTA significantly inhibits the persistence of NaOCl treated biofilms, though not entirely, indicating possibilities for secondary endodontic infections.

3.9 Conclusions

This first of its kind study reports a strain dependent impact on efficacy of endodontic irrigants. The *in vitro* data suggest that existing endodontic irrigant regimens are not 100% efficient, and that biofilm tolerant cells are permitted to repopulate their immediate physical surroundings. The clinical implications for these observations are the potential for a secondary infection driven by initial ineffectual endodontic treatment. Clinicians conducting endodontic therapies should be aware of this microbiological phenomenon, and support the development of alternative augmentative treatment

4 Comparison of three endodontic irrigant regimens against dual-species interkingdom biofilms: considerations for maintaining the status quo

4.1 Preface

In the previous chapters, we showed that *C. albicans* isolates are able to persist and regrow following NaOCl treatment. Sequential irrigation with NaOCl followed by EDTA has significantly inhibited the regrowth of *C. albicans*. 1-hydroxyethane 1,1-diphosphonic acid (HEDP) is a clinically used chelating agent that has been advocated for its continuous chelating effect during root canal treatment. Continuous chelation is advantageous in terms of reducing the irrigation time compared with EDTA and therefore, shortening the treatment time. It is also less erosive to the dentine. In the light of these benefits, we hypothesized that HEDP has the same inhibitory effect as EDTA on *C. albicans* regrowth potential. Therefore, this chapter aims at comparing the effect of three irrigation regimens, NaOCl only, NaOCl with EDTA and NaOCl with HEDP. *E. faecalis*, a commonly isolated bacteria from endodontic infections was also included in the investigation. The response of *C. albicans* and *E. faecalis* to endodontic irrigants were explored in single and dual-species biofilms.

This chapter is presented as a published journal article:

Alshanta, O.A., Alqahtani, S., Shaban, S., Albashaireh, K., McLean, W. and Ramage, G., 2020. Comparison of three endodontic irrigant regimens against dual-species interkingdom biofilms: Considerations for maintaining the status quo. *Antibiotics*, *9*(9), p.634.

4.2 Author declaration and contribution

Conceptualisation: The concept of the presented work in this publication is totally of my conception.

Data Curation: I had the complete responsibility of data curation.

Formal Analysis: I completed all the formal analysis presented in this publication.

Investigation: I conducted 95% of all the laboratory experiments presented in this publication. Mr Saeed Alqahtani, Mrs Suror Shaban and Ms Khawlah Albashaireh contributed by supporting some aspects of the laboratory experiments.

Methodology: I designed all the experiments presented in this publication.

Project Administration: Prof Gordon Ramage and Dr William McLean were responsible for project administration.

Visualisation: I was responsible for all the preparation and presentation of data presented in this publication.

Writing – original draft: I wrote the complete original draft for this publication.

Writing – review and editing: I completed the all the editing requested by the journal. Prof Gordon Ramage and Dr William McLean provided review and feedback for the original draft.

4.3 Materials and methods

This section covers in details the materials and methods used in this chapter.

4.3.1 Microbial growth conditions and standardisation

E. faecalis strains used throughout this thesis are listed in Table 4.1

Table 4.1: *E. faecalis* laboratory strains and clinical isolates.

Name	Reference
ATCC-29212	(Kim et al., 2012)
NCTCC 5957	National Collection of Type Cultures, Public Health Laboratory Service (www.phe-culturecollections.org.uk).
E1	(Sedgley et al., 2004)
E2	(Sedgley et al., 2004)
E3	(Sedgley et al., 2004)
ER5/1	(Johnson et al., 2006)
ER35	GDS culture collection
OS-16	(Sedgley et al., 2004)
V583	(Sahm et al., 1989)
OGX-1	(Sedgley et al., 2004)
J 42-7	GDS culture collection
AA-OR 34	(Sedgley et al., 2006)

E. faecalis laboratory strains and clinical isolates were maintained at -80^{o}C as described for *C. albicans*. Strains were subcultured on Columbia blood agar [CBA (BA + 5% defibrinated horse blood) (Fisher Chemicals, UK)] and plates were incubated at 37^{o} C in 5% CO_2 for 48 hours. Afterwards, plates were kept at 4^{o}C. Overnight cultures were then prepared in 10 mL of brain heart infusion (BHI)

growth medium [Sigma–Aldrich, Dorset, UK] and incubated at 37° C, 5% CO_2. Cells were then pelleted by centrifugation at 3,000 rpm for 5 minutes, washed twice with PBS and standardised using spectrophotometry at OD_{600}. Previously, Miles and Misra colony counting method was used to determine the correct absorbance and the corresponding bacterial cell number (Miles et al., 1938). By employing serial dilution of pure cultures, it was identified that absorbance of 0.3 at OD_{600nm} corresponds to a cellular density of $2x10^8$ cells/mL.

4.3.2 *C. albicans* and *E. faecalis* dual-species biofilm development

Overnight cultures were standardised to the desired density ($1x10^6$ cells/mL for *C. albicans* and $1x10^7$ cells/mL for *E. faecalis*) in 1:1 v/v mixture of RPMI/ Todd Hewitt broth (THB) (Sigma–Aldrich, Dorset, UK) growth media (THB:RPMI). THB media was supplemented with 0.01 mg/mL hemin and 2 µg/mL menadione before being sterilized by autoclaving and subsequently mixed with pre-sterilized RPMI. Biofilms were then formed onto sterile Thermanox™ Coverslips (Thermo Fisher Scientific, Paisley, UK) contained in a polystyrene, 24-well microtiter plates (Corning Incorporated, Corning, NY, USA) and plates incubated at 37° C, 5% CO_2 for the indicated time. After incubation, biofilms were washed and assessed as indicated.

4.3.3 DNA extraction

Following treatment of mono- and dual- species biofilms, samples were sonicated at 35 kHz in an ultrasonic water-bath (Fisher Scientific, Paisley, UK) for 10 minutes to dislodge attached cells and vortexed for 30 seconds before collecting samples in sterile Eppendorf tubes. DNA was extracted from samples using the QIAamp DNA mini kit, as per manufacturer's instructions (Qiagen, Crawley, UK). Biofilm samples

were centrifuged for 10 minutes at 13,000 rpm and supernatant was then discarded. Each cell pellet was suspended in a mixture of 180 µL ATL buffer and 20 µL proteinase K and incubated in a water bath at 56°C for 20 minutes. Afterwards, samples were transferred to a bead beating tube containing 0.5 mm sterile glass beads and 3 cycles for 30 seconds at 400 rpm was applied. Following centrifugation at 7,000 rpm for 10 minutes, supernatant was transferred to a new Eppendorf tube and 200 µL of buffer AL was added. Samples were then vortexed for 15 seconds, incubated at 70°C for 10 minutes and 200 µL of 100% ethanol was then added. Samples were centrifuged for 1 min at 7000 rpm before applying mixture to spin columns followed by centrifugation for 1 minute at 8,000 rpm. The flow-through was discarded and spin column membranes were washed with 500 µL of buffer AW1 followed by 500 µL of AW2 with centrifugation for 3 minutes at 14,000 rpm. The spin column was then transferred into a fresh RNase free microcentrifuge tube, 100 µL AE elution buffer added and the filtrate was re-eluted. In both elution and re-elution steps, spin columns were centrifuged for 1 minute at 8,000 rpm. qPCR was carried out as described in chapter 2 using primers *ITS3* and *ITS4* for *C. albicans* and *DDL* for *E. faecalis*. Serial dilutions of bacterial/fungal DNA extracted from $1x10^8$ cells/mL were also included to create a standard curve for each species against which colony forming equivalents (CFE) were calculated Figure 4.1.

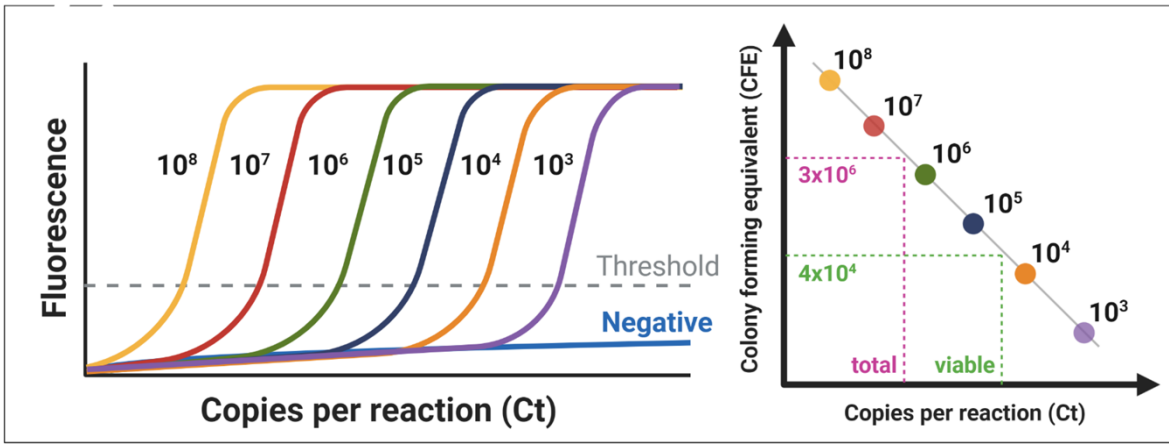


Figure 4.1: Standard curve of *C. albicans* and *E. faecalis* was created using DNA extracted from standardised cellular count. Taken from (Brown et al., 2022).

4.4 Comparison of three endodontic irrigant regimens against dual-species interkingdom biofilms: considerations for maintaining the status quo

Om Alkhir Alshanta, Saeed Alqahtani, Suror Shaban, Khawlah Albashaireh, William McLean and Gordon Ramage*

Glasgow Endodontology Group, Glasgow Dental School, School of Medicine, Dentistry and Nursing, College of Medical, Veterinary and Life Sciences, Glasgow, United Kingdom.

*Correspondence: Corresponding Author: Gordon Ramage, Glasgow Endodontology Group, Glasgow Dental School, School of Medicine, Dentistry and Nursing, College of Medical, Veterinary and Life Sciences, University of Glasgow, 378 Sauchiehall Street, Glasgow, G2 3JZ, UK. Phone: +44(0)141 211 9740. e-mail: gordon.ramage@glasgow.ac.uk

4.5 Abstract:

Background: Endodontic infections are often interkingdom biofilms, though current clinical management rarely considers this phenomenon. This study aimed to evaluate new and standard endodontic antimicrobial regimens against simple and complex *Candida albicans* and *Enterococcus faecalis* mono- and dual-species biofilms. **Methods:** *C. albicans* and *E. faecalis* mono- and dual-species biofilms were grown upon Thermanox™ coverslips and treated for 5 min with 3% NaOCl, 3% NaOCl followed by 17% EDTA, or 9% HEDP dissolved in 3% NaOCl. The number of cells remaining immediately after treatment at 0h and after 72h of regrowth were assessed using real-time quantitative PCR. **Results:** All three treatment arms showed a similar positive antimicrobial effect on *C. albicans* and *E. faecalis* in both mono- and dual-species biofilms following initial treatment, resulting in ≥98% reduction in colony forming equivalent (CFE). Regardless of species or biofilm type (mono- or dual-species), the antimicrobial effect of NaOCl:HEDP mixture was comparable to that of NaOCl alone, with both showing significant regrowth after 72h, whereas sequential treatment with NaOCl and EDTA consistently prevented significant regrowth. **Conclusions:** Our data suggest that sequential irrigation with NaOCl and EDTA remains the antimicrobial strategy of choice as it significantly reduces biofilm persistence and regrowth in our experimental dual-species biofilm conditions.

Keywords: Interkingdom; biofilm; *Candida albicans*; *Enterococcus faecalis*; EDTA; NaOCl; HEDP; persistence

4.6 Introduction

Apical periodontitis arises from micro-organisms mainly organized as microbial biofilm in the intricate anatomy of the root canal system (Kakehashi et al., 1965, Moller et al., 1981). It is now widely accepted that biofilms rarely exist as single species entities. In fact, they exist as complex, diverse and heterogeneous cellular communities of organisms spanning different phylogenetic kingdoms (O'Donnell et al., 2015). Interactions between the contributing organisms can have a negative impact on health through intensification of pathogenicity and increased tolerance to antimicrobial challenge (Kong et al., 2016). *Enterococcus faecalis* has been classically associated with endodontic infections, and whilst occasionally detected in primary infections, it is more frequently associated with post-treatment disease (Rôças et al., 2004b). Microbially less well recognised with post-treatment disease, but arguably as important is the yeast *Candida albicans*. Its prevalence in canals of failing root filled teeth varies from one study to another (Persoon et al., 2017b, Persoon et al., 2017a), though it has been reported to be as high as 36.7% (Ashraf et al., 2007). Interestingly, it is frequently co-isolated from polymicrobial biofilm infections and can interact with a number of different bacteria in a variety of ways (Delaney et al., 2018), particularly driving enhanced antimicrobial tolerance. Given its phenotypic plasticity to change from yeast cells to form long hyphal elements, it creates a physical structure to support and protect bacterial biofilms (Ovchinnikova et al., 2012).

The main goal of endodontic treatment is to eliminate or reduce the microbial biofilm burden to a subcritical level that promotes healing of periapical lesions. It is recognised that mechanical debridement and chemical disinfection should be utilised to achieve this aim. A number of chemical disinfection agents have been explored; however, sodium hypochlorite (NaOCl) remains the most widely used irrigant for root canal disinfection. NaOCl is considered the gold standard disinfectant in endodontic applications due to its proven effectiveness against biofilms and its ability to dissolve organic material (Zehnder, 2006). It is important to recognise that despite employing robust mechanical and chemical regimens, the complete eradication of microorganisms from the root canal system is difficult to achieve (Byström and Sunvqvist, 1985). This is mainly due to the complexity of the root canal system, the ability of microorganisms to invade the dentinal

tubules, and the overriding biofilm lifestyle (Brook, 2003). The complex anatomy of the root canal system has been well described by Vertucci (Vertucci, 2005). The presence of lateral canals, multiple foramina, deltas, isthmus and c-shaped canals are some forms of the complex anatomy that may render root canal disinfection more challenging. Recent work from our group has highlighted notable persistence of microorganisms following the use of standard endodontic irrigants (sequential application of NaOCl and EDTA), which has potential clinical relevance when considering polymicrobial biofilms (Alshanta et al., 2019). Recently 1-hydroxyethane 1,1-diphosphonic acid (HEDP) has been suggested as an alternative chelating agent (Zehnder et al., 2005). HEDP is a mild chelating agent that, unlike others can be used simultaneously with NaOCl. This has led to the development of the concept of 'continuous chelation' (Neelakantan et al., 2012), which is in contrast to the sequential chelation that is required when other chelators such as EDTA are utilised. This single chemotherapeutic strategy has the potential to mitigate any tolerance and persistence observed in conventional approaches. Notably, there have been few studies actively addressing the effectiveness of HEDP, and these have been limited to single *E. faecalis* biofilm studies (Zehnder et al., 2005, Arias-Moliz et al., 2014, Morago et al., 2016). In light of the convenience of HEDP as a potential replacement for EDTA, the aim of the present study was to explore the effect of the use of a continuous or sequential chelator on persistence and regrowth of dual-species *C. albicans* and *E. faecalis* biofilms; biofilms representing a tougher and more meaningful challenge.

4.7 Results

4.7.1 Effect of endodontic irrigants on planktonic cells

Initially, the effect of treatment regimens on planktonic mono-species and dual-species microorganisms was established through planktonic minimum inhibitory concentration (PMIC) tests (Table 4.2). Data indicated that *C. albicans* was most sensitive to all agents tested compared to *E. faecalis*, except HEDP dissolved in NaOCl. Moreover, apart from this treatment, all dual-species inoculums were equivalent to the MICs of *E. faecalis*. Both organisms were most sensitive to NaOCl alone or along with HEDP. These data provided reassurances that the endodontic irrigants have antimicrobial activity for subsequent experiments, except for HEDP alone (PMIC = 4.5%).

Table 4.2: Susceptibility of planktonic *Candida albicans*, *Enterococcus faecalis* and their co-cultures to sodium hypochlorite, EDTA and HEDP. Numbers represent PMIC values.

	C. albicans	*E. faecalis*	Co-culture
NaOCl	0.093%	0.187%	0.187%
EDTA	0.13%	0.265%	0.265%
HEDP:NaOCl	0.093%	0.093%	0.187%
HEDP:saline	2.25%	4.5%	4.5%

4.7.2 Effect of endodontic irrigants on single species biofilms

Next, we tested each endodontic irrigant against *C. albicans* mono-species biofilms, where it was shown that all the three treatments have a comparably high initial killing effect at 0h, resulting in > 99% reduction in CFE compared to untreated controls (Figure 4.2a). The median number of CFE/mL in the control group was 8.7×10^6, reducing to approximately $\leq3.5\times10^4$ CFE/mL for all treatment arms (Table 4.3). After 72h NaOCl+EDTA was significantly the most effective at inhibiting subsequent regrowth ($p<0.05$). Only residual yeast cells can be observed when viewed by Calcofluor staining (Figure 4.3). Conversely, NaOCl alone and the NaOCl:HEDP mixture exhibited a significant regrowth ($p<0.05$), showing a 17- and 23.5-fold increase in CFE/mL at 72h compared to 0h respectively. There was no significant difference between NaOCl alone and NaOCl:HEDP at both these time points ($p>0.05$).

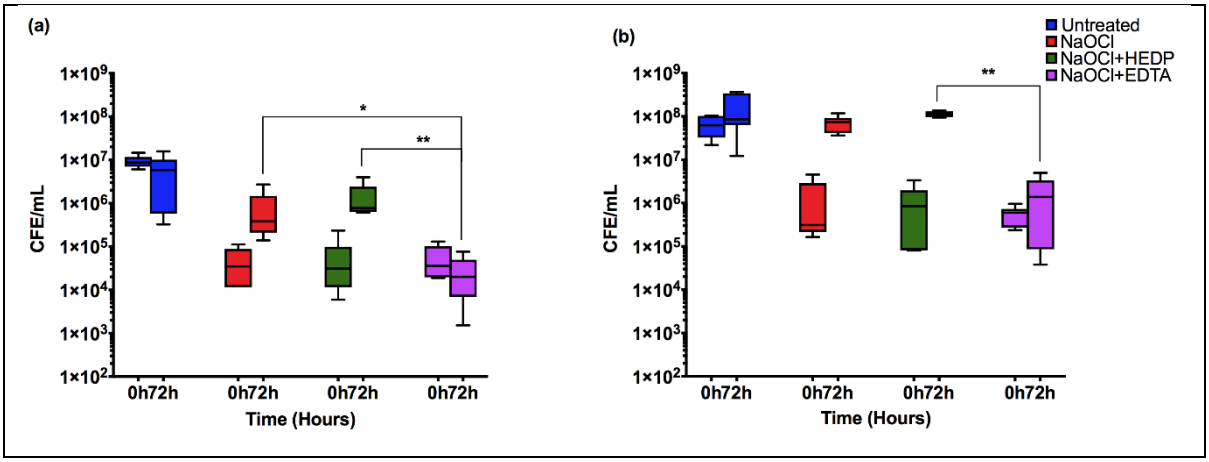


Figure 4.2: The effect of the three endodontic irrigants on pre-formed 24h mono-species biofilms. NaOCl alone, NaOCl:HEDP and NaOCl+EDTA were used to treat **(a)** *C. albicans* and **(b)** *E. faecalis*. CFE at 0h and 72h are presented as box and whisker plot of each treatment on $\log_{10}$ Y axis. Statistical significance between the three treatment conditions at 72h was presented as * $p < 0.05$, ** $p < 0.01$. Results represent data from three independent experiments.

For *E. faecalis*, the immediate killing effect of the three treatments at 0h was comparable to that of *C. albicans*, resulting in ≥98% reduction in CFE compared to untreated controls (Figure 4.2b). The median number of CFE/mL was reduced from 6.22×10^7 in the control group to $\leq8.5\times10^5$ for all treatments. There was no statistically significant difference between the three treatments at 0h (Table 4.3). However, at 72h, *E. faecalis* was more resilient and showed a greater ability to regrow with the NaOCl and NaOCl:HEDP treatments compared to the effect on *C. albicans*. There was approximately a 54- and 66-fold increased cell number at 72h compared to 0h with NaOCl alone and NaOCl:HEDP treatments, respectively. Again, NaOCl+EDTA was the most effective in inhibiting regrowth at 72h, with only a 3-fold increase in CFE/mL ($p < 0.01$) (Figure 4.2b). Small aggregates of cells can be observed retained on the surface of coverslips when viewed by fluorescence microscopy (Figure 4.3).

Table 4.3: The median number and range of CFE/mL for untreated controls, NaOCl, NaOCl:HEDP and NaOCl+EDTA treatment at 0h and 72h.

Biofilm type	Time point	Treatment	Median CFE/mL	Range (minimum to maximum)
C. albicans **mono-species**	0h	Untreated control	8.70×10^6	(6.05×10^6 to 1.46×10^7)
		NaOCl	3.47×10^4	(1.16×10^4 to 1.13×10^5)
		NaOCl:HEDP	3.10×10^4	(5.97×10^3 to 2.34×10^5)
		NaOCl+EDTA	3.57×10^4	(1.89×10^4 to 1.32×10^5)
	72h	Untreated control	5.83×10^6	(3.26×10^5 to 1.58×10^7)
		NaOCl	3.82×10^5	(1.40×10^5 to 2.71×10^6)
		NaOCl:HEDP	7.76×10^5	(6.07×10^5 to 3.99×10^6)
		NaOCl+EDTA	2.01×10^4	(1.53×10^3 to 7.65×10^4)
E. faecalis **mono-species**	0h	Untreated control	6.22×10^7	(2.19×10^7 to 1.04×10^8)
		NaOCl	3.11×10^5	(1.64×10^5 to 4.53×10^6)
		NaOCl:HEDP	8.51×10^5	(8.01×10^4 to 3.36×10^6)
		NaOCl+EDTA	5.97×10^5	(2.37×10^5 to 9.57×10^5)
	72h	Untreated control	8.61×10^7	(1.22×10^7 to 3.64×10^8)
		NaOCl	7.41×10^7	(3.62×10^7 to 1.18×10^8)
		NaOCl:HEDP	1.10×10^8	(9.44×10^7 to 1.36×10^8)
		NaOCl+EDTA	1.38×10^6	(3.81×10^4 to 4.989×10^6)
C. albicans **dual-species**	0h	Untreated control	9.24×10^5	(5.472×10^5 to 1.60×10^6)
		NaOCl	1.01×10^4	(2.92×10^3 to 5.77×10^4)
		NaOCl:HEDP	1.14×10^4	(4.724×10^3 to 2.23×10^4)
		NaOCl+EDTA	5.70×10^3	(1.60×10^3 to 5.54×10^4)

	72h	Untreated control	2.31×10^6	(1.37×10^6 to 5.01×10^6)
		NaOCl	3.52×10^4	(1.62×10^4 to 1.01×10^5)
		NaOCl:HEDP	1.10×10^5	(5.59×10^3 to 4.49×10^5)
		NaOCl+EDTA	7.85×10^3	(4.34×10^3 to 4.50×10^4)
***E. faecalis* dual-species**	0h	Untreated control	5.54×10^7	(2.935×10^7 to 6.61×10^7)
		NaOCl	6.22×10^5	(2.081×10^5 to 4.81×10^6)
		NaOCl:HEDP	5.10×10^5	(1.029×10^5 to 1.89×10^6)
		NaOCl+EDTA	2.76×10^5	(1.05×10^5 to 8.70×10^5)
	72h	Untreated control	1.46×10^8	(1.03×10^8 to 2.39×10^8)
		NaOCl	7.24×10^7	(6.44×10^7 to 8.06×10^7)
		NaOCl:HEDP	9.76×10^7	(6.21×10^7 to 2.02×10^8)
		NaOCl+EDTA	1.39×10^6	(3.73×10^5 to 1.89×10^6)

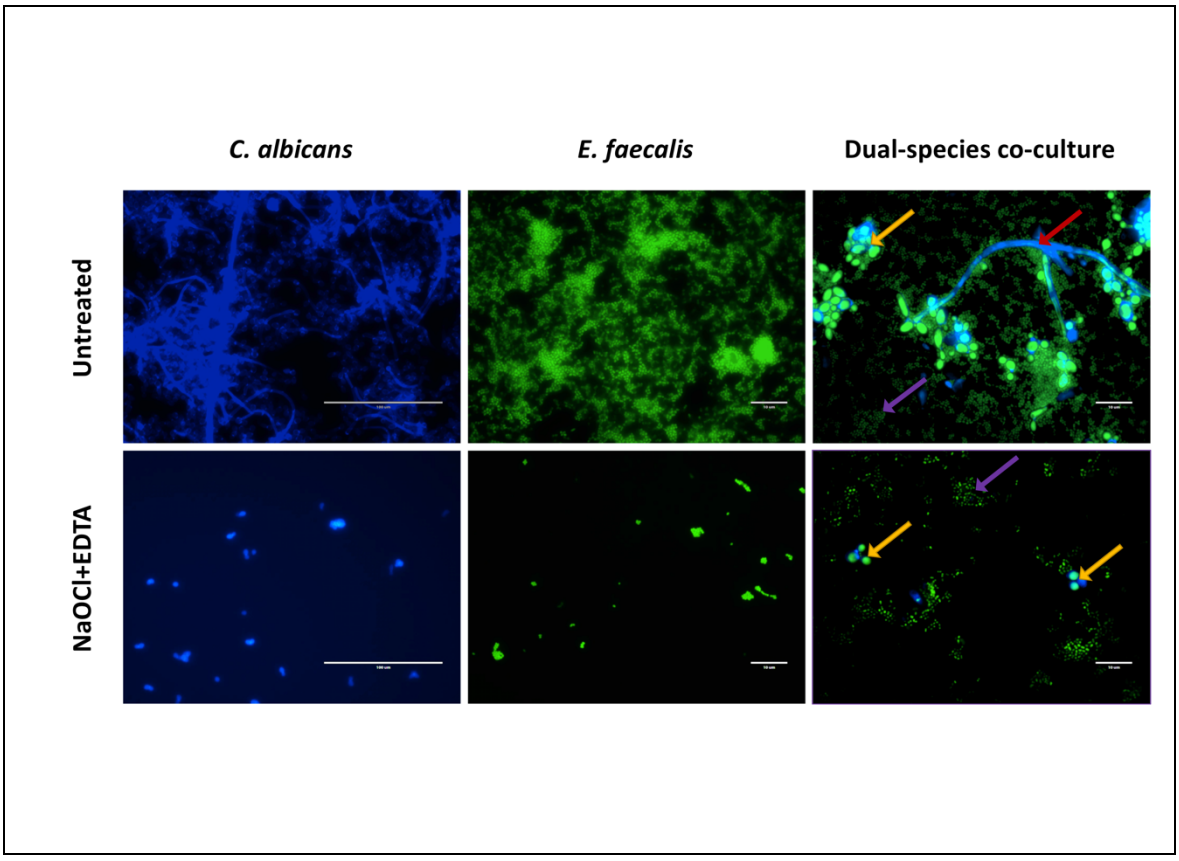


Figure 4.3:Fluorescence microscopic examination of *C. albicans* mono-species, *E. faecalis* mono-species and dual-species biofilms. Upper panel represents untreated biofilms at 72h. Lower panel represents regrowth of biofilms treated with NaOCl+ EDTA at 72h. *C. albicans* in mono-species and dual-species biofilms was stained blue with calcofluor white while the green fluorescence SYTO 9 stained both *C. albicans* and *E. faecalis* cells. Yellow arrows show *C. albicans* yeast cells, red arrow shows *C. albicans* hyphae and purple arrows show *E. faecalis*. Scale bars are 100 µm in *C. albicans* mono-species and 10 µm in *E. faecalis* mono-species and dual-species co-culture.

4.7.3 Effect of endodontic irrigants on dual-species biofilms

Next, dual-species biofilms were treated and assessed as described above for mono-species biofilms, and *C. albicans* quantified (Figure 4.4). The effect of the three treatments at 0h (immediately after treatment) was similar to that of mono-species biofilms, with all treatments significantly reduced CFE by ≥98.4% compared to controls (9.24 x 10^5 CFE/mL, $p<0.05$). NaOCl+EDTA was the most effective regimen overall, but also notably with no significant regrowth detected after 72h (7.8x10^3 CFE/mL) compared to the initial treatment (5.7x10^3 CFE/mL). Conversely, despite their initial effectiveness both NaOCl alone and NaOCl:HEDP were associated with increased residual biofilm by 3- and 12-fold between initial treatment at 0h compared to 72h post-treatment, respectively. Notably, the residual *C. albicans* cells were significantly lower in NaOCl+EDTA at 72h compared to NaOCl:HEDP treatment ($p<0.05$, Figure 4.4).

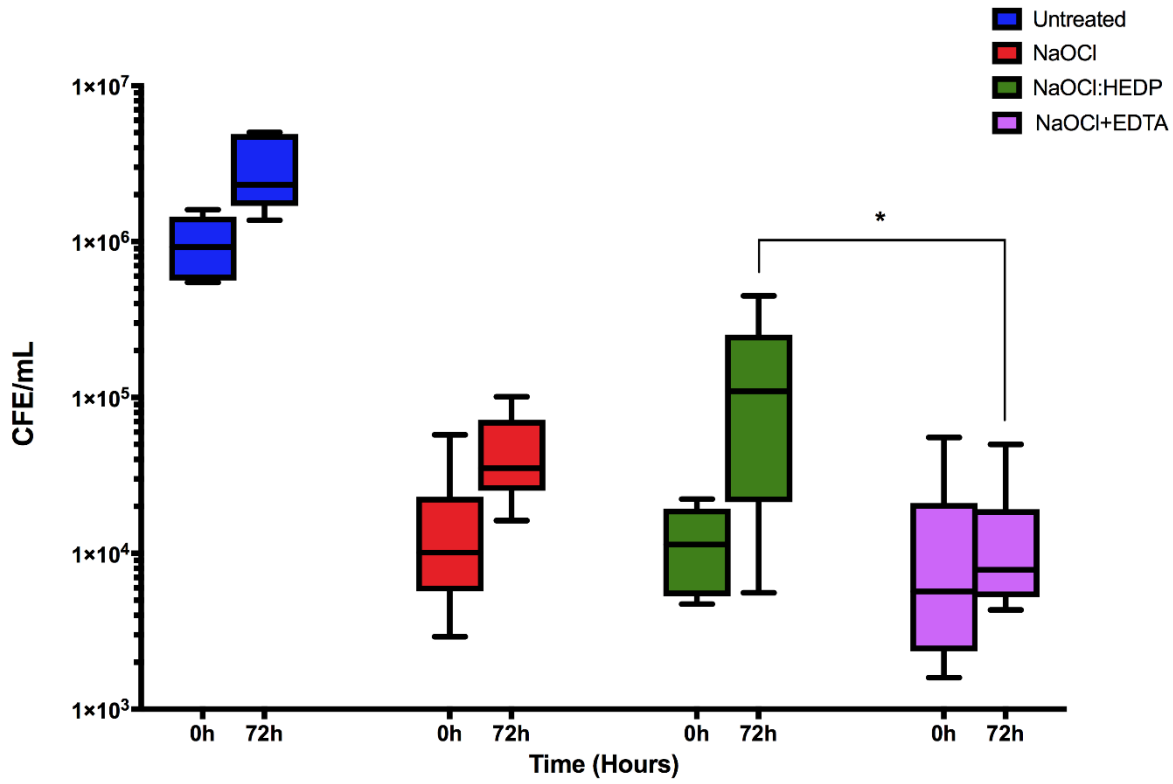


Figure 4.4: The effect of the three endodontic irrigants on *C. albicans* in pre-formed 24h dual-species biofilm. NaOCl alone, NaOCl:HEDP and NaOCl+EDTA were used to treat mixed species biofilms and *C. albicans* quantified. Box and whisker plot of CFE at 0h and 72h for each treatment. Values were plotted as $\log_{10}$ on the Y axis. Statistical significance between the three treatment conditions at 72h was presented as * $p < 0.05$. Results represent data from three independent experiments.

Finally, dual-species biofilms were treated and assessed as described above for mono-species biofilms, and *E. faecalis* quantified. In dual-species, *E. faecalis* was significantly reduced immediately after all three treatments by ≥97.4% ($p<0.01$), though neither treatment was more effective than one another (Figure 4.5). However, both NaOCl and NaOCl:HEDP treatments showed significant levels of regrowth after 72h ($p<0.01$), returning to baseline control levels of up to and greater than $7x10^7$ CFE/mL (55- and 137-fold increase, respectively) (Table 4.3). Conversely, for the NaOCl+EDTA treatment after 72h only a 5-fold increase was observed, which was significantly reduced compared to NaOCl ($p>0.05$) and NaOCl:HEDP ($p<0.01$). This is visually evident when microscopic examination is performed (Figure 4.3). Here we show that compared to the untreated dual-species biofilm where coccus, yeasts and hyphae are observed, that when treated and observed at 72h then very low levels of representative morphotypes are evident.

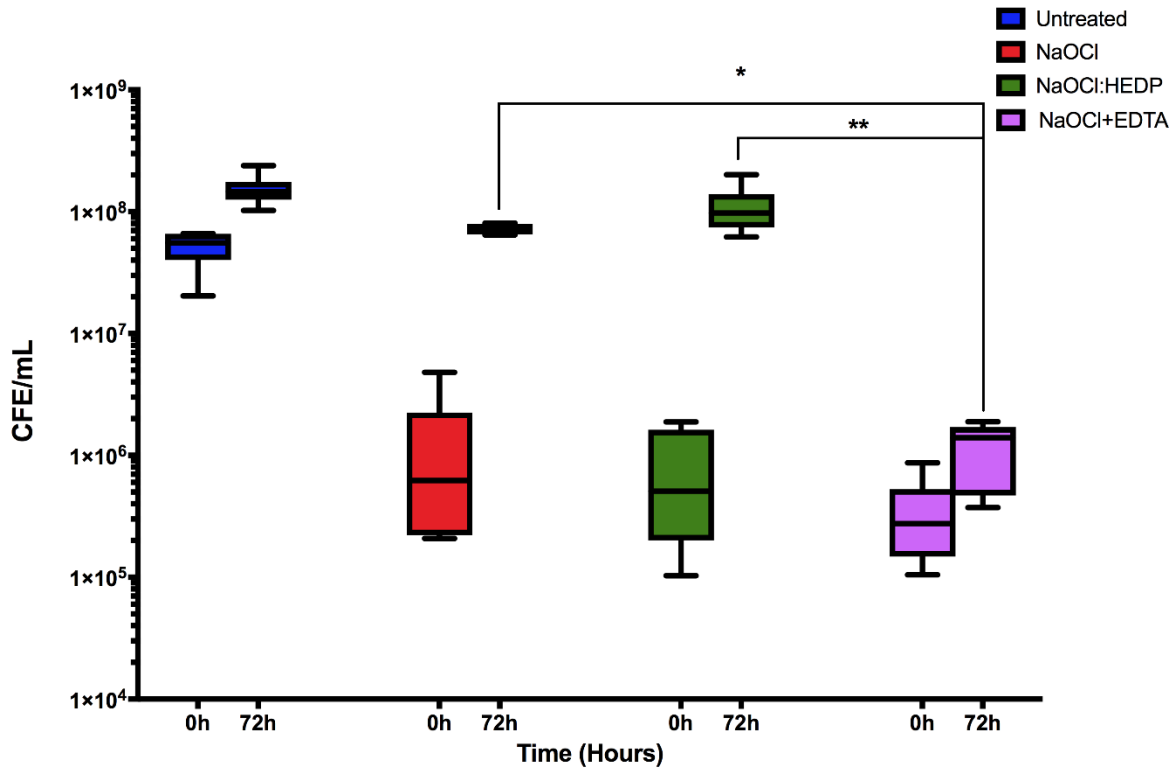


Figure 4.5: The effect of the three endodontic irrigants on *E. faecalis* in pre-formed 24h dual-species biofilm. NaOCl alone, NaOCl:HEDP and NaOCl+EDTA were used to treat mixed species biofilms and *E. faecalis* quantified. Box and whisker plot of CFE at 0h and 72h for each treatment. Values were plotted as log10 on the Y axis. Statistical significance between the three treatment conditions at 72h was presented as * $p < 0.05$, ** $p < 0.01$. Results represent data from three independent experiments.

4.8 Discussion

In the present study we tested the anti-biofilm properties of HEDP, which in endodontics is used as a mild chelating agent to remove the smear layer. In contrast to other chelating agents such as EDTA, it can be mixed with NaOCl to simplify chemical cleaning. It can be also dissolved in normal saline and used separately to remove the smear layer. Its effectiveness in smear layer removal and its effect on radicular dentine has been previously evaluated and shown to be as effective as NaOCl followed by 17% EDTA, but resulted in less dentine erosion and is less toxic (Lottanti et al., 2009). However, augmentative antimicrobial properties of chelating agents would also be highly desirable in root canal disinfection. Here, its antimicrobial properties were compared with standard endodontic irrigants such as NaOCl alone, and more importantly those of NaOCl followed by EDTA. This latter sequential chelation system is considered the gold standard in irrigation protocols as it allows for not only biofilm disruption but also smear layer removal. Results of the present study show that HEDP has no significant impact on the antimicrobial activity of NaOCl, either positively or negatively, within a simple mono-species and more complex dual-species challenge. Indeed, our studies have indicated that treatment with NaOCl followed by EDTA is consistently more effective, though caveats exist with respect to resilient biofilm populations.

Our direct post-treatment data (0h) for both mono- and dual-species *E. faecalis* biofilms did not show a significant difference between either of the treatment arms. In contrast, when considering regrowth after treatment over time, NaOCl+EDTA was significantly more effective in inhibiting regrowth. HEDP alone (dissolved in normal saline) has no antimicrobial effect under the experimental conditions of the present study. Contrary to this, comparison of the antimicrobial effect of combined NaOCl and dual rinse HEDP to a standard NaOCl+EDTA irrigating sequence against *E. faecalis* grown inside root canals from extracted human teeth showed that a NaOCl:HEDP mixture resulted in a significantly lower residual bacterial viability (Giardino et al., 2019). However, Zehnder et al (Zehnder et al., 2005), found that HEDP did not affect *E. faecalis* viability and did not impact the

antibacterial activity of NaOCl, findings echoed elsewhere (Arias-Moliz et al., 2014, Morago et al., 2016). These experimental conclusions are likely explained by differences in the *in vitro* substrates used for these studies, so some caution must be exercised in making bold clinical judgements based on these findings.

Irrespective of treatment regimen tested, there were still remaining populations of *C. albicans* and *E. faecalis* following initial treatment which were able to persist and regrow. The antifungal effect of the traditional chelator EDTA is well-documented in the literature (Ates et al., 2005, Sen et al., 2000), and as discussed in our previous work EDTA significantly reduced *C. albicans* regrowth (Alshanta et al., 2019). Nevertheless, it has limited or no antibacterial activity on *E. faecalis* at any time or concentration (Arias-Moliz et al., 2009, Arias-Moliz et al., 2008). Therefore, it may be somewhat unexpected to see that EDTA significantly inhibited *E. faecalis* regrowth after NaOCl treatment. It is possible that this observed effect of EDTA is related to its membrane permeabilising effect. Lipopolysaccharide molecules have a strong affinity for divalent cations which are required for the thermodynamic stability of the cell membrane (Hancock, 1984). EDTA disrupts the outer membrane of gram-negative bacteria by removing divalent cations (Leive, 1968). This membrane destabilising effect may be potentiated by the prior treatment of the bacterial cells with NaOCl. HEDP in our experiments did not show an effect which may be a result of its weaker chelating activity.

Perhaps the most well studied mechanism of Candida-bacteria interactions is physical attachment. A scaffold of hyphae within a biofilm provides a potential niche for the colonisation of various Gram-positive and Gram-negative bacteria, a phenomenon that has been termed as a 'mycofilm' (Kean et al., 2017). Increasing evidence suggests that *E. faecalis* and *C. albicans* are usually found co-isolated in persistent endodontic infections (Pinheiro et al., 2003). Understanding the inter-kingdom interaction between bacteria and fungi in endodontic infections might help in developing more effective treatment strategies. We have previously demonstrated that *C. albicans* is resilient with high regrowth potential following NaOCl treatment (Alshanta et al., 2019). These findings were confirmed in the present study for mono-species biofilms. However, when co-cultured with *E. faecalis*, *C. albicans* became more susceptible to treatment with decreased ability

for regrowth in comparison to their mono-species biofilms. This can be explained due to the interaction between the two species. *E. faecalis* has been shown to inhibit hyphal morphogenesis and biofilm formation in *C. albicans* (Cruz et al., 2013). Cruz and colleagues also showed that *C. albicans* and *E. faecalis* attenuate each other's virulence in a *Caenorhabditis elegans* model. This might also explain the low fungal prevalence in endodontic infections, although this effect may be also modified by the conditions of the environment in which this interaction occurred. *C. albicans* virulence attenuation was largely due to the inhibition of hyphal morphogenesis, a major virulence factor in *C. albicans*. However, we did not find that *C. albicans* affected *E. faecalis* response to irrigants in dual-species biofilms. Ishijima et al. (2014) also showed that heat killed *E. faecalis* EF2001 (commercially available probiotic) administered orally has a protective effect against oral candidiasis of mice tongue. Although the effect of *C. albicans* on *E. faecalis* is partially revealed, it is still unknown how *C. albicans* affects *E. faecalis* virulence and response to treatment. Future work should explore the molecular basis for the bidirectional modulation of *E. faecalis* and *C. albicans* behaviour and how that would affect these microorganisms' responses to endodontic treatment.

As outlined previously, the yeast *C. albicans* and bacterium *E. faecalis* have been frequently isolated from secondary endodontic infection. Historically, both have been used as model endodontic pathogens to test various endodontic antimicrobials. It is clear from the findings of the present study that the use of mono-species models is no longer appropriate. Any model system must recognise the potential for inter-species/inter-kingdom interactions. The impact of *E. faecalis* on *C. albicans* ability to persist and regrow is both interesting and important, as these have a significant impact on how we may view the effectiveness of tested irrigants or medicaments. As such, further studies are required to explore and validate model systems of endodontic disease. This study also highlights the importance of persistence and regrowth in endodontic disease, albeit in a limited number of clinical strains. The present model represents an open system with no anatomical irregularities or complex topography for biofilm to persist, yet we still demonstrate persistence. This is a significant problem and further work is required to optimise irrigation strategies and other adjuncts to achieve adequate disinfection.

These results indicate the need for additional work to elucidate the potential clinical implications should a continuous chelation strategy be adopted. *C. albicans* is significantly more susceptible to NaOCl and NaOCl:HEDP treatment when co-cultured with *E. faecalis*, suggesting some level of interkingdom antagonism. Future work should investigate the effect of different endodontic antimicrobials against multi-species biofilms on dentine substrate to allow for wider interspecies interactions to be assessed.

4.9 Materials and Methods

4.9.1 Microbial Growth Conditions and Standardisation

A clinical isolate of *C. albicans* from an oral rinse of a patient attending the restorative clinic at Glasgow Dental Hospital and School for routine dental care was used (Coco et al., 2008). This isolate has been previously characterised in terms of antifungal susceptibility, proteolytic activity and biofilm formation ability (Ramage et al., 2012a, Ramage et al., 2011, Alshanta et al., 2019). The strain was maintained on Sabouraud's dextrose agar [SAB (Sigma–Aldrich, Dorset, UK)] plates at 30^{o}C for 48h. An *E. faecalis* root canal clinical isolate (ER5/1) was used (Johnson et al., 2006). The isolate was cultured on 5% horse blood Columbia agar plates and incubated in 5% CO_2 at 37^{o}C for 24h. An overnight culture was then prepared in yeast peptone dextrose [(YPD) (Sigma-Aldrich, Dorset, UK)] for *C. albicans* and in Brain Heart Infusion [(BHI) Sigma–Aldrich, Dorset, UK] for *E. faecalis*. *C. albicans* culture was incubated at 30^{o}C at 120 rpm in an orbital shaker (IKA KS 4000 i control, Berlin, Germany) for 18h while *E. faecalis* culture was incubated in 5% CO_2 at 37^{o}C for 18h. Cells in cultures were then pelleted by centrifugation for 5 min at 3000 rpm and washed twice with PBS. Finally, yeast cells were counted using a haemocytometer and bacterial cells were standardised to OD_{600} of 0.3 which was equivalent to 2×10^{8} cells/mL using a colorimeter.

4.9.2 Planktonic Minimum Inhibitory Concentration

First, to ensure the active agents were effective against planktonic (free-floating cells), we determined the PMIC for mono-species and dual-species microorganisms. 3% NaOCl (Parcan, Septodont, Saint-Maur-des-Fosses, France), 17% EDTA (Endo-Solution, CERKAMED, Stalowa Wola, Poland) and 9% HEDP [(Dual

Rinse HEDP® Medcem GmbH, Weinfelden, Switzerland) dissolved in either 3% NaOCl or normal saline as per manufacturer's instructions] were tested using a broth microdilution method according to the M27-A3 standard for fungi (CLSI, 2008) and the M07-A10 standard for *E. faecalis* (CLSI, 2015). Briefly, *C. albicans* was adjusted to a cellular density of 2×10^4 cells/mL into 1:1 *v/v* mixture of Roswell Park Memorial Institute (RPMI)-1640 (Sigma–Aldrich, Dorset, UK)/ Todd Hewitt broth (THB) (Sigma–Aldrich, Dorset, UK) growth media. *E. faecalis* cells were adjusted to a density of 2×10^5 cells/mL. Serial double-fold dilutions of each treatment were performed in 96-well round-bottom microtiter plates (Corning Incorporated, Corning, NY, USA) using 1:1 *v/v* RPMI/THB media. Treatment concentrations ranged from 1.5%-0.0029% for NaOCl, 8.5%-0.066% for EDTA and 4.5%-0.035% for HEDP dissolved in 3% NaOCl or normal saline. After serial dilution of treatments, 100 µl of standardised microorganisms were added to 100 µl of each concentration and plates were incubated in 5% CO_2 at 37ºC for 24h. All PMIC tests contained 4 technical repeats and were performed on 3 different occasions. Finally, the PMIC concentration was determined as the lowest concentration of each treatment that prevents visible growth or colony formation at the bottom of the plate wells.

4.9.3 Biofilm Development, Treatment and Regrowth Assessment

Next, treatments were tested against *C. albicans* and *E. faecalis* mono-species biofilms (surface attached cells). A cellular density of (1×10^6 cells/mL) for *C. albicans*, (1×10^7 cells/mL) for *E. faecalis* was prepared in 1:1 *v/v* RPMI / THB growth media. 24h biofilms of mono-species and dual-species were then formed onto sterile Thermanox™ Coverslips (Thermo Fisher Scientific, Paisley, UK) contained in polystyrene, 24-well microtiter plates (Corning Incorporated, Corning, NY, USA) and incubated in 5% CO_2 at 37°C. Afterwards, unattached cells were removed by washing with PBS and biofilms treated for 5 min by adding 500 µl of either 3% NaOCl only, 3% NaOCl followed by 17% EDTA (5 min each) or 9% HEDP dissolved in 3% NaOCl. Untreated controls of each biofilm were also included. After the 5 min treatment, 500 µl of 5% sodium thiosulfate (Fisher Chemicals, London, UK) was used for 10 min at room temperature to inactivate NaOCl and NaOCl:HEDP mixture, while 500 µl of Dey Engley Neutralising broth (Sigma-Aldrich, London, UK) was used for 15 min at 37°C to inactivate EDTA.

Treatments and neutralizers were removed, and coverslips were washed with PBS. Neutralizers were also added to untreated controls to ensure that the observed effect is solely due to applied treatments and not neutralizers. To further assess the effect of treatments on biofilms' ability to persist and regrow, 500 µl of a fresh 1:1 *v*/*v* RPMI / THB growth media was added to the treated and washed biofilms and plates re-incubated for a further 72h in 5% CO_2 at 37°C. As an outcome measure, the number of cells remaining immediately after treatment (0h) and those regrowing after 72h of re-incubation with growth media (72h) was assessed using real-time quantitative polymerisation chain reaction (qPCR). All treatment experiments were performed in duplicate and repeated at 3 different occasions.

4.9.4 Quantitative Analysis using Real-time quantitative PCR

Thermanox™ coverslips were first washed with PBS and sonicated in 1 mL of PBS at 35 kHz in an ultrasonic water-bath (Fisher Scientific, Paisley, UK) for 10 min to dislodge cells. DNA was extracted from sonicated samples using the QIAamp DNA mini kit, as per manufacturer's instructions (Qiagen, Crawley, UK). PCR was carried out as previously described (O'Donnell et al., 2016), using the Step-One plus real time PCR machine and StepOne software V2.3 (Life Technologies, Paisley, UK). Briefly, *C. albicans*/E. *faecalis* DNA was added to a PCR mastermix containing Fast SYBR® Green (Thermo Fisher Scientific, Paisley, UK) and specific forward and reverse primers (Table 4.4) within irradiated RNase-free water. Serial dilutions of bacterial/fungal DNA extracted from 1×10^8 cells/mL were also included to create a standard curve for each species. The applied thermal cycles were as follow: 50°C for 2 min, 95°C for 2 min, 40 cycles of 95°C for 3 s and 60°C for 30 seconds. Finally, colony forming equivalents (CFE) was calculated in relation to each species standard curve.

Table 4.4: *C. albicans* and *E. faecalis* primers for qPCR.

Primer	Sequence (5′– 3′)	References
C. albicans	ITS3—GCA TCG ATG AAG AAC GCA GC ITS4—TCC TCC GCT TAT TGA TAT GC	(Fujita et al., 1995)
E. faecalis	F—CAAACTG TTGGCATTCCACAA R—TGGATTTCCTTTCCAGTC ACTTC3	(Rathnayake et al., 2011)

4.9.5 Fluorescence Microscopic Imaging

Thermanox™ coverslips from each treatment condition in the two time points (0h and 72h) were washed with PBS. 5 μM calcofluor white (Invitrogen, Paisley, UK) which specifically stains chitin and beta-glucans of fungal cell wall was used to stain *C. albicans* while 5 μM SYTO9 (Thermo Fisher Scientific, Paisley, UK) was used to stain both bacterial and fungal cells with high affinity to nucleic acids. After incubating the samples in the dark for 20 min, excess stain was washed away with sterile water. Afterwards, stained samples were fixed with 2% paraformaldehyde for 1h and imaged using EVOS FL Cell Imaging system (Thermo Fisher Scientific, Waltham, MA, USA).

4.9.6 Statistical Analysis

GraphPad Prism (version 7.0 d) was used for graph production, data distribution and statistical analysis. D'Agostino-Pearson omnibus normality test was used to test the normal distribution of the data. For statistical analysis, non-parametric Kruskal–Wallis test with Dunn's multiple comparison test was used to compare CFE values of the three treatment conditions at 0h and 72h. Mann-Whitney test was also used to compare mono- and dual-species biofilms of the same treatment.

4.10 Conclusions

Although continuous chelation is an admirable goal, simplifying protocols and potentially minimising the risk of deleterious interactions between irrigants, the current work raises questions of whether we should depart from sequential irrigation. The use of NaOCl followed by EDTA has an enhanced effect on biofilm disruption and significantly reduces *C. albicans* and *E. faecalis* persistence and regrowth. The current study also highlights the need for appropriate biofilm model choices. Both inhibitory and promoting effects can be observed between the participating microorganisms within the biofilm, evidenced here where *C. albicans* is significantly more susceptible to NaOCl and NaOCl:HEDP treatment when co-cultured with *E. faecalis*. It may be that with the addition of further complexity within the biofilm model that the interactions between microorganisms modify to an even greater extent irrigation effectiveness.

5 *Candida albicans* and *Enterococcus faecalis* biofilm frenemies: when the relationship sours

5.1 Preface

In the previous chapters the characterisation of *C. albicans* oral isolates and their response to endodontic therapeutics were discussed. We have also shown the importance of using a chelating agent following NaOCl treatment to inhibit regrowth and, in this regard, we showed that EDTA is superior to HEDP. We then tested the effect of endodontic irrigants on *C. albicans* and *E. faecalis* (the commonly co-isolated endodontic pathogens in recurrent infections) in mono- and dual-species biofilms. Interestingly, *C. albicans* was more susceptible to endodontic irrigants when cocultured with *E. faecalis* while the latter remained largely unaffected by *C. albicans*. Therefore, we hypothesized that *E. faecalis* has an inhibitory effect on *C. albicans*. This component of this thesis aimed at investigating the effect of *E. faecalis* on *C. albicans* phenotype, growth and gene expression. Moreover, we employed RNA seq to investigate the mechanism of this interaction and proposed an *E. faecalis* driven pH dependent effect as an unreported possible mechanism of interaction.

This chapter is presented as published journal article:

Alshanta, O.A., Albashaireh, K., McKloud, E., Delaney, C., Kean, R., McLean, W. and Ramage, G., 2022. *Candida albicans* and *Enterococcus faecalis* biofilm frenemies: When the relationship sours. *Biofilm*, p.100072.

A supplementary folder containing high resolution images of hard to read figures is provided separately as a part of thesis submission.

5.2 Author declaration and contribution

Conceptualisation: The idea of the pH driven mechanism was conceived by myself and Ms Khawlah Albashaireh following the analysis of shared RNA seq data. All the subsequent idea formulation and hypothesis was my own.

Data Curation: Myself and Dr Christopher Delaney had shared responsibility of data curation.

Formal Analysis: I completed all the formal analysis presented in this publication except for quality control, sequence trimming and read alignment and enumeration of the RNA seq raw data which was performed by Dr Christopher Delaney.

Investigation: I conducted 95% of all the laboratory experiments presented in this publication. Ms Khawlah Albashaireh contributed by generating data for Figure 5.5 panel b.

Methodology: I designed all the experiments presented in this publication except for RNA seq experiment which was designed by Oral Sciences Research Group including myself.

Project Administration: Prof Gordon Ramage, Dr William McLean and Dr Ryan Kean were responsible for project Administration.

Visualisation: I was responsible for all the preparation and presentation of data presented in this publication except for Supplementary figure 5.1 which was generated by Dr Christopher Delaney.

Writing – original draft: I wrote the complete original draft for this publication.

Writing – review and editing: I completed the all the editing requested by the journal. Prof Gordon Ramage, Dr William McLean and Dr Ryan Kean provided review and feedback for the original draft.

5.3 Materials and methods

This section covers in detail the materials and methods used in this chapter.

E. faecalis supernatant was used in four different conditions (normal supernatant, boiled normal supernatant, ultrafiltered supernatant and boiled ultrafiltered supernatant) as illustrated in Figure 5.1.

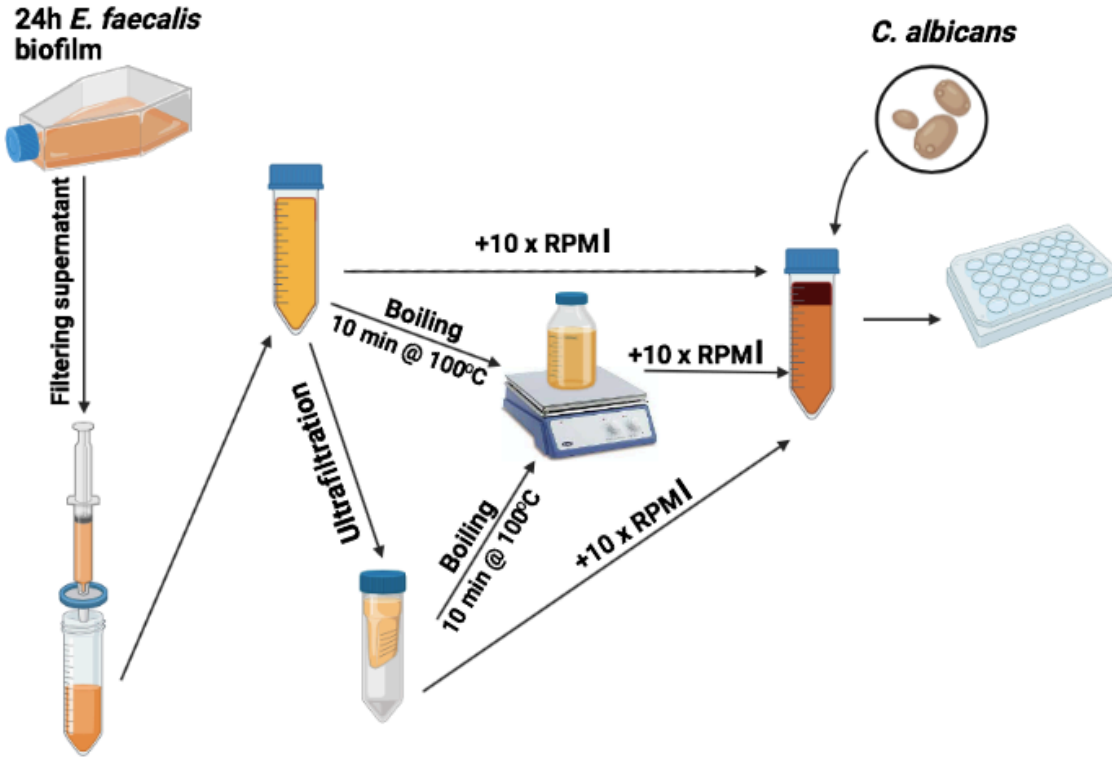


Figure 5.1: Schematic illustration of different *E. faecalis* supernatant conditions. Diagram created with Biorender.com.

5.3.1 Growth kinetics

E. faecalis supernatant was obtained as above and mixed with 10xRPMI in 9:1 ratio. *C. albicans* SC5314 was standardised to a cellular density of $1x10^6$ cells/mL in either *E. faecalis* supernatant or in THB:RPMI media. Two hundred µL of cell suspension was dispensed in each well of a 96 well flat-bottom plate. The plate was incubated at 37°C for 24h with absorbance measured at 570 nm every 15 minutes after shaking at 100 rpm for 10 seconds.

5.3.2 Transcriptional analysis

For *C. albicans* related gene expression in dual-species biofilm, MasterPure™ Yeast RNA Purification Kit (Cambio, Cambridge, UK) for yeast was used as per the manufacturer's protocol. Biofilms were scraped into 1 mL of PBS and cells pelleted by centrifugation at 13,000 rpm for 10 minutes. Supernatant was discarded and 300 µL of extraction reagent containing 1 µL of 50 µg/µL Proteinase K was then added to each sample. Following thorough vortexing, samples were incubated at 70º C for 15 minutes with vortexing every 5 minutes. Afterwards, samples were kept on ice for 5 minutes and 175 µL of MPC Protein Precipitation Reagent was added and samples vortexed for 5 seconds. Supernatant containing RNA was then separated by centrifugation at 13,000 rpm for 10 minutes at 4°C and transferred to a new microcentrifuge tube. Five hundred µL of ice-cold isopropanol was added and samples inverted 30-40 times. RNA was then pelleted by centrifugation at 13,000 rpm for 10 minutes at 4°C and isopropanol was discarded.

Removal of DNA contaminants from extracted RNA was achieved as follow. Two hundred µL of DNase solution containing 20 µL of 10X DNase Buffer, 175 µL of deionised water and 5 µL of RNase-Free DNase I was added to each sample and mixed by pipetting. Following incubation at 37°C for 10 minutes, 200 µL of 2X T and C Lysis Solution was added and vortexed for 5 seconds. Once again, 200 µL of MPC Protein Precipitation Reagent was added and samples vortexed for 5 seconds. Supernatant containing RNA was then separated by centrifugation at 13,000 rpm for 10 minutes at 4°C and transferred to a new microcentrifuge tube. Five hundred µL of ice-cold isopropanol was added and samples inverted 30-40 times. RNA was then pelleted by centrifugation at 13,000 rpm for 10 minutes at 4°C and the isopropanol was discarded. Pellets were carefully washed twice with 70% ethanol. After removal of ethanol, RNA was suspended in 35 µL of TE Buffer and finally 1 µL of RiboGuardTM RNase Inhibitor was added. cDNA synthesis and real time PCR analysis were performed as described in chapter 2.

5.3.3 Whole-genome transcriptional analysis using RNA sequencing

5.3.3.1 Sample preparation and RNA extraction

Samples were prepared by growing *C. albicans* SC5314 of cellular density $1x10^6$ cells/mL in 1:1 THB:RPMI media in T-75 cell culture flasks (Corning Incorporated, NY, USA) at 5 % CO_2 and 37°C for 4h. Following incubation, the medium was removed, and biofilms washed with PBS and *E. faecalis* ER5/1 was standardised to a cellular density of $1x10^7$ cells/mL in THB:RPMI and added to 4h *C. albicans* biofilms. After 2h, 4h or 20h of additional incubation, supernatant was discarded, biofilms washed with PBS and scraped into 1 mL of RNA*later* (ThermoScientific, Loughborough, UK). Graphical presentation of sample preparation is illustrated in Figure 5.2. RNA extraction was carried out using RiboPureTM RNA Purification Kit for yeast (ThermoScientific, Loughborough, UK), as per manufacturer's protocol. Cells harvested in RNA*later* were pelleted by centrifugation at 12,000g for 5 minutes and supernatant discarded. Pellets were then suspended in 480 µL lysis buffer, 48 µL of 10% SDS and 480 µL of Phenol:Chloroform:IAA and vortexed for 15 seconds. Samples were then transferred to screw-cap tubes containing about 0.75 mL of ice- cold zirconia beads. Cells were beaten in 3 cycles of 30 seconds using a BeadBugTM microtube homogenizer (Merck, Gillingham, UK). To separate the aqueous layer containing the RNA, samples were centrifuged at 16,100g for 5 minutes and then transferred to a new microcentrifuge tube. Afterwards, 1.9 mL of binding buffer and 1.25 mL of 100% ethanol was added and thoroughly mixed. The samples were then passed through a filter cartridge assembled in a collection tube and the eluate was discarded. The filter was then first washed with 700 µL wash solution 1 and then twice with 500 µL wash solution 2/3. The filter was then transferred to a new collection tube and RNA was eluted in 25 µL of the pre-heated elution solution and RNA was collected.

DNase digestion was achieved by adding 5 µL of 10X DNase 1 Buffer and 4 µL of DNase I to the RNA samples and heated at 37° C for 30 minutes. 11 µL DNase inactivation reagent was then added, vortexed and incubated for 5 minutes at room temperature. Finally, samples were centrifuged at 10,000g for 3 minutes and the supernatant containing RNA was transferred to a fresh tube. The quality and quantity of RNA was checked using a Bioanalyser system (Aligent, USA).

Samples with a minimum RNA integrity number (RIN) of 7.0 and a minimum quantity of 2.5 µg were deemed acceptable and sent for sequencing using Illumina NOVASeq6000 sequencing platform at (Edinburgh Genomics).

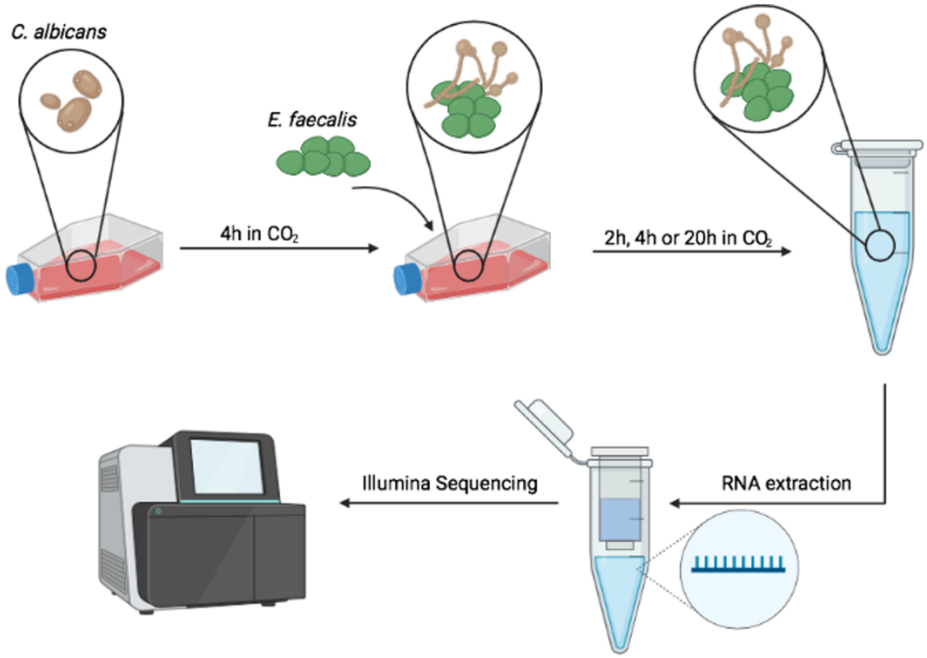


Figure 5.2: Overview of experimental design of RNA sequencing analysis. Diagram created with Biorender.com.

5.3.3.2 Analysis pipeline

The received raw fastq reads were first quality controlled by implementing FastQC and trimmomatic software to remove adaptors and low quality reads of <30 bases (Bolger et al., 2014). Hisat2 was then used to map the trimmed high-quality reads to *C. albicans* SC5314 genome (Candida Genome database). Reads that were mapped to a feature on *C. albicans* genome were counted with the use of the program HTSeq-count and imported to R for further analysis. R based DESeq2 package was utilised to perform differential expression analysis. Dr Christopher Delaney (University of Glasgow) kindly conducted the quality control, sequence trimming and read alignment and enumeration of the raw reads. A summary of the analysis pipeline is illustrated in Figure 5.3.

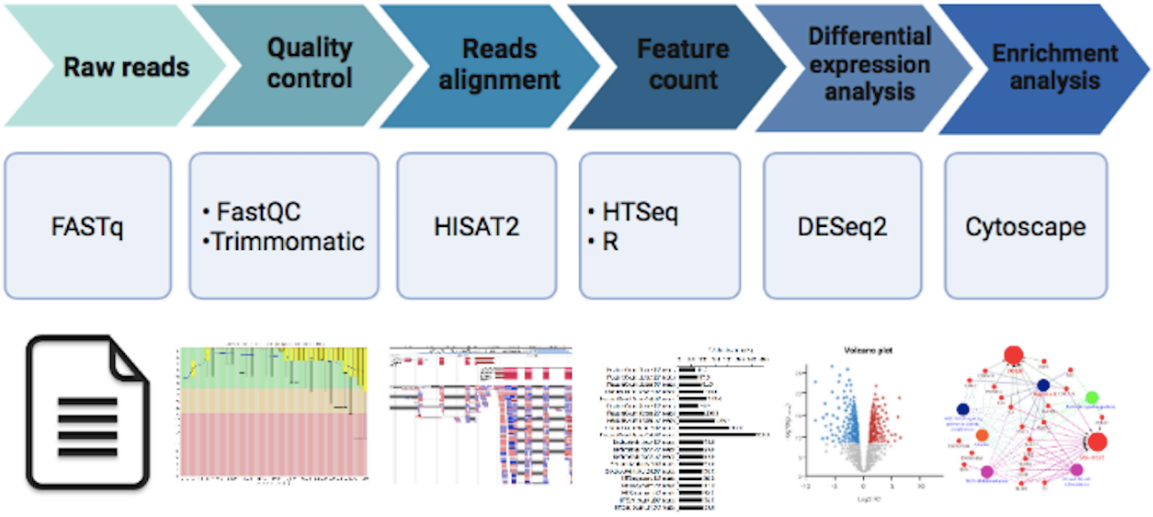


Figure 5.3: Summary of the data analysis pipeline. Diagram created with Biorender.com.

5.4 *Candida albicans* and *Enterococcus faecalis* biofilm frenemies: when the relationship sours

Om Alkhir Alshanta[1†], Khawlah Albashaireh[1†], Emily McKloud[1], Christopher Delaney[1], Ryan Kean[2], William McLean[1] and Gordon Ramage[1*]

[1]Glasgow Endodontology and Oral Sciences Research Group, Glasgow Dental School, School of Medicine, Dentistry and Nursing, College of Medical, Veterinary and Life Sciences, Glasgow, United Kingdom.

[2]Department of Biological and Biomedical Sciences, School of Health and Life Sciences, Glasgow Caledonian University, Glasgow, UK

*****Corresponding: gordon.ramage@glasgow.ac.uk; +44(0)141 211 9740.

[†]Om Alkhir Alshanta and Khawlah Albashaireh contributed equally to this work.

5.5 Abstract

The opportunistic yeast *Candida albicans* and lactic acid bacteria *Enterococcus faecalis* are frequently co-isolated from various infection sites on the human body, suggesting a common interkingdom interaction. While some reports suggest an antagonism, the reason for their co-isolation therefore remains unclear. The purpose of this study was to undertake a detailed characterisation of this dual-species interaction. We used standard biofilm characterisation methodologies alongside an RNASeq analysis to assess the response of *C. albicans* to *E. faecalis*. We evaluated the relevance of pH to dual-species biofilm interactions and demonstrated that *E. faecalis* rapidly and significantly impacted *C. albicans* morphogenesis and biofilm formation, which was mirrored by levels of gene expression. These transcripts were enriched in amino acids biosynthesis and metabolism pathways in co-cultures, a finding that guided our investigation into pH related mechanism. We were able to demonstrate the direct role of *E. faecalis* induced low pH, which inhibited *C. albicans* hyphal morphogenesis and biofilm formation. The results suggest that the anti-candidal effect of *E. faecalis* is not based solely on a single mechanism. Instead, it may involve various mechanisms which collectively reflect the complexity of interaction between *C. albicans* and *E. faecalis* and impact treatment outcomes.

Keywords: Interkingdom; biofilm; *Candida albicans*; *Enterococcus faecalis*; supernatant; pH.

5.6 Introduction

Candida albicans biofilm infections are often polymicrobial, highlighting the importance of interkingdom interactions. These relationships may take the form of synergism, antagonism, or simply coexistence. It is well documented that *C. albicans* has the capacity to interact with various bacterial species, including *Staphylococcus aureus*, *Streptococcus mutans* and *Pseudomonas aeruginosa* (Kean et al., 2017, He et al., 2017, Méar et al., 2013). Beyond the basis of these interactions, *C. albicans* influences the composition, virulence and dysbiosis of the bacterial microbiome (Janus et al., 2017, Bertolini and Dongari-Bagtzoglou, 2019, Zhai et al., 2020). Reciprocally, it has also been shown that *C. albicans* is in turn influenced by bacteria or bacterial products in terms of growth, virulence, resistance and phenotypic switch (Dutton et al., 2016, Kim et al., 2018, Xu et al., 2014). The pleiomorphic fungus *C. albicans* and *Enterococcus faecalis* are opportunistic pathogens that colonize various niches of the human body, such as the skin, oral cavity, vagina and gastrointestinal tract (Kashem and Kaplan, 2016, Rindum et al., 1994, Achkar and Fries, 2010, Mason et al., 2012, Higuita and Huycke, 2014).

Endogenous in nature, *C. albicans* and *E. faecalis* can become pathogenetic due to impairment in host immunity or dysbiosis in microflora (Hube, 2004, Mason et al., 2011). Infections caused by the opportunistic yeast *C. albicans* can range in severity from superficial oropharyngeal candidiasis to a serious life-threatening candidemia (Yapar, 2014). Likewise, translocation of *E. faecalis* from the gut to other body sites is associated with various infections, including endocarditis, meningitis, urinary tract infections and blood-stream fatal sepsis (Mason et al., 2011, Krishnamoorthy et al., 2020). In particular, it has been observed that the oral microbiome of immunocompromised mice in an oropharyngeal candidiasis model showed a shift toward *E. faecalis* dominance (Bertolini et al., 2021). In the gut, *C. albicans* inoculated mice remained dominated by enterococci (mainly *E. faecalis*) compared with the non-infected candidal animals, in which the *Enterococcus* species were replaced by lactobacilli (Mason et al., 2012). Therefore, it can be inferred that *C. albicans* and *E. faecalis* may favour the same ecological environment or interact in a way that supports the survival of one another. Indeed, both microorganisms are frequently co-isolated from the root

canals of the teeth and oral mucosa (Kovac et al., 2013, Dahlén et al., 2012). Notably, in the endodontic literature these two pathogens are often considered the paradigm fungal and bacterial model to study, test and evaluate the effectiveness of endodontic antimicrobials or techniques (de Freitas Lima et al., 2015, Vaghela et al., 2011, Ghivari et al., 2017, Noites et al., 2014).

A variety of environmental conditions and microbial molecules have been shown to influence *C. albicans* hyphal morphogenesis and biofilm formation (Kean et al., 2018a). These include nutrient source, stressors, such as pH and temperature, and direct and indirect bacterial interactions (O'Donnell et al., 2015). RNASeq has been extensively used to analyse how *C. albicans* responds under these circumstances, which has dramatically enhanced our understanding of the transcriptional profiles of *C. albicans* response to weak organic acids (Cottier et al., 2015), growth media, oxidative and nitrosative stresses (Bruno et al., 2010), *Lactobacillus* (McKloud et al., 2021), *Streptococcus gordonii* (Dutton et al., 2016), *Pseudomonas aeruginosa* quorum sensing molecules (Bandara et al., 2020), amongst others. The interaction between *C. albicans* and *E. faecalis*, and more specifically the effect of *E. faecalis* and its supernatant on *C. albicans* morphogenesis, biofilm formation and virulence has been the subject of several investigations (Cruz et al., 2013, Graham et al., 2017, Garsin and Lorenz, 2013, Brown et al., 2019, Ishijima et al., 2014, Hassan et al., 2018, Bachtiar et al., 2016, Krishnamoorthy et al., 2020, Shekh and Roy, 2012). It was proposed that *E. faecalis* secretes an anti-candidal protein (Shekh and Roy, 2012), and that it is the EntV bacteriocin that is specifically linked to the observed *C. albicans* inhibition (Graham et al., 2017). Nevertheless, despite the well documented adverse effect of low pH and acid producing bacteria, such as lactobacilli, on *C. albicans* hyphal formation (McKloud et al., 2021), the influence of the acidifying potential of the lactic acid bacterium *E. faecalis* remains largely unexplored. Therefore, the present *in vitro* study aimed to examine the phenotypic and transcriptomic biofilm interaction of *C. albicans* when co-cultured with *E. faecalis*. To the best of our knowledge this the first study that reveals *E. faecalis* induced changes in *C. albicans* biofilm transcriptome using RNA sequencing, and the impact that low pH has on this phenomenon.

5.7 Materials and Methods

5.7.1 Microbial growth conditions and standardisation

All *C. albicans* and *E. faecalis* strains used in this study are listed in Table 5.1.*C. albicans* strains were incubated at 30°C for 48h on Sabouraud dextrose agar (SAB [Sigma–Aldrich, Dorset, UK]). *E. faecalis* strains were maintained on 5% v/v horse blood Columbia agar plate in 5% CO_2 at 37°C for 24h. *C. albicans* overnight cultures were grown in yeast peptone dextrose ([YPD - Sigma-Aldrich, Dorset, UK]) with shaking at 120 rpm in an orbital shaker (IKA KS 4000 i control, Berlin, Germany) at 30°C. For *E. faecalis*, Brain Heart Infusion ([BHI] Sigma–Aldrich, Dorset, UK) with incubation in 5% CO_2 for 18h at 37°C was used. Cells were then harvested by centrifugation for 5 min at 3000 rpm and washed twice with PBS. Yeast cells were counted using a hemocytometer and standardised to 1×10^6 yeasts/mL. Bacterial cells were standardised using a spectrophotometer at an OD_{600nm} of 0.3 (~ 2×10^8 cells/mL).

Table 5.1: List of used *C. albicans* and *E. faecalis* strains.

C. albicans		*E. Faecalis*	
Name	**Reference**	**Name**	**Reference**
SC5314	(Fonzi and Irwin, 1993)	ATCC-29212	(Kim et al., 2012)
BC023 (LBF) BC146 (HBF)	(Coco et al., 2008)	NCTCC 5957	National Collection of Type Cultures, Public Health Laboratory Service (**www.phe-culturecollections.org.uk**).
		E1, E2, E3	(Sedgley et al., 2004)
		ER5/1	(Johnson et al., 2006)
		ER35	GDS culture collection
		OS-16	(Sedgley et al., 2004)
		V583	(Sahm et al., 1989)
		OGX-1	(Shelburne et al., 2007)
		J 42-7	GDS culture collection
		AA-OR 34	(Sedgley et al., 2006)

HBF = high biofilm former; LBF = low biofilm former; GDS = Glasgow Dental School

5.7.2 Assessment of *Candida albicans* characteristics when co-cultured with *Enterococcus faecalis*

For phenotypic evaluation, *C. albicans* SC5314, high biofilm formers (HBF) and low biofilm formers (LBF) (O'Donnell et al., 2017, Sherry et al., 2014), and *E. faecalis* ER5/1, were standardised to 1×10^6 cells/mL for *C. albicans* and 1×10^7 cells/mL for *E. faecalis* in Todd-Hewitt broth (THB; Merck UK) supplemented with 10mM menadione and 10mg/mL hemin (Thermo Fisher). These were subsequently mixed 1:1 v/v with Roswell Park Memorial Institute (RPMI-1640 [Sigma–Aldrich, Dorset, UK]) (THB:RPMI), a medium which has been shown to support the co-culture of *C. albicans* and bacterial species (Montelongo-Jauregui et al., 2016). Mono-cultures of *C. albicans* and co-cultures of *C. albicans* with *E. faecalis* were created in a 24 well microtiter plates (Corning Incorporated, Corning, NY, USA) for 24h in 5% CO_2 at 37ºC. After incubation, biofilms were washed with PBS and stained with 5 µM calcofluor white (Invitrogen, Paisley, UK), a stain which specifically stains the chitin and beta-glucans of fungal cell wall (Gil-Bona et al., 2016). Biofilms were incubated in the dark for 20 min and excess stain was washed away with sterile water. 2% paraformaldehyde was used for 1h to fix the stained biofilms which were then imaged using EVOS FL Cell Imaging system (Thermo Fisher Scientific, Waltham, MA, USA). Biofilm biomass of the same biofilms was also quantified using crystal violet stain as previously described (Jose et al., 2010).

Table 5.2: *C. albicans* and *E. faecalis* primers used for quantitative and real time qPCR

Primer	Gene name	Sequence (5′– 3′)	Function
C. albicans		ITS3 - GCATCGATGAAGAACGCAGC	
		ITS4 - TCCTCCGCTTATTGATATGC	
	ACT1	F - AAGAATTGATTTGGCTGGTAGAGA	Housekeeping
		R - TGGCAGAAGATTGAGAAGAAGTTT	
	HWP1	F- GCTCAACTTATTGCTATCGCTTATTACA	Hyphal wall protein
		R - GACCGTCTACCTGTGGGACAGT	
	ALS3	F - CAACTTGGGTTATTGAAACAAAAACA	Adhesion
		R - AGAAACAGAAACCCAAGAACAACCT	
	ECE1	F - GCTGGTATCATTGCTGATAT	Hyphae specific protein
		R - TTCGATGGATTGTTGAACAC	
	YWP	F - TCCGGTTCTGGTTCTGATTC	

		R - TACCGTGGACCGTAGTGACA	Yeast wall protein
	SAP2	F-GAATTAAGAATTAGTTTGGGTTCAGTTGA	Secreted aspartyl proteases
		R - CCACAAGAACATCGACATTATCAGT	
	SAP5	F - CCAGCATCTTCCCGCACTT	Secreted aspartyl proteases
		R - GCGTAAGAACCGTCACCATATTTAA	
	PLB1	F - GGTGGAGAAGATGGCCAAAA	Phospholipase
		R - AGCACTTACGTTACGATGCAACA	
	HSP90	F - GGTTGCTGATCACGTCCAAGTT	Heat shock protein
		R - AACTTACCACCAGCGTTAGATTCC	
	CDR1	F - GTACTATCCATCAACCATCAGCACTT	Efflux pump
		R - GCCGTTCTTCCACCTTTTTGTA	
	MDR1	F - TCAGTCCGATGTCAGAAAATGC	Efflux pump
		R - GCAGTGGGAATTTGTAGTATGACAA	
	ARG1	F - CTTTGGTTTGTGCCACTGGG	Arginine synthesis
		R - TGCCGTTTCTCACGGTTGTA	
	ARG3	F - TGCGTCTTCACAAACACCAC	Arginine and citrulline biosynthesis
		R - ATGATGCCGCTCCTTCAGTA	
	CPA1	F - TGAAATGGTGCCTTGGTGGT	Arginine synthesis
		R - AAACGTTCTGGTGTTGCTGC	
	CPA2	F - TGCTCAAGGTGTGGTGGTTT	Arginine synthesis
		R - TGGCATCACCGGAATGAACA	
	GPT1	F - CAGTTCGGCTGGTACCACTT	Transmembrane activity
		R - CGATACTGACATCACCCCCG	
	AAP1	F - CTCGTCAACGGTCAACCAGA	Transmembrane transport
		R - TGCGCTATTGGGGCATTACA	
E. faecalis	DDL	F - CAAACTG TTGGCATTCCACAA	housekeeping
		R - TGGATTTCCTTTCCAGTC ACTTC	
	EntV	F - AGCTGCACAAAAGAAAGCCTG	Secreted bacteriocin
		R- GCTTAGCCCACATTGAACTGC	

5.7.3 Quantitative analysis using quantitative PCR

Mono- and dual-species biofilms of *C. albicans* SC5314 and *E. faecalis* ER5/1 and E2 were grown as described above in 6 well microtiter plates for 4, 6, 8 and 24h in 5% CO_2 at 37°C. Biofilms were then removed by scraping into 1mL of PBS before DNA extraction using QIAamp DNA mini kit, as per manufacturer's instructions (Qiagen, Crawley, UK). For qPCR, a mastermix containing Fast SYBR GreenER™ (Thermo Fisher Scientific, Paisley, UK), forward/reverse primers (Table 5.2), and UV treated RNase-free water was prepared, to which extracted DNA was added. Standard curves for each strain were also included. The used thermal cycles were

50°C for 2 min, 95°C for 2 min, 40 cycles of 95°C for 3 s and 60°C for 30 seconds using Step-One plus real time PCR machine and StepOne software V2.3 (Life Technologies, Paisley, UK). Colony forming equivalents (CFE) were calculated in relation to each species standard curve, as previously described (O'Donnell et al., 2016). Data obtained is from triplicates from three independent experiments.

5.7.4 Gene expression analysis of *Candida albicans* genes and *Enterococcus faecalis EntV* gene

For assessment of *C. albicans* related genes, biofilms of *C. albicans* SC5314 alone or in co-culture with *E. faecalis* ER5/1 were developed for 2h, as described above. For *E. faecalis EntV* gene expression, biofilms of 12 *E. faecalis* strains were grown for 4h in THB:RPMI 5% CO_2 at 37ºC. After incubation, biofilms were removed by scraping, and RNA extracted using the MasterPure™ Yeast RNA Purification Kit (Cambio, Cambridge, UK) for yeast, as per the manufacturer's protocol, and with a TRIzol extraction for bacteria (Thermo Fisher Scientific, Paisley, UK). Extracted RNA was converted to cDNA with a high-capacity cDNA reverse transcription kit (Thermo Scientific, Loughborough, UK). Real-time PCR was carried out as described above. The list of primers for genes assessed are located in Table 5.2. Gene expression was analysed for *C. albicans* using $\Delta\Delta$CT method, after normalising CT values to ACT1 housekeeping gene (Livak and Schmittgen, 2001). *EntV* gene expression in *E. faecalis* strains was calculated as a percentage of expression in relation to $\Delta\Delta$CT of the housekeeping gene. No-reverse transcription samples were included. Data obtained is from triplicates from three independent experiments.

5.7.5 Assessing the transcriptional response of *Candida albicans* to *Enterococcus faecalis*

Transcriptional analysis of *C. albicans* SC5314 in response to co-culture with *E. faecalis* ER5/1 was performed using RNA sequencing as previously described (McKloud et al., 2021, Short et al., 2021). Briefly, *C. albicans* mono-cultures were grown in THB:RPMI media in T-75 cell culture flasks (Corning, USA) at a cellular density of $1x10^6$ cells/mL for 4h in 5% CO_2 at 37ºC. Next, biofilms were washed with PBS and *E. faecalis* in cellular density of $1x10^7$ cells/mL was added for additional 2, 4 or 20h. After each time point, biofilms were washed and scraped

into 1mL of RNAlater (Thermo Scientific, Loughborough, UK). RNA was extracted using RiboPure™ RNA purification kit for yeast (Thermo Scientific, Loughborough, UK). The quantity and quality of extracted RNA were evaluated using a Bioanalyzer system. RNA samples were submitted to Edinburgh Genomics facility where RNA sequencing was performed using the Illumina NOVASeq6000 sequencing platform. The obtained raw fastq reads were trimmed to remove adaptors and poor-quality reads using Trimmomatic (V0.38). Reads were then aligned to a reference *C. albicans* genome (http://www.candidagenome.org) and the number of aligned reads per gene were counted. Differential expression analysis was then performed using DESeq2 in RStudio which was also used to generate principal component analysis (PCA), volcano and heatmap plots.

To further evaluate transcriptional changes based on key findings, amino acid biosynthesis/metabolism related gene expression were analysed using RT-PCR. In this experiment, *C. albicans* SC5314 was grown in THB:RPMI in 6 well plates in 5% CO_2 for 4h. After incubation, supernatant was removed, and biofilms were washed with PBS. *E. faecalis* ER5/1 suspended in 3 different media conditions was then added to *C. albicans* biofilms. The media conditions used were THB:RPMI, THB:RPMI supplemented with amino acids (Sigma-Aldrich, Dorset, UK) and THB:RPMI supplemented with glucose (Sigma-Aldrich, Dorset, UK). Amino acids (L-glutamine, L-arginine and L-histidine) were added to THB:RPMI to reach a final concentration of 0.03, 0.4 and 0.6g/L respectively. The final glucose concentration in THB:RPMI with glucose was 5g/L. Supplemented media without *E. faecalis* was also added to *C. albicans* biofilms for mono-species biofilms. After incubation for an additional 2h in 5% CO_2 at 37°C, biofilms washed, removed by scraping, RNA extracted and RT-PCR performed as described above using primers specific for ARG1, ARG3, CPA1, CPA2, GPT1 and AAP1 genes (Table 5.2).

5.7.6 Assessment of *Candida albicans* biofilm biomass with *Enterococcus faecalis* supernatant

Cell free supernatant from 12 *E. faecalis* strains was obtained by growing *E. faecalis* to a cellular density of 1×10^7 cells/mL in THB:RPMI in T-15 cell culture flasks (Corning, USA) for 24h in 5% CO_2 at 37°C. Supernatant was centrifuged at 4000 rpm for 5 min before filtering with a 0.2µm syringe filter (Sartorius™ Minisart®, Fisher Scientific, Loughborough, UK). Next, a 9:1 v/v of

supernatant to 10 times concentrated RPMI (10×RPMI) media was then used to grow *C. albicans* SC5314, HBF and LBF in 24 well plate for 24h in 5% CO_2 at 37ºC (10×RPMI was used to ensure any effects were not a result of nutrient depletion). Following incubation, biofilms were washed with PBS and dried at room temperature overnight. The biofilm biomass of the dry biofilms were assessed with crystal violet stain.

Next, *E. faecalis* E2, ER5/1 and V583 were selected based on their differential level of *EntV* expression. *E. faecalis* supernatant from these strains was ultrafiltered using an Amicon Ultra-0.5 Centrifugal Filter Unit with 3 kDa NMWCO pore size (Merck Life science, Gillingham, UK). As *E. faecalis* EntV was indicated to be larger than 3–10 kDa (Graham et al., 2017), the 3 kDa pore size was selected to ensure removal of any proteins larger than 3 kDa NMWCO from the supernatant. Following ultrafiltration, both normal and ultrafiltered supernatant were boiled for 10 min at 100°C to heat inactivate any active proteins in the supernatant. Afterwards, the supernatant from each condition was mixed with 10×RPMI at 9:1 ratio and used to grow *C. albicans* SC5314, HBF and LBF, as described above. The pH of each supernatant was also measured using a pH meter (Fisher Scientific, Loughborough, UK). *C. albicans* biofilm biomass was assessed as described above.

5.7.7 pH modification of *Enterococcus faecalis* supernatant and growth media

The pH of *E. faecalis* E2, ER5/1 and V583 supernatant was quantified and shown to be ~5 for all three strains. The pH of the supernatant was then adjusted to 6.0 and 7.0 using 1 M sodium hydroxide. The pH of THB:RPMI (normally has pH of 7.0) was also adjusted to 5.0 and 6.0 using 35% hydrochloric acid (Sigma–Aldrich, Dorset, UK) and 90% lactic acid (Sigma–Aldrich, Dorset, UK). pH modified supernatant was mixed with 10×RPMI and used along with pH modified media to grow *C. albicans* SC5314, HBF and LBF. *C. albicans* biofilm biomass was assessed as described above.

5.7.8 Assessment of *Enterococcus faecalis* inhibition of *Candida albicans* hyphal morphogenesis

Mono- and dual-cultures of *C. albicans* SC5314, HBF, LBF and *E. faecalis* E2, ER5/1 and V583 were prepared, as described above. Biofilms were washed with PBS and stained and imaged with calcofluor white at 2, 4, 6, 8 and 24h, as described above. The pH of the supernatant from each biofilm at the indicated time were also measured.

5.7.9 Statistical analysis

Figures and statistical analysis were performed using GraphPad Prism (version 7.0 d, GraphPad, La Jolla, CA, USA). To compare data sets and calculate significance, non-parametric Kruskal–Wallis test with Dunn's multiple comparison test was used. The ClueGO application in Cytoscape (Version: 3.8.0) software was used to generate gene ontology networks.

5.8 Results

5.8.1 *Enterococcus faecalis* impacts biomarkers of *Candida albicans* biofilms

First, we investigated the general effect of *E. faecalis* on *C. albicans* hyphal morphogenesis, biofilm formation, growth, and expression of key genes related to biofilm formation, virulence and drug resistance. *C. albicans* SC5314, HBF and LBF were grown in mono-species biofilms or co-cultured with *E. faecalis* ER5/1 for 24h. As reported by various studies (Cruz et al., 2013, Graham et al., 2017, Brown et al., 2019, Hassan et al., 2018, Bachtiar et al., 2016), *C. albicans* hyphal morphogenesis was shown to be clearly inhibited in co-cultures, as shown in calcofluor stained images (Figure 5.4a). *C. albicans* SC5314 (panel a [i]) and HBF (panel a [iii]) are known for their ability to form dense biofilm with tangled networks of hyphae (Ramage et al., 2002, Alshanta et al., 2019). This was obvious in mono--species biofilms, though in the presence of *E. faecalis*, less abundant and shorter hyphae are evident (panel a [ii], [iv]). The phenotype of *C. albicans* LBF (panel a [v], [vi]) remained unaltered. This phenotype of *C. albicans* usually grows in yeast form with occasional hyphae, therefore, it was not influenced by *E. faecalis*. To assess the effect of *E. faecalis* on *C. albicans* biofilm biomass, cell

free *E. faecalis* supernatant supplemented with 10×RPMI was used instead of live *E. faecalis* to ensure that the measured biofilm biomass is purely *C. albicans* biomass. *E. faecalis* supernatant was reported to have similar inhibitory effect on *C. albicans* as live *E. faecalis* (Cruz et al., 2013). The biofilm biomass, assessed using crystal violet assay, showed that *E. faecalis* supernatant significantly inhibited biofilm formation of *C. albicans* SC5314 and HBF compared with *C. albicans* only biofilms. Again, LBF remained unaffected (Figure 5.4b).

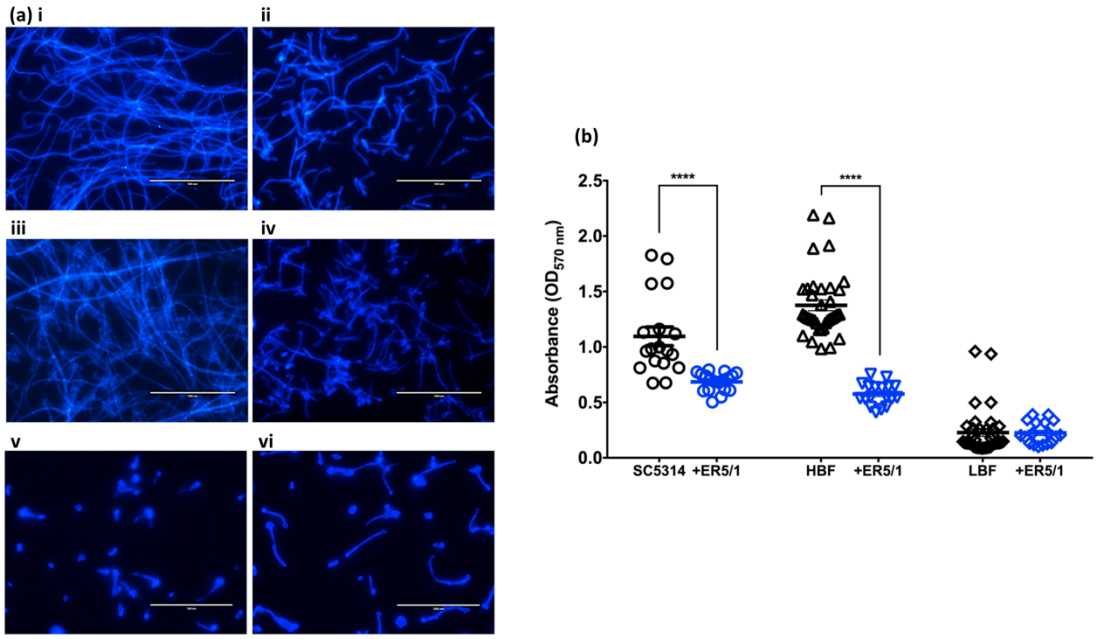


Figure 5.4: *E. faecalis* inhibits *C. albicans* hyphal morphogenesis and biofilm formation in *in vitro* coculture. (a) *C. albicans* laboratory strain SC5314 **(i, ii)**, HBF clinical isolate BC146 **(iii, iv)** and LBF clinical isolate BC023 **(v, vi)** were grown in THB:RPMI media in mono-culture **(I, iii, v)** or in co-culture with *E. faecalis* strain ER5/1 **(ii, iv, vi)** for 24h. Biofilms were stained with calcofluor white. Scale bars are 100 µm. **(b)** *C. albicans* biofilm biomass measured using crystal violet stain after 24h of incubation in mono-culture and with ER5/1 supernatant supplemented with 10xRPMI. Statistical significance was presented as **** $p < 0.0001$. Data obtained from triplicates of three independent experiments.

Next, qPCR analysis was used to further quantify cell numbers during these interactions. *E. faecalis* was confirmed to inhibit the growth of *C. albicans* SC5314 compared with mono-species (Figure 5.5a). This inhibition was notably more significant with *E. faecalis* ER5/1 than with that of E2. Whereas, the growth of *E.*

faecalis remained unaffected by the presence of *C. albicans*, as there was no significant difference in *E. faecalis* CFE/mL between mono- and dual-species cultures at all time points for both strains, although there was a trend toward increased growth of ER5/1 in co-cultures at 24h. Similar growth inhibition was observed in the kinetic growth curve of *C. albicans* grown in *E. faecalis* supernatant compared with *C. albicans* only at 24h (Figure 5.5b). The growth kinetics of *C. albicans* were characterised by steadily increasing growth up to 8h, followed by a plateau where the growth remained stable over the remaining hours. *C. albicans* grown with *E. faecalis* supernatant showed a higher growth rate between the 8 and 20h time points, after which the growth decreased sharply at 24h compared with *C. albicans* only control. This matches the growth inhibition observed in qPCR analysis at 24h in Figure 5.5a(i).

At the single transcript level, co-culturing *C. albicans* SC5314 with *E. faecalis* ER5/1 resulted in down-regulation of the hyphal related genes *HWP1* and *ECE1* in relation to *C. albicans* only at 2h. Conversely, the adhesion gene *ALS3* was upregulated, which may indicate that *E. faecalis* affects hyphal formation of *C. albicans* without affecting its adhesion to surfaces (Figure 5.5c). *SAP5* encoding SAP5 enzyme, which are expressed upon hyphal formation (White and Agabian, 1995), is also downregulated in dual-species biofilms. In contrast, *SAP2* and *YWP*, yeast typical genes, were upregulated. The gene expression patterns support the above-mentioned inhibition levels observed microscopically and by crystal violet assay. In addition, the effect of *E. faecalis* on *C. albicans* virulence was assessed using genes encoding proteolytic enzymes, *PLB1* and *SAP2* and those associated with drug resistance *CDR1*, *MDR1* and *HSP90*. The upregulation of the genes *PLB1* and *SAP2* indicates enhanced virulence of *C. albicans* via the increase in proteolytic enzymes' activities in the dual-species biofilms. Similarly, the efflux pump genes, *CDR1*, *MDR1* and *HSP90*, which are directly linked to drug resistance were also upregulated. This upregulation in efflux pump expression may refer to *C. albicans* attempt to remove intracellular toxic substances produced by *E. faecalis*, such as quorum sensing molecules, bacteriocins or metabolites. Overall, the above analysis indicates that *E. faecalis* inhibits *C. albicans* hyphal morphogenesis, biofilm formation and growth, while positively affecting enzymatic activity and drug resistance.

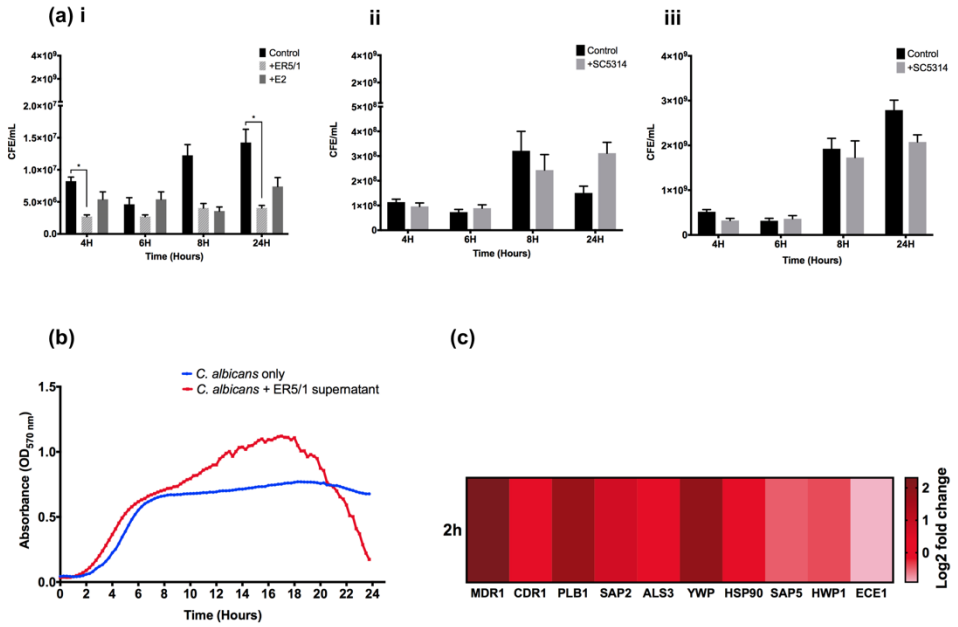


Figure 5.5: *E. faecalis* inhibits *C. albicans* growth and induce change in *C. albicans* SC5314 genes associated with biofilm, virulence and resistance. (a) The growth of *C. albicans* SC5314 **(i)** *E. faecalis* ER5/1 **(ii)** and *E. faecalis* E2 **(iii)** in mono- and dual-species was assessed using qPCR at 4h, 6h 8h and 24h. Statistical significance was presented as * $p < 0.05$, **** $p < 0.0001$. **(b)** 24h kinetic growth curve of *C. albicans* SC5314 only (blue) and *C. albicans* cultured in *E. faecalis* ER5/1 supernatant (red) at 570 nm absorbance **(c)** Heatmap of *C. albicans* SC5314 genes associated with biofilm, virulence and resistance was measured in mono-culture and in presence of *E. faecalis* at 2h. Control = *C. albicans* only biofilms. Data shown is the mean log fold change relative to mono-species biofilms. Results represent data from three independent experiments. Panel (b) was generated by Ms Khawlah Albashaireh.

5.8.2 Transcriptomic analysis of *Candida albicans* response to *Enterococcus faecalis* demonstrated the importance of early interactions

We have shown *E. faecalis* to have a significant effect on various biological functions of *C. albicans*. In order to gain a more comprehensive overview of the molecular mechanisms of this interaction, a whole transcriptome sequencing (RNA-Seq) approach was employed. For this purpose, *C. albicans* SC5314 as mono-cultures and as co-cultures with *E. faecalis* ER5/1 were prepared at 2, 4 and 20h, after the addition of *E. faecalis* to 4h *C. albicans* biofilms, to enable a temporal analysis. This approach enabled us to capture gene expression levels at early, intermediate and late time points of microorganisms' interaction.

The principal component analysis demonstrates the level of variance between *C. albicans* mono- and co-cultures at the different time points (Figure 5.6a). The variance in clustering was the highest at 6h (*E. faecalis* was added to 4h pre-established *C. albicans* biofilms and samples were harvested after 2h of further incubation). This indicates that *C. albicans* shows a quick transcriptional response to *E. faecalis*. The degree of variance then decreased at 8 and 24h. Therefore, the subsequent analysis will be focused on 6h time point. In total, 186 genes were significantly and differentially expressed between mono- and dual-species biofilms at 6h (with cut-off of 1.5 for fold change and 10e-3 for p-adjusted value). Of these, 112 genes were upregulated in dual-species and 74 were downregulated (Figure 5.6b). The top 50 differentially expressed genes are shown in Figure 5.6c, accompanied by a list of the top 10 upregulated genes in *C. albicans* mono- and co-cultures, and their functions (if known) are shown in Table 5.3. However, most of these genes are of unknown function and were not involved in the enriched biological pathways within the subsequent enrichment analysis. The full list of all significantly differentially expressed genes in *C. albicans* mono- and dual-species biofilm are available in Supplementary Table 5.1.

Table 5.3: List of the top 10 upregulated genes in *C. albicans* mono- and dual-species biofilms at 6h.

	Gene ID	Gene Name	P value (adj)	Log2FC
Up in *C. albicans* mono-species biofilms	CR_03580C_A	CR_03580C_A	0.00140825	-5.9714945
	CR_04510W_A	HXK2	4.07E-15	-5.4994791
	C2_01000W_A	HGT7	4.44E-05	-5.436272
	C3_01540W_A	C3_01540W_A	8.47E-05	-4.7324912
	C4_00450C_A	PGA10	4.08E-07	-4.3971164
	C4_04720W_A	C4_04720W_A	9.05E-09	-4.1599839
	C1_07160C_A	C1_07160C_A	0.00283371	-4.1157133
	CR_07170W_A	CR_07170W_A	2.39E-06	-4.0613455
	C6_00150W_A	ARD	2.09E-09	-4.0025723
	CR_01910C_A	CR_01910C_A	0.02841275	-3.8845479
Up in *C. albicans* - *E. faecalis*	C4_05580C_A	C4_05580C_A	1.65E-13	14.967598
	C1_02520W_A	SCW4	2.64E-22	14.6848791
	C1_13080W_A	OP4	5.11E-18	14.2265626
	C1_07580C_A	PRY1	4.27E-13	12.9814901

dual-species biofilms	C1_05890W_A	C1_05890W_A	2.05E-18	12.4438686
	C2_09800C_A	C2_09800C_A	2.21E-13	12.1176629
	CR_10320W_A	CR_10320W_A	1.21E-07	12.052965
	C2_02230C_A	C2_02230C_A	6.16E-19	11.4784176
	C6_00070C_A	PGA25	8.75E-26	10.9038636
	C1_09580C_A	LIP1	0.04085374	10.6107591

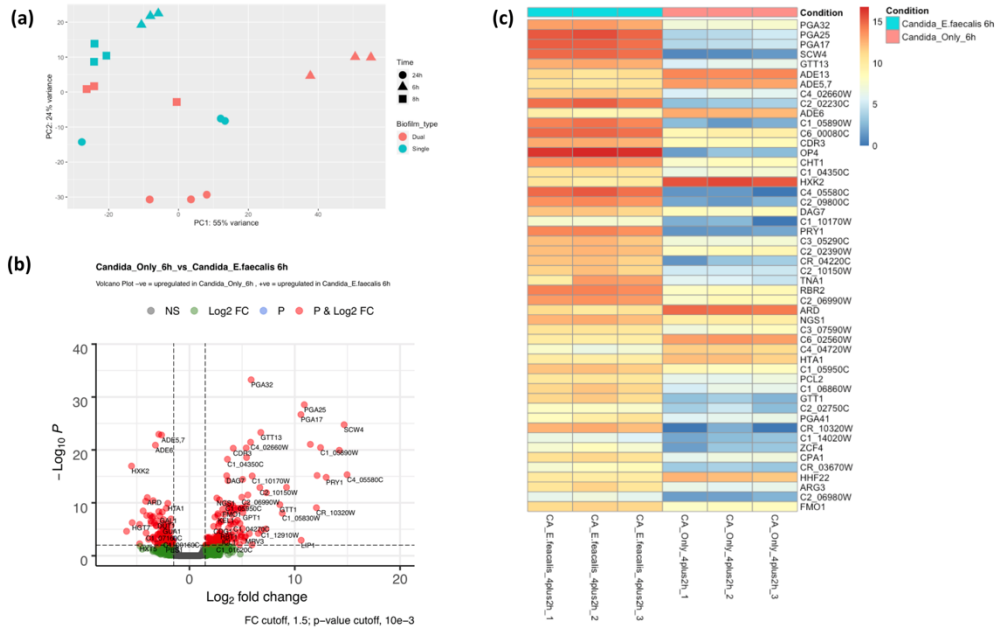


Figure 5.6: Overview of the transcriptional analysis of *C. albicans* SC5314 with *E. faecalis* ER5/1 at 4h, 6h and 24h. (a) The principal component analysis of data at all time points. **(b)** Volcano plot of the significantly differentially expressed genes of mono- and dual-species at 6h. Genes located on the negative x-axis are the up regulated genes in mono- species biofilms. Genes on the positive x-axis are the up regulated genes in dual-species biofilms. **(c)** Heatmap of the top 50 significantly differentially expressed genes of mono- and dual-species biofilms at 6h.

5.8.3 Amino acid biosynthesis and metabolism genes are upregulated by *Enterococcus faecalis*, regardless of glucose and amino acids concentration in the media

Gene ontology (GO) term analysis was then carried out to investigate the biological functions of the differentially expressed genes in mono- and dual-species biofilms. The upregulated genes in the dual-species were mainly responsible for amino acids biosynthesis/metabolism and transmembrane transport activity (Figure 5.7a). For the downregulated genes, the main biological functions enriched were purine nucleotide inosine monophosphate (IMP)/ nucleotides/purine related biosynthetic processes (Figure 5.7b). A simple presentation of the GO term analysis is illustrated in Supplementary figure 5.1. The increased amino acid uptake, biosynthesis and metabolism in response to *E. faecalis* can be an indication of amino acids or glucose starvation, or a stress response. It was also evident that *C. albicans* down-regulated the purine nucleotide biosynthesis pathway, an essential precursor for the nucleic acids DNA and RNA (Daignan-Fornier and Pinson, 2019). This may indicate an inhibition in *C. albicans* growth when co-cultured with *E. faecalis*, as shown previously in Figure 5.5a, b.

Next, we aimed to investigate whether the up-regulated amino acid biosynthesis and metabolism was a result of *E. faecalis* induced amino acids or glucose starvation. To achieve this, *C. albicans* SC5314 was grown in THB:RPMI media for 4h. Afterwards, *E. faecalis* was standardised to the desired cellular density in either THB:RPMI only, THB:RPMI supplemented with amino acids or with glucose and added to *C. albicans* biofilms. *C. albicans* mono-species biofilms with the three different media were also included. After additional incubation of 2h, mono- and dual-species biofilms were analysed for the expression of amino acids biosynthesis and metabolism genes; *ARG1*, *ARG3*, *CPA1*, *CPA2* and transmembrane transport related genes *GPT1* and *AAP1*. Despite supplementation with amino acids and glucose, all the investigated genes were still upregulated in all the conditions in the dual-species biofilms (Figure 5.7c). However, the level of expression of all genes was the highest in THB:RPMI media and lower in glucose supplemented THB:RPMI. Furthermore, the *CaGcn4* gene, *C. albicans* functional homologue of *S. cerevisiae* Gcn4, which mediates *C. albicans* response to amino acids starvation (Tripathi et al., 2002), was not upregulated at any time point, as

indicated in RNASeq analysis. These findings suggest that increased amino acid uptake, biosynthesis and metabolism is not caused by amino acids starvation, but instead, it is a direct stress response to *E. faecalis*, its released products, or the environment that it generates.

As recently published by our group (McKloud et al., 2021), amino acid biosynthesis and breakdown related genes were also upregulated in dual-species biofilms of *C. albicans* and *L. crispatus* at 24h. Indeed, there were 21 shared genes that were upregulated by both *E. faecalis* and *L. crispatus* at 24h (Figure 5.7d). Of note, the transcriptional response of *C. albicans* to *L. crispatus* was delayed (at 24h) compared to its early response to *E. faecalis* (at 6h).

Moreover, 10 out of 27 of the enriched GO biological processes in our dual-species biofilms, and 20 out of 22 of the enriched GO biological processes in our mono-species biofilms are also enriched in response to the weak organic acids (Cottier et al., 2015). In addition, many genes involved in arginine and lysin biosynthesis were induced in *C. albicans* under mild oxidative stress (Bruno et al., 2010, Dutton et al., 2016, Lorenz et al., 2004). Considering that *E. faecalis* is a lactic acid bacteria (Mehmeti et al., 2013), the similarities observed in *C. albicans* transcriptional response to *E. faecalis*, *L. crispatus*, and weak organic acids, and the fact that acidic environment favours yeast growth (Davis, 2003, Vazquez-Munoz and Dongari-Bagtzoglou, 2021, Dos Santos, 2010), then we hypothesized that *C. albicans* biofilm and hyphal morphogenesis inhibition observed in dual-species biofilms is a pH dependent phenomenon.

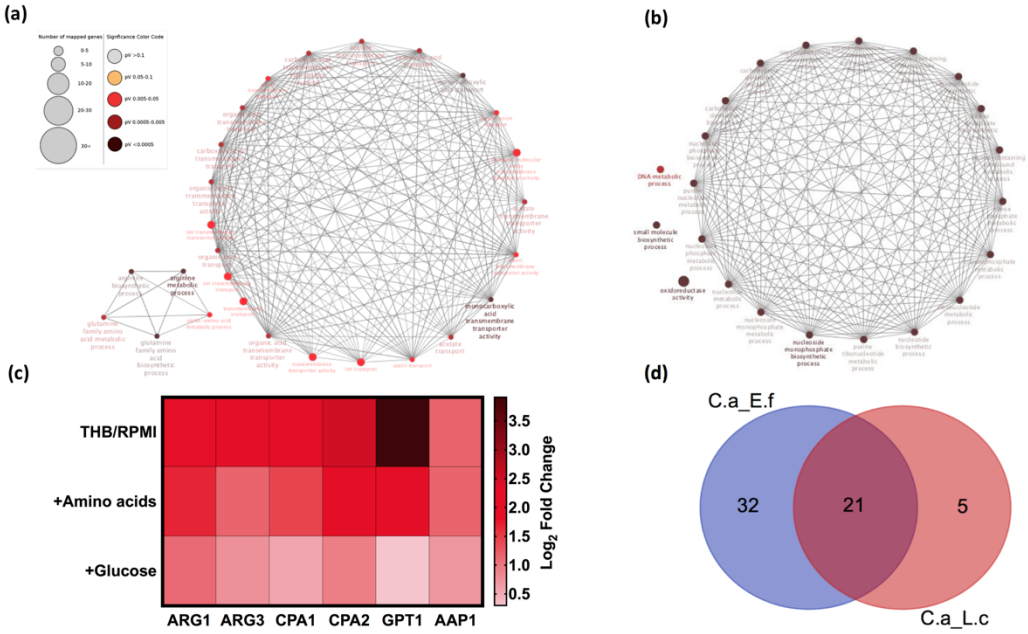
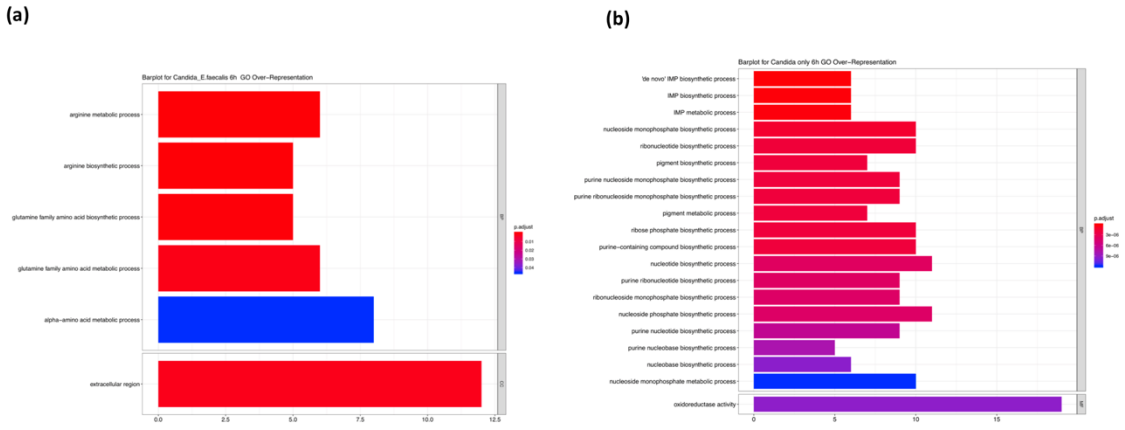


Figure 5.7: Gene ontology (GO) enrichment analysis of significant biological, cellular, and molecular functions of mono- and dual-species biofilms at 6h. Amino acids biosynthesis/metabolism and purine related biosynthesis *C. albicans* genes expression in mono- and dual-species biofilms at 6h. **(a)** Enriched biological processes within the upregulated genes in dual-species biofilms. **(b)** Enriched biological processes within the upregulated genes in mono-species biofilms with ≥ 10 associated genes. Only enriched pathways of a significant ≤ 0.05 are displayed. **(c)** Heatmap of key Amino acids biosynthesis/metabolism and transmembrane transport *C. albicans* genes in THB:RPMI media (media), THB:RPMI supplemented with glucose (+Glucose) or THB:RPMI supplemented with amino acids (+Amino acids) at 6h. Data shown is the mean log fold change relative to mono-species biofilms. **(d)** Number of the unique and overlapped genes that are upregulated at 24h in *C. albicans* co-cultured with *E. faecalis* or with *L. crispatus*.



Supplementary figure 5.1: Barplot of GO over representation of significant biological, cellular, and molecular functions of mono and dual species biofilms at 6h. (a) Enriched pathways in dual species biofilms. (b) Enriched pathways in *C. albicans* mono species biofilms. This figure was generated by Dr Christopher Delaney.

5.8.4 *Enterococcus faecalis* induced *Candida albicans* biofilm formation inhibition is not dependent on bacteriocin *EntV* expression.

E. faecalis inhibition of *C. albicans* hyphal morphogenesis, biofilm formation and virulence are reported in various studies (Cruz et al., 2013, Graham et al., 2017, Brown et al., 2019, Hassan et al., 2018, Bachtiar et al., 2016). However, few investigated the mechanism of this interaction. Shekh and Roy (2012) first characterized and purified an anti-candida protein produced by *E. faecalis*, which was heat stable up to 90°C with molecular weight of approximately 43 KDa. Graham et al. (2017) also identified the bacteriocin EntV produced by *E. faecalis* as a potent inhibitor of *C. albicans* biofilm formation and virulence. Therefore, we investigated the expression of *EntV* gene in 12 different *E. faecalis* strains and correlated the level of *EntV* expression with the degree of *C. albicans* biofilm inhibition, as quantified by the crystal violet assay. There was a wide variation in

the expression of *EntV* among the screened *E. faecalis* strains at 4h (Figure 5.8a). *E. faecalis* V583, showed the highest level of expression, followed by *E. faecalis* E1 and ER5/1. All other *E. faecalis* strains showed comparable expression levels. The inhibition of biofilm from *C. albicans* SC5314 (Figure 5.8b), HBF (Figure 5.8c) and LBF (Figure 5.8d) was then assessed using the cell free supernatant from the 12 *E. faecalis* strains. Here the biomass inhibition was not correlated with the quantified *EntV* expression, and there was not a statistically significant difference in *C. albicans* biomass inhibition induced by the different *E. faecalis* strains. Moreover, the linear regression analysis showed that there is no correlation between the level of *EntV* expression of different *E. faecalis* strains and the biofilm biomass of *C. albicans* grown in the supernatant of the corresponding *E. faecalis* strains (Supplementary figure 5.2).

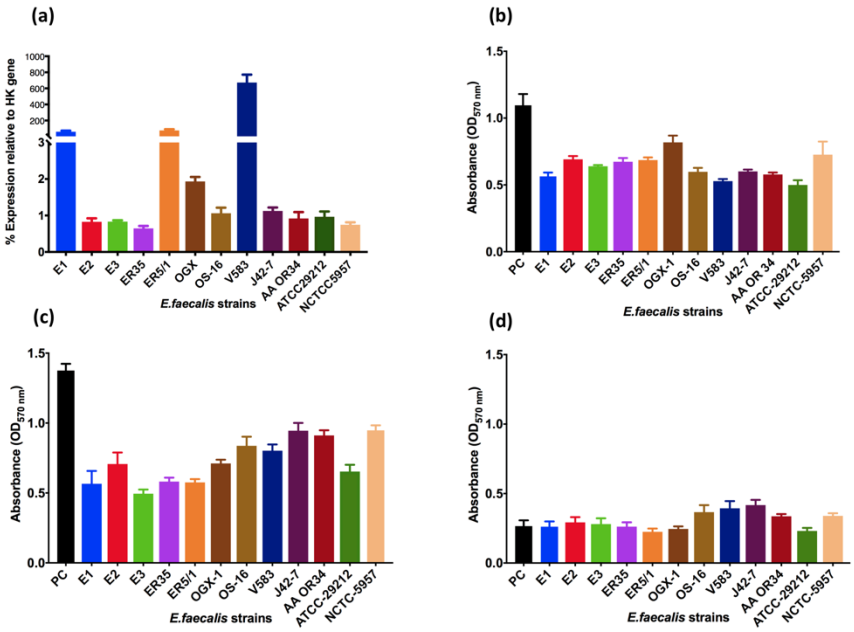
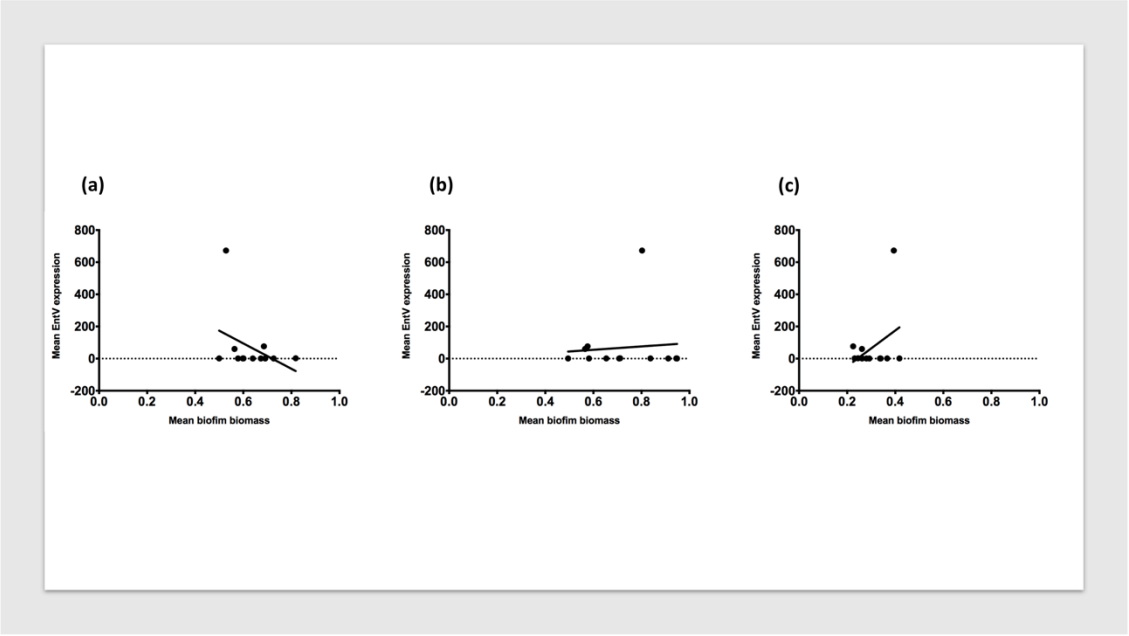


Figure 5.8: Inhibition of *C. albicans* hyphal morphogenesis when cocultured with *E. faecalis* is not dependent on bacteriocin EntV expression. (a) Percentage of *EntV* expression in 12 *E. faecalis* strains relative to ddl housekeeping gene at 4h with real time qPCR. The expression of ddl housekeeping gene was stable cross all the 12 *E. faecalis* strains with a variation within 2 cycle threshold (Ct) values. **(b-d)** Cell free *E. faecalis* supernatant obtained from 24h incubation of E2, ER5/1 or V583 in THB:RPMI was supplemented with 10xRPMI. *C. albicans* biofilm biomass of the mono-culture (control) and *C. albicans* grown in 12 *E. faecalis* strains supernatant was assessed by crystal violet at 24h **(a)** SC5314 **(b)** HBF (BC146) **(c)** LBF (BC023). Data obtained from triplicates of three independent experiments.



Supplementary figure 5.2: Linear regression analysis for the correlation of *EntV* expression in 12 *E. faecalis* strains and biofilm biomass of *C. albicans* grown in the corresponding *E. faecalis* supernatant. (a) SC5314 **(b)** HBF (BC146) **(c)** LBF (BC023). The p-value for SC5314, HBF and LBF was 0.2352, 0.7894 and 0.2273 respectively.

In order to further investigate the effect of the EntV peptide on *C. albicans*, we employed ultrafiltration membranes with 3kD cut-off (EntV was identified as a 7.2-kDa peptide) (Graham et al., 2017) and heat inactivation of *E. faecalis* supernatant. We aimed to separate any proteins larger than 3kD and heat inactivate the supernatant. *E. faecalis* E2, ER5/1 and V583 were selected for the subsequent analysis based on their variation in *EntV* expression. The supernatant was then used in combination with 10xRPMI to grow *C. albicans*. Despite the ultrafiltration and the heat inactivation of *E. faecalis* supernatant, a significant inhibition in *C. albicans* biofilm biomass was still evident in SC5314 (Figure 5.9a) and HBF (Figure 5.9b). Furthermore, ultrafiltration resulted in increased inhibition compared with normal supernatant in *C. albicans* SC5314 and HBF. Of note, the pH of the normal, ultrafiltered and heat inactivated supernatant was similar around 5.45 before incubation with *C. albicans* (Supplementary figure 5.3). *C. albicans* is known for its ability to raise extracellular pH (Vylkova et al., 2011), and since the RPMI used in this work was unbuffered, the pH of all the conditions

increased to around 6 - 6.3 after 24h of incubation. Despite this rise in pH, the conditions remained in the acidic range and the biofilm biomass of *C. albicans* SC5314 and HBF was significantly lower in all supernatant containing conditions compared with the *C. albicans* only controls.

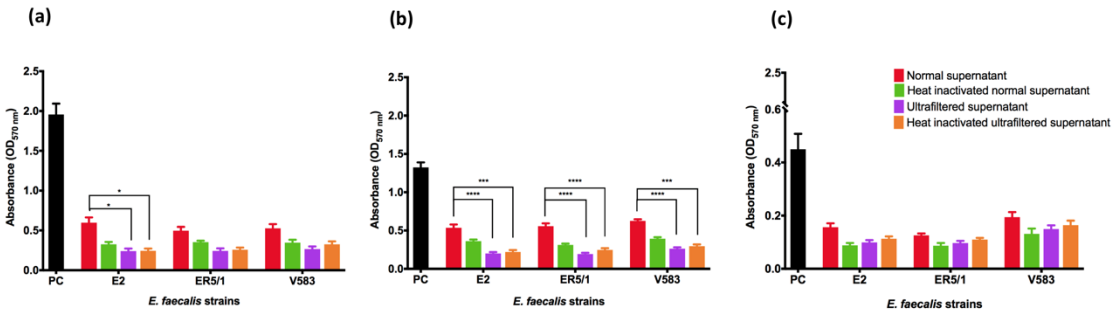
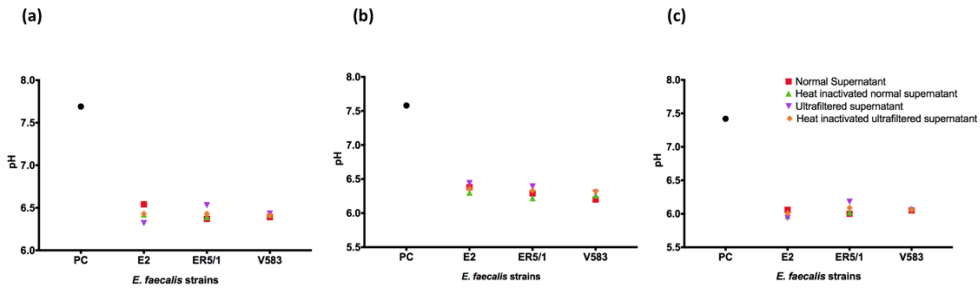


Figure 5.9: Inhibition of *C. albicans biofilm* formation by *E. faecalis* normal, ultrafiltered and boiled supernatant. *C. albicans* biofilm biomass of the mono-culture (control) and *C. albicans* grown in *E. faecalis* E2, ER5/1 and V583 supernatant supplemented with 10xRPMI as assessed by crystal violet at 24h. Four types of each strain supernatant were used; normal, boiled, ultrafiltered and boiled ultrafiltered supernatant **(a)** SC5314 **(b)** HBF **(c)** LBF. Statistical significance was presented as * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$. Results represent data from three independent experiments. PC = *C. albicans* only control.



Supplementary figure 5.3: pH measurements of normal, boiled, ultrafiltered and boiled ultrafiltered supernatant of *E. faecalis* after incubation with *C. albicans* for 24h. (a) SC5314 **(b)** HBF **(c)** LBF.

5.8.5 *Candida albicans* biofilm inhibition is pH dependent

As suggested above, we aimed to investigate the effect of *E. faecalis* supernatant pH on *C. albicans* hyphal morphogenesis and biofilm formation. Firstly, we measured the pH of the *E. faecalis* cell free supernatant obtained from 24h *E. faecalis* biofilms grown in THB:RPMI media. The pH of the supernatant was approximately 5.0. The pH of this supernatant and THB:RPMI media was then modified to different pH levels. Following the growth of *C. albicans* in various pH conditions, the biofilm biomass was assessed. Interestingly, there was a positive correlation between raising the pH of the *E. faecalis* supernatant and increased *C. albicans* biofilm biomass of SC5314 and HBF (Figure 5.10a and b, respectively). *C. albicans* biofilm biomass was also increased with a pH rise of pH modified media. In all conditions tested, the biomass was significantly higher at pH 7.0 compared to that of pH 5.0. More importantly, *E. faecalis* supernatant showed a similar effect trend on *C. albicans* biofilm formation to that of the pH modified media at the same pH value. This similarity is further phenotypically confirmed using calcofluor white images of the same conditions (Figure 5.10d). More

abundant and longer *C. albicans* hyphae can be observed with the rise in pH of both the ER5/1 supernatant and the media. As shown, with pH 5.0, yeast is the predominant phenotype of *C. albicans*. With a pH increase to 6.0, more hyphae are evident, which becomes the dominant phenotype at pH 7.0. The biofilm biomass of LBF was not affected by pH modification of the supernatant and media except for lactic acid modified media, in which the biofilm biomass was significantly higher in pH 7.0 compared with pH 5.0 (Figure 5.10c). These observations indicate the potential role of *E. faecalis* supernatant pH on *C. albicans* hyphal morphogenesis and biofilm formation.

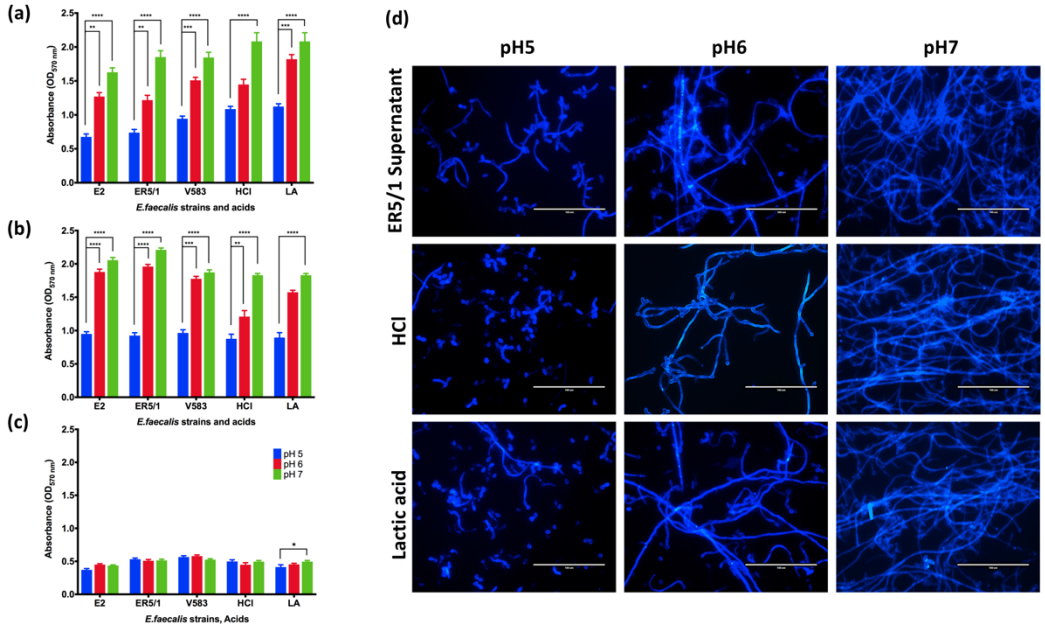


Figure 5.10: The effect of pH modified *E. faecalis* supernatant and acid modified media on *C. albicans* biofilm biomass. Cell free *E. faecalis* supernatant of E2, ER5/1 or V583 supplemented with 10xRPMI used with its original pH 5 or adjusted to pH 6 and pH 7 using NaOH. THB:RPMI media also used at its normal pH 7 or adjusted to pH5 and pH6 using hydrochloric acid (HCl) or lactic acid (LA). pH modified supernatant and media was used to grow *C. albicans* SC5314 **(a)** HBF **(b)** and LBF **(c)** for 24h and biofilm biomass assessed using crystal violet. **(d)** *C. albicans* SC5314 biofilms stained with Calcofluor white following incubation in the above-mentioned conditions for 24h. Scale bars are100 µm. Statistical significance was presented as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Results represent data from three independent experiments.

5.8.6 *Enterococcus faecalis* induced pH drop correlates with inhibition of *Candida albicans* hyphal morphogenesis

To gain insight into the chronology of *C. albicans* hyphal and biofilm formation in mono- and dual-species cultures with *E. faecalis*, the biofilm of both conditions was stained and imaged at 2, 4, 6, 8 and 24h. The pH of the supernatant of each condition was also measured at the corresponding time points. It was shown that *C. albicans* starts to form germ tubes in the first hour of incubation in both conditions when the pH was 7.0 (Figure 5.11). No differences were observed between the two conditions at 2h, where true hyphae start to form and elongate whilst the pH of the mono-species remains neutral, but has dropped to 6.5 in the co-culture. From 4h onwards, the difference between the two conditions becomes visibly evident. Here the mono-species *C. albicans* biofilms showed denser and longer hyphae that continued to increase over time, whilst the pH remained neutral across all time points. Conversely, the abundance and length of the hyphae appeared to be unchanged from 4 to 24h in the co-cultures. Interestingly, the 4h time point also represents the point in which the media becomes more acidic and drops to 5.5 in the co-cultures. The pH of the co-culture remained acidic (pH 5.5) at 4, 6, and 8h, and starts to rise slightly at 24h to reach 6.0, which indicates some buffering attempts exerted by *C. albicans*. The pH values of *C. albicans* mono-species, *E. faecalis* mono-species and their co-culture at the different time points is available in Supplementary figure 5.4. Collectively, these observations further confirm that *E. faecalis* induced inhibition of *C. albicans* hyphal morphogenesis and biofilm formation is based on the ability of *E. faecalis* to acidify the environment which in turns drives a predominantly yeast phenotype (Davis et al., 2000).

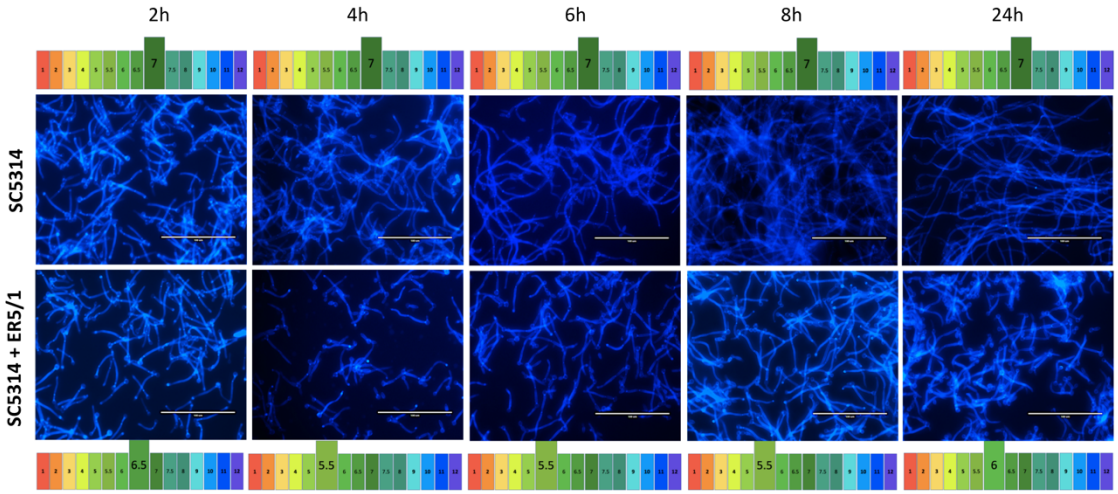
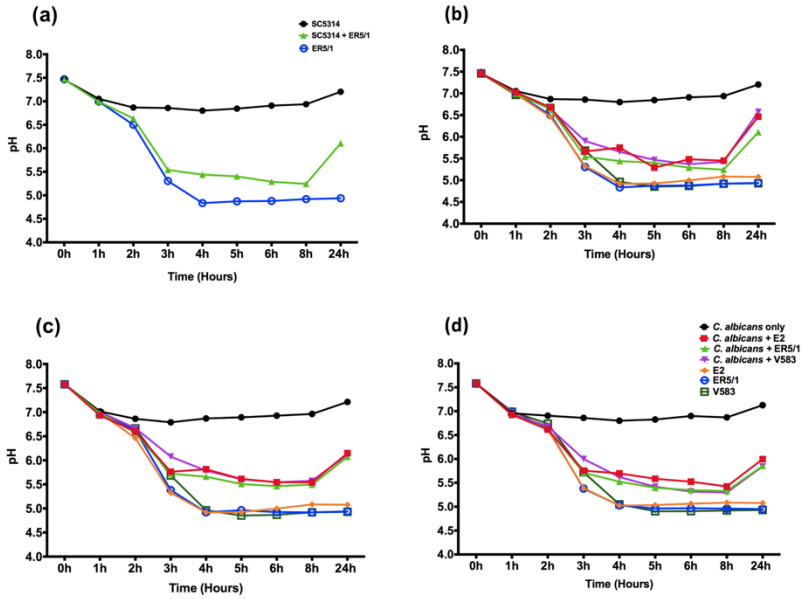


Figure 5.11: *C. albicans* hyphal formation and elongation in mono-culture and coculture with *E. faecalis* at 2, 4, 6, 8 and 24h. *C. albicans* SC5314 grown in THB:RPMI in mono- and dual-species biofilms with *E. faecalis* ER5/1. At every time point, supernatant removed, biofilms washed with PBS and stained with calcofluor white and supernatant's pH is measured. Images are for SC5314 and ER5/1 with Scale bars of 100 µm and pH measurements of the supernatant of each condition at the relevant time point.



Supplementary figure 5.4: pH change of the *C. albicans and E. faecalis* monocultures and dual species cultures at 2h, 4h, 6h, 8h and 24h. (a) pH measurements of *C. albicans* SC5314, *E. faecalis* ER5/1 and their coculture. **(b-d)** pH values of 3 *C. albicans* and 3 *E. faecalis* mono and dual species cultures at the indicated time points. **(b)** *C. albicans* SC5314 with *E. faecalis* E2, ER5/1 and V583. **(c)** *C. albicans* HBF. **(d)** *C. albicans* LBF.

5.9 Discussion

There is a growing fascination of how *C. albicans* interacts with bacterial species within complex interkingdom communities (Short et al., 2021, Pérez, 2021, McKloud et al., 2021, Santus et al., 2021). These studies have been instrumental in furthering our understanding of how this opportunistic yeast co-operates, or otherwise, with key bacterial species. Our previous work has shown how interactions with different species of *Lactobacillus* lead to differential health outcomes in patients with recurrent vulvovaginal candidiasis, and that denture wearing patients have resilient populations of *C. albicans* that support bacterial biofilm populations (McKloud et al., 2021, Delaney et al., 2019). In this new study we describe changes in *C. albicans* growth, phenotype, virulence and transcriptome when co-cultured with *E. faecalis in vitro*. In agreement with previous *in vitro* and *in vivo* studies, our preliminary investigation showed an

inhibition of *C. albicans* growth, hyphal morphogenesis and biofilm formation in dual-species biofilms (Cruz et al., 2013, Graham et al., 2017, Garsin and Lorenz, 2013, Brown et al., 2019, Ishijima et al., 2014, Hassan et al., 2018, Bachtiar et al., 2016, Krishnamoorthy et al., 2020, Shekh and Roy, 2012). Subsequent RNASeq showed that *E. faecalis* rapidly and significantly alters *C. albicans* gene expression. Our enrichment analysis findings and the degree of overlapping with responses observed to *lactobacilli* and weak organic acids directed our interest toward the role of pH and stress response in this inter-kingdom interaction.

At the outset of this work we reviewed the published mechanisms of interaction between *C. albicans* and *E. faecalis*, where the secreted bacteriocin EntV was reported as a potent inhibitor of *C. albicans* hyphal morphogenesis, biofilm formation, and virulence, albeit with limited strain selection (Graham et al., 2017, Shekh and Roy, 2012). Despite this carefully characterised phenomenon we were unable to demonstrate a correlation between the expression of *EntV* and the level of inhibition of *C. albicans* biofilm biomass, suggesting that an alternative additional interaction was important. It has been reported that for optimal functionality EntV requires post-translational gelatinase processing (Brown et al., 2019), though our own assessment of gelatinase activity showed there was no difference observed between the strains with positive activity, and those lacking this property in supressing *C. albicans* biofilm (data not shown). Moreover, despite the heat inactivation and the removal of any *E. faecalis* supernatant proteins of molecular weight of more than 3kDa, *C. albicans* hyphal and biofilm formation was not recovered, again suggestive of another mechanism. EntV is a heat stable peptide, therefore, recovery of *C. albicans* biofilm activities was not anticipated following heat treatment. However, the increased inhibition associated with ultrafiltered supernatants was surprising. There is no clear explanation of why ultrafiltration enhanced biofilm inhibition, however, it is possible that the proteins and molecules from the supernatant had a subtle buffering capacity, so removal of these exposed *C. albicans* solely to a pH effect. We surmised that differences in growth media and the *E. faecalis* strains used in our work could account for the difference in findings. The ability of *E. faecalis* to metabolise nutrients and thereby produce organic acids may differ according to the media used. Metabolising THB:RPMI by *E. faecalis* resulted in a significant drop in pH after 24h. However, a similar drop may be not be achieved with other growth

media such as artificial saliva used in EntV based studies. Indeed, post-translational modification following gene expression may explain the absence of correlation between the high *EntV* gene expression in three of the *E. faecalis* strains and the levels of *C. albicans* biofilm inhibition. It is also possible that the low expression of *EntV* gene in most *E. faecalis* strains was sufficient, or reached an expression threshold, beyond which any extra expression fails to cause more inhibition of *C. albicans* biofilm formation. We cannot rule out the possibility that EntV requires undefined optimal conditions for full functionality, including a specific pH range. Therefore, it is possible that raising the supernatant pH may have impaired the functionality of *EntV* and renders it less effective in inhibiting *C. albicans* biofilm formation. However, the evidence gathered does not support previous reports showing the singular importance of EntV in preventing *C. albicans* hyphal growth and biofilm formation (Graham et al., 2017).

To investigate for alternative mechanisms, we conducted a large-scale transcriptional analysis, and in doing so revealed the possibility of pH dependent mechanisms. Amino acid starvation was excluded for the reasons explained above. Some studies reported enrichment of *C. albicans* amino acids biosynthesis/metabolism in response to acids (Cottier et al., 2015), lactic acid producing bacteria (McKloud et al., 2021) and to oxidative stress (Bruno et al., 2010, Lorenz et al., 2004). Moreover, the vast majority of enriched pathways in *C. albicans* mono-species biofilms were also enriched in the response to the weak organic acids (Cottier et al., 2015). Upregulation in arginine biosynthesis and oxidative stress related genes were also shown in *C. albicans* when internalized by macrophages (Lorenz et al., 2004). However, none of the reported oxidative stress genes in their study was upregulated in our dual-species biofilm. This further focussed the subsequent investigation to pH dependent mechanisms.

C. albicans tolerates and adapts to a wide pH range, and this dynamic feature plays an important role in changing gene expression and morphology (Davis, 2003, Hollomon et al., 2016). In an acidic environment, *C. albicans* grows as yeast cells, while neutral and alkaline environments induce filamentation (Davis et al., 2000). In low pH, *C. albicans* uses amino acids as a carbon source in glucose limiting environments and produces ammonia which in turn raises the extracellular pH and induces hyphal morphogenesis. This ability of *C. albicans* to raise the extracellular

pH was also observed in our results. As shown in Figure 5.11 and Supplementary figure 5.3, despite the early drop, the pH of dual-species biofilms started to rise at 24h. Therefore, *E. faecalis* induced inhibition of *C. albicans* can be time and media dependent where the ability of *E. faecalis* to acidify the media and the time required for *C. albicans* to raise the extracellular pH and restore hyphal morphogenesis play a major role. *C. albicans* environment alkalisation was also associated with upregulation in arginine biosynthesis (Vylkova et al., 2011). This finding further supports the close association between amino acid biosynthesis upregulation and pH response in *C. albicans*. Moreover, *C. albicans* utilisation of amino acids for environment alkalization is repressed by glucose. This may explain the reduced expression of amino acid biosynthesis and transport genes in glucose supplemented media noted in our data shown in Figure 5.7c.

As *E. faecalis* favours the growth in the yeast form of *C. albicans*, it suppresses the latter's virulence. We showed previously that *C. albicans* is more susceptible to endodontic irrigants when co-cultured with *E. faecalis* compared with its mono-species biofilm (Alshanta et al., 2020). Ishijima and colleagues (2014) also showed that oral administration of heat killed *E. faecalis*, in the form of the commercially available probiotic, protected immunocompromised mice from oral candidiasis (Ishijima et al., 2014). They reasoned this protection to the direct binding of heat killed *E. faecalis* to *C. albicans* yeast, pseudohypha and hyphae, and therefore reduces *C. albicans* adherence to surfaces. This is another proposed mechanism of *C. albicans* and *E. faecalis* interaction. Likewise, it has been shown that the survival of *Caenorhabditis elegans* was significantly higher when co-infected with *C. albicans* and *E. faecalis* compared with the infection with single species. Similar results were also reported by Cruz and colleagues (Cruz et al., 2013), which indicates that *C. albicans* and *E. faecalis* are inhibiting one another's virulence, and instead enhance commensalism. When considering endodontic infections of the root canals of teeth where these two microorganisms are frequently co-isolated from recurrent infections, this interkingdom interaction may influence their persistence in the root canals. The standard endodontic treatment regimen of root canals involves dressing the canals with calcium hydroxide as antimicrobial medicament between treatment visits. The antimicrobial efficiency of calcium hydroxide is attributed to its high alkalinity (pH 12.5) (Siqueira Jr and Lopes, 1999). Although, most endodontic pathogens are sensitive to calcium hydroxide,

C. albicans and *E. faecalis* are shown to be tolerant (Waltimo et al., 1999b). The ability of *E. faecalis* to produce lactic acid may account for its ability, *and C. albicans* through coexistence, to neutralize and survive the high alkalinity of calcium hydroxide.

Finally, we show the importance of *E. faecalis* induced pH change as inducer of *C. albicans* inhibition of hyphal and biofilm formation. However, the interkingdom interaction between *C. albicans* and *E. faecalis* is a dynamic and complicated process that is further influenced by the environment in which this interaction takes place. Therefore, it is expected that this interaction between the two microorganisms can vary in different environments such as in the root canals of teeth and the gut. It is plausible to consider that the biofilm microenvironment within root canals has a positive influence on their ability to coexist and take advantage of the dentinal tubules (Ran et al., 2015). The low pH can drive dissolution and enlargement of the tubules, which can then enhance invasive capacity. When *C. albicans* elevates the pH within this microenvironment, then its ability to induce hyphae and invade into the tubules is enhanced (Al-Nazhan et al., 2014). Furthermore, it cannot be claimed that the *E. faecalis* effect is based solely on pH and subsequently, the other reported mechanisms namely, *EntV* bacteriocin (Graham et al., 2017), anticandidal protein (Shekh and Roy, 2012) and direct binding (Ishijima et al., 2014) cannot be excluded. Notably, we observed that despite a downregulation of hyphal regulated genes *HWP1* and *ECE1*, we did not observe an expected reciprocal downregulation of the key adhesin *ALS3*. This may indicate that the presence of *E. faecalis* stimulates *C. albicans* to maintain its extracellular adhesins to support potential interactions, or at least the organisms within the same microenvironments.

Overall, it is more reasonable to consider that the effect of *E. faecalis* on *C. albicans* is a collective effect of a combination of different mechanisms that ultimately manifest as *C. albicans* inhibition. How the interaction between *C. albicans* and *E. faecalis* fits for potential antimicrobial strategies remains unclear and should be further explored. In addition, to gain a full picture of this interkingdom interaction, the impact of *C. albicans* on the biology of *E. faecalis* should be also evaluated.

5.10 Conclusions

This study highlights the importance that the microenvironment can make in driving dysbiosis or rebiosis within complex interkingdom interactions. We cannot assume antagonism from a simple phenotypic change, but instead we need to carefully assess whether this change benefits the competing species. We have shown that modulation of the pH microenvironment influences these perceived enemies to become friends (frenemies) and drive co-existence to support invasion of dentinal tubules.

Acknowledgements

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Author contributions

OAA, KA, EM and CD participated in study design, experimental procedures and data analysis, and were responsible for preparation of the manuscript. RK, WM and GR conceived the study, participated in study design, and were responsible for producing the final manuscript. All authors have read and approved the final manuscript.

Data availability

Sequenced transcriptome data has been deposited to the NCBI sequence read archive (SRA) database and can be found under accession number PRJNA731052.

Competing interests

The authors declare no competing interests.

6 General discussion

In review, the key findings of this thesis are as follows and are illustrated in Figure 6.1.

- *C. albicans* heterogeneity influences the response to endodontic treatment

- *C. albicans* tolerates NaOCl treatment and is able to regrow.

- *C. albicans* is more susceptible to endodontic irrigants when cocultured with *E. faecalis*.

- Sequential treatment with NaOCl and EDTA is more effective in controlling *C. albicans* and *E. faecalis* regrowth compared to NaOCl combined with HEDP.

- *E. faecalis* rapidly and significantly changes the *C. albicans* transcriptome.

- *E. faecalis* inhibits *C. albicans* hyphal morphogenesis and biofilm formation through a pH dependent mechanism.

Although the included publications discuss the nature of the work and the relevance of the findings to existing knowledge, there are some questions that arise in response to the work contained within the thesis.

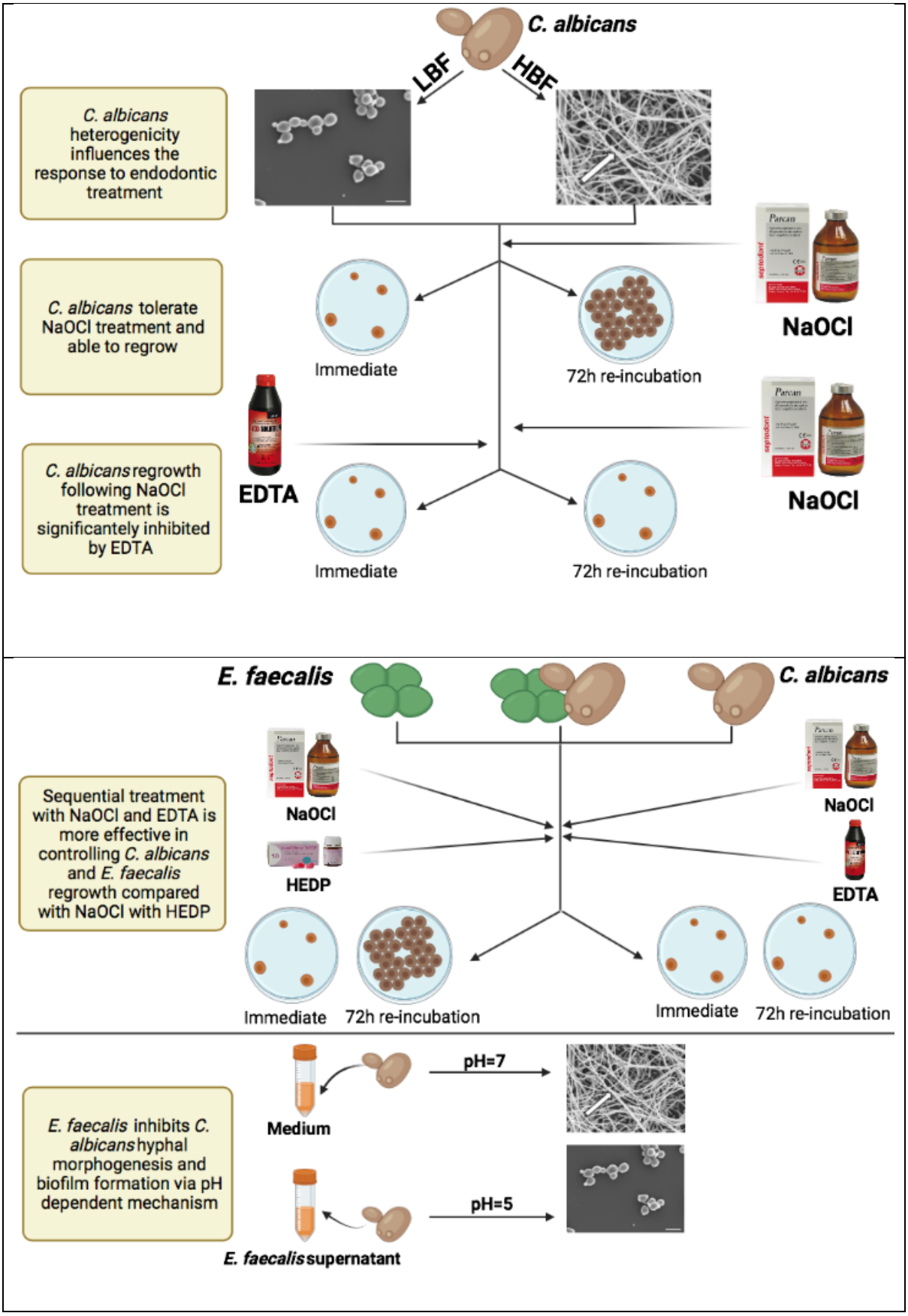

Figure 6.1: Illustration of thesis key findings. Diagram created with Biorender.com.

What is the optimal microbial biofilm model?

This thesis focused on *C. albicans* and *E. faecalis* in single and dual-species models to test endodontic irrigants. One may question why these two microorganisms were selected to represent a polymicrobial endodontic microbiota. To answer this question, it seems that the first fundamental fact to consider is that no single, or group of microorganisms, is clearly implicated in endodontic infection causation. Indeed, no single microorganism has been consistently demonstrated to be present in teeth with endodontic infection. As a result, the question arises to what extent endodontic microorganisms can be represented in an *in vitro* model. The occurrence of biofilms in root canal infections has been confirmed by microscopic histo-bacteriologic techniques with the occurrence of intraradicular biofilm being reported in 80% of studied untreated teeth with endodontic infection (Sen et al., 1995, Molven et al., 1991, Ricucci and Siqueira, 2010). In these biofilms, culture-based techniques have identified 258 taxa in endodontic infections, and 317 taxa by molecular techniques (Swimberghe et al., 2019). Clearly, in light of the large diversity of microorganisms in endodontic biofilms, modelling for *in vitro* testing is a significant challenge and in truth somewhat arbitrary.

Are multispecies models optimal?

When considering available model systems that have been used in endodontics, mono-species models have been used in the majority of studies (Swimberghe et al., 2019). Few have used dual-species or multispecies models of either defined or undefined composition. Looking at the composition of microorganisms in these studies, there is significant heterogeneity in the selected microorganisms. This variation limits the making of comparisons between these studies. Moreover, some studies have used bacteria of limited relevance to endodontic infections such as *Staphylococcus epidermidis* (Niazi et al., 2014). Undefined multispecies models using inocula from oral sites are even more variable. Samples may have been affected by various factors, such as saliva, food, and although retrieved from the oral environment, this may not mimic the endodontic environment (Swimberghe et al., 2019). Indeed, such studies lack reproducibility, and the results can be significantly different if the bacterial inoculum is obtained from other oral sites or individuals.

However, despite the aforementioned limitations in our understanding of and consistency in the make-up of the endodontic microbiota it would be reasonable to include a group of the most commonly isolated microorganism in a model system and use it for *in vitro* testing. Recently, Abusrewil et al. (2020b), reported the optimisation and use of a multi-species biofilm model for endodontic testing using common endodontic species reported by NGS studies. The model was composed of *C. albicans, Streptococcus gordonii*, *Porphyromonas gingivalis*, and *Fusobacterium nucleatum*. Abusrewil and colleagues were able not only to optimize the model to ensure optimal atmospheric conditions, media, and incubation times, but also to test it with conventional and novel therapies. The extensive optimisation work undertaken in this study sets it apart from most other studies utilising multispecies models and reflects the difficulty of designing multispecies models. It also highlights the fact that establishing optimal and testable multispecies models represents a more complex task than simply growing representative microorganisms together. Despite the extensive optimisation, it was obvious that *S. gordonii* dominated the biofilm making up ≥91.23% of organisms by number and the representation of the two anaerobes *P. gingivalis* and *F. nucleatum* was very low (0.001% and 0.63% respectively). This was attributed to the selection of CO_2 as optimal atmosphere for the model as a whole. Generally, multi-species models are more representative of the endodontic microbiota than a single species model. However, they require extensive optimisation to define an optimal environment that supports the growth of the included microorganisms. However, in the light of the limitations of endodontic sampling procedures, heterogeneity of microbiological methodology, variations in taxa and genera reported by various studies, the choice of organisms will always be controversial.

Are single species models still useful?

Single species models may be less reflective of the endodontic biofilm, but they have advantages in terms of simplicity, reproducibility, time and cost effectiveness, and the ability to create high throughput systems. The selection of a microorganism for use in a single species model can be based upon their susceptibility to a given therapy. By using this approach, resistant rather than sensitive organisms can be selected to be incorporated into multispecies models. This has resulted in *E. faecalis* being extensively used as a model organism (92%

of the mono-species studies and 79% of dual- or multispecies studies)(Swimberghe et al., 2019). The extensive use of *E. faecalis* in endodontic models is not limited to its high isolation rate from secondary infections, but also due to its high resistance to the commonly used endodontic therapeutics; mainly $Ca(OH)_2$. It was shown that *E. faecalis* can remain viable for up to 12 months in root canals *ex vivo* (Sedgley et al., 2005a). Therefore, to prove or disprove efficiency of an endodontic intervention, using resistant species such as *E. faecalis* seems more reasonable than using other more susceptible bacterial species. Resistant species are more likely to cause recurrence of the infection. Despite what seems to be a reasonable rationale for the use of *E. faecalis*, namely its high prevalence and antimicrobial resistance, some have suggested it is merely a coincidence, driven by research activity and based on preferences of research groups. Irrespective, it does serve as a rational and convenient mono-species endodontic model, or as a part of multispecies model.

C. albicans has also been isolated from root canal infections, although it is studied less thoroughly compared to bacteria. The understanding of the role of fungi in endodontic infections is in its infancy and is often a forgotten kingdom in endodontic studies. However, the presence of *C. albicans* has been shown to significantly influence the microbiome composition at different body sites including root canals (Persoon et al., 2017a). Similar to *E. faecalis*, *C. albicans* shows resistance to the intracanal medicament $Ca(OH)_2$ (Waltimo et al., 1999a). Therefore, the use of *C. albicans* to represent the fungal kingdom along with bacteria is an appropriate choice.

As highlighted early in the literature review, *C. albicans* and *E. faecalis* are often co-isolated from teeth with endodontic infections (Kovac et al., 2013, Dahlén et al., 2012). So, collectively they are members of the endodontic biofilms and their interaction may influence the disease or treatment outcome. So, in answer to the question “Are single species models still useful?”, yes, they are. However, it is clear that although they represent an appropriate starting point for investigations, they cannot match the usefulness of multi-species models and the richness of understanding that can be drawn from studies of such systems.

Do reference strains represent real *in vivo* pathogenicity?

In most research, including endodontic studies, reference laboratory strains are used (Fux et al., 2005). Using reference strains allows for standardisation and comparability, however, reference strains may be genetically different from clinical isolates. It was reported that clinical isolates of *Saccharomyces cerevisiae* were more virulent in an *in vivo* mice model compared with nonclinical laboratory strains (Clemons et al., 1994). Similarly, that clinical strains of *Mycobacterium tuberculosis* were more virulent in an infected mice model than the reference strain (Fursov et al., 2021). In addition to the present study, Alnuaimi et al. (2013) showed variability in biofilm formation, the major virulence trait of *C. albicans*, by clinical isolates and laboratory strains. Sherry et al. (2014) also revealed that *C. albicans* clinical isolates with high biofilm forming ability have higher resistance to antifungal drugs and are associated with higher virulence in an *in vivo* model. This variation in virulence and pathogenicity between reference laboratory and clinical isolates is attributed to the extraordinary plasticity of the organisms' genome. Reference strains have been sub-cultured unlimited times since their first isolation, and therefore, important pathophysiological characteristics may have been lost. Clinical isolates, on the other hand, may develop strategies to adapt to the *in vivo* environment via genetic alterations (Fux et al., 2005). Therefore, inclusion of clinical isolates is advisable to ensure that results obtained with reference strains are applicable to the *in vivo* environment. This applies to the present work where LBFs showed higher tolerance to NaOCl. Similarly, the expression of the bacteriocin *EntV* showed high variability between different *E. faecalis* strains including reference strains.

Is biofilm substrate important?

When it comes to substrates, there are several substrates used for endodontic testing. The most commonly used among the studies are extracted teeth, dentine or hydroxyapatite discs or materials. To maximise similarity with *in vivo* environment, dentine substrate would be the optimal *in vitro* choice. However, dentine is biologically active. It is shown to buffer and significantly inhibit the action of NaOCl and CHX, the commonly used endodontic irrigants (Karale et al., 2016). The presence of collagen as an organic component of dentine and adhesion target for some bacteria, such as *streptococci* influence the adhesion and biofilm formation of the tested organisms (Love et al., 1997). Furthermore, dentine structure is characterized by the presence of dentinal tubules, which were shown

to be deeply invaded by endodontic microbes including *C. albicans* and *E. faecalis*, therefore, these present a confounding factor that may affect the test results (Yoo et al., 2020, Waltimo et al., 2003, Şen et al., 1997). Extracted teeth are obtained from various individuals of different age groups, but as the dentine composition and diameter of dentinal tubules change with increase in age (Ozdemir et al., 2010), this may potentially introduce variability in results. In addition to dentinal tubules, extracted teeth have anatomical complexities such as presence of lateral canals and isthmuses which influences the outcome of tested interventions. When considering the work presented in chapter 2, chapter 3, and chapter 4, the presence of dentinal tubules and anatomical complexity would have significantly influenced our results. If extracted teeth or dentine substrate were used in this work, it would not be possible to differentiate whether the observed regrowth has arisen from tolerant treated populations or from those which evaded treatment by shielding in dentinal tubules, or inaccessible root canal complex anatomy. Although they mimic the *in vivo* scenario, dentine substrates have some limitations that should be considered when interpreting experimental results. Non-biological substrates, on the other hand, are less representative of the clinical conditions. The adhesion of organisms to these substrates can differ significantly from their adhesion to inorganic and organic components of dentine. However, they have the advantage of simplicity, standardisation and reproducibility. In contrast to opaque dentine, transparent non-biological substrates allow for direct real time visualisation of biofilms and effectiveness of an intervention. Thus, they can be useful for preliminary *in vitro* investigation and for simple testing of materials that can be subsequently verified using more relevant and complex models if required. Moreover, their use may be essential when designing experiments where any substrate introduced variation is likely to significantly influence the outcome.

Are there relevant pre-clinical models?

To establish the clinical significance of an intervention, randomised clinical trials are optimal. However, they are time consuming, pose risks, and may be associated with ethical issues (Swimberghe et al., 2019). Therefore, preceding *in vitro* and *in vivo* testing is usually essential. Animal models have been historically used for vaccines, diagnostic and therapeutic testing and it was estimated that 192 million animals were used for research purposes in 2015 (Taylor and Alvarez, 2019). Using

animal models is associated with ethical issues, however, their use may be indispensable before clinical use on humans. In endodontics, small animal models such as mice and rats are the most commonly used models (Nagendrababu et al., 2019). They are beneficial to study the pathogenesis of periapical periodontitis and the associated host response. However, the small size of teeth in these animals limits their use in root canal treatment when the use of endodontic instruments such as files is required. Large animals, such as dogs, cats, guinea pigs and rabbits have been also used, however their use is currently limited for ethical considerations (Nagendrababu et al., 2019). Animal models have been used to study pulpitis (Aubeux et al., 2021), for regenerative endodontics (Nakashima et al., 2019) and periapical periodontitis (Yoneda et al., 2017, Au - Goldman et al., 2019, Tagger and Massler, 1975, Liu and Peng, 2013, Hao et al., 2015). These studies investigated the pathogenesis, host response and treatment modalities for periapical periodontitis, including the landmark paper by Kakehashi et al. (1965). In most of these studies, the tooth pulp of the animal model was exposed to the oral microbiota to allow for the development of periapical periodontitis. Apical periodontitis involves inflammation of the periapical tissue, and in most cases resorption of the surrounding bone. Therefore, the main advantage of using animal models in studying periapical periodontitis and endodontic procedures is the ability to assess the associated host immune response that is lacking in *in vitro* models. While the interaction between microorganisms can be evaluated *in vitro* (for example *C. albicans* and *E. faecalis* of the present work), the ultimate effect of this interaction on the host and how it affects the immune response and disease progression requires *in vivo* evaluation. Among the animal models, germ free animals would be the optimal model for endodontic investigations. It allows for the introduction of preselected microorganisms, assessment of their virulence, their interaction with the host and with each other and measurement of the effectiveness of an intervention without the influence of other confounding factors.

What is the clinical significance of the presented work?

There is a wide belief in endodontics that the persistence of infection is mainly due to anatomical complexity that impedes the complete disinfection of root canals. Tolerance of the treated microorganism to endodontic irrigants is underestimated, and it is clear from the preceding, that it may influence the

persistence and recurrence of periapical infection. As shown in chapter 3 and chapter 4, access to a nutritional source enables tolerant populations to proliferate. Clinically, leaking coronal restorations or periapical inflammatory exudate may act as a nutritional source and enable persistent populations to reinfect the root canal. Occurrence of persisters is well reported for bacteria (Miyaue et al., 2018) and *C. albicans* (Wuyts et al., 2018). While the inability to disinfect the inaccessible areas of the root canals can be still considered as the principal reason for infection persistence, tolerance of the treated population should be also considered.

Clinically, in order to adopt a new treatment protocol over an existing one, the former should have superior effectiveness and superior or similar safety profile. In endodontics, as shown in chapter 4, the different endodontic irrigants may show a comparable efficacy immediately following treatment. However, expanding evaluation to test their effect on persistence and regrowth of the treated cells showed significant variation between the tested irrigants. Therefore, newly introduced procedures or agents should be critically evaluated, and multiple analyses should be conducted to capture a complete picture of their effectiveness.

Will the identification of "guilty microorganisms" change current endodontic practice?

Currently, endodontic treatment is based on non-specific killing of endodontic microbes using broad spectrum agents, mainly NaOCl. Therefore, isolation and identification of species is less likely to change current treatment protocols. Moreover, it has been found that some periapical lesions can heal despite the presence of bacteria in the root canal at the time of obturation. Sjögren et al. (1997) showed that 68% of teeth that yielded a positive culture, at the time of obturation, healed after root canal treatment. However, despite the superior antimicrobial properties of NaOCl, it has some limitations, mainly; tissue toxicity, emphysema, allergy and undesirable taste and smell (Mohammadi, 2008). There is also strong evidence suggesting that NaOCl adversely alters the mechanical properties of dentine when used as root canal irrigant (Pascon et al., 2009). Therefore, identification of novel agents with comparable antimicrobial properties and enhanced biocompatibility compared to NaOCl would be advantageous. For this purpose, identifying potentially important endodontic

pathogens would be advantageous as it can be used to test potential novel antimicrobials and compare with the gold standard, NaOCl.

Looking to the future, management of endodontic disease may well move away from the use of artificial materials to fill the root canal space. Instead, regenerative endodontics may represent the future for endodontic treatment (Zbańska et al., 2021). Regenerative endodontic procedures are based upon the concept of tissue engineering to restore the root canal system to a healthy state, by generating a functional dentine-pulp complex (Elnawam et al., 2022). Regenerative endodontics currently is the treatment of choice for immature teeth due to its high success rate (Rizk et al., 2019) and is under investigation as a potential replacement for conventional root canal treatment in mature teeth (Arslan et al., 2019). The first key element in regenerative endodontics is stem cells (Bakhtiar et al., 2018). NaOCl at the clinically used concentrations has been shown to have a profound negative effect on the survival and differentiation of stem cells of the apical papilla (Martin et al., 2014). Likewise, bacteria can directly affect stem cells and may result in inflammation in the generated tissues (Maisonneuve et al., 2020). Overall, the identification of "guilty endodontic pathogens" may not change current practice but will definitely be required when searching for alternatives to NaOCl and in testing bacterial insults to stem cells.

Does interkingdom interaction matter?

There is a potential for synergy or antagonism between microorganisms of dual- and multi-species biofilms, which in turn, may influence the response to therapeutics. As revealed in chapter 4, *C. albicans* was more susceptible to endodontic irrigants when co-cultured with *E. faecalis*. This interaction is likely to be influenced further by the endodontic environment and anatomy and may well lead to an altered host response. Furthermore, there is potential that the *C. albicans'* complex network of hyphae may provide physical support and act as a shield for the immotile *E. faecalis*, facilitating the latter's penetration into dentinal tubules. Such a phenomenon has previously been reported for *C. albicans* with *S. aureus* (Kean et al., 2017). Reciprocally, the acidic environment created by the lactic acid producing *E. faecalis* may result in dissolution of the peritubular dentine, enlarging the diameter of dentinal tubules and enhancing invasion of both organisms.

Interkingdom interactions between *C. albicans* and other bacteria have also been reported for *Pseudomonas aeruginosa* and *Lactobacilli*. *P. aeruginosa* was shown to attach and form a dense biofilm on *C. albicans* hyphae and subsequently kill the fungus. In contrast, when *C. albicans* was in the yeast form, *P. aeruginosa* neither binds to nor kills *C. albicans* (Hogan and Kolter, 2002). The same phenomenon can be applied to the observed interaction of *C. albicans* and *E. faecalis* where *E. faecalis* promotes the resistant yeast form of *C. albicans* that enhances commensalism. Furthermore, in a *Caenorhabditis elegans* model where *C. albicans* and *E. faecalis* were the sole colonizers of the gut, they enhanced each other's growth but diminished each other's virulence (Zeise et al., 2021). This finding was also reported by Garsin and Lorenz (2013).

In addition to the endodontic environment, *C. albicans* and *E. faecalis* occupy overlapping niches in the oral cavity, gastrointestinal tract and urogenital tract (Lebreton et al., 2014, Oever and Netea, 2014). There is a growing evidence that *C. albicans* can play an important role in modulating the ecology of the human gut microbiome and its associated immune response. In the gut, *C. albicans* was shown to interact with lactic acid producing bacteria, *Lactobacillus* species and *Enterococcus* species, resulting in a marked change in the dysbiotic state of these species. In particular, there was an antagonism between *Lactobacillus* species and both *Enterococcus* species and *C. albicans*. On the other hand, *E. faecalis* and *C. albicans* were shown to exhibit a mutualistic relationship.

Organism to organism interaction may not only impact the response to intervention, it may also modify the state of any restorative dental material with which they come into contact. Mineral trioxide aggregate (MTA) and Biodentine are calcium silicate based dental materials with extensive use in endodontics for various purposes including pulp capping, root end filling material and perforation sealing (Tang et al., 2019). These materials have a higher solubility in low pH (Pushpa et al., 2018). Therefore, an acidic environment may result in material dissolution and compromise material sealing properties. Furthermore, the antimicrobial characteristic of these important materials is based on their high alkaline pH (Farrugia et al., 2017). pH drop observed with *E. faecalis* cultures can negatively impact their antimicrobial properties. On the other hand, some other active antimicrobials like chitosan require acidic pH to exhibit their antimicrobial activities and pH rise results in reduced effectiveness (Fei Liu et al., 2001). For

these particular agents, acidic pH driven by *E. faecalis* may be advantageous. Abusrewil et al. (2021), showed enhanced antimicrobial properties of Biodentine when incorporated with chitosan. This combination may undergo dissolution in acidic pH in endodontic infections, resulting in continuous release of chitosan thus enhancing the antimicrobial activity of these materials.

Is *E. faecalis* induced inhibition of *C. albicans* hyphal morphogenesis dependent on EntV or pH?

It was clear that removal of EntV from *E. faecalis* supernatant through the ultrafiltration did not restore hyphal morphogenesis of *C. albicans* as the pH was still acidic. Indeed, adjusting the pH of *E. faecalis* containing EntV to pH 6 and pH 7 restored *C. albicans* hyphal morphogenesis. Considering the well reported pH dependent inhibition of *C. albicans* by the acid producing *Lactobacilli* species in the context of vulvovaginal candidiasis, it was surprising to find that the pH effect of the well-known lactic acid producing bacteria, *E. faecalis*, was largely understudied. Moreover, in EntV based studies, the pH of the supernatant was not reported.

While the acidic pH is more likely to be the mechanism by which *E. faecalis* inhibits *C. albicans* hyphal formation, the role of EntV cannot be ruled out for the following reasons. Firstly, there is a chance that Entv requires specific pH range to be functional, and therefore, pH adjustment in the present work may have resulted in loss of functionality. Secondly, in EntV based studies, artificial saliva was used and *E. faecalis* supernatant was shown to inhibit *C. albicans* hyphal formation. In the present work, the effect of artificial saliva on *E. faecalis* was not investigated. As a result, it cannot be excluded that the different conditions may drive very different modes of hyphal inhibition.

6.1 Future work

The work presented in this thesis has identified some important aspects of key microorganisms' response to endodontic irrigants. The interesting observation of the occurrence of persisters with endodontic treatment requires further analysis. How persisters are different from other populations, the frequency of their occurrence with different treatment protocols or with various strains will be

important questions to answer. Secondly, this thesis was focused upon the effect of *E. faecalis* on *C. albicans*. The other direction of this interaction is equally important. How *C. albicans* affects *E. faecalis* is worthy of investigation. The present work lacks an essential component of endodontic treatment, the mechanical aspect, whether it is debridement or activation of irrigant. Endodontic treatment is based on the chemo-mechanical disinfection of the root canals. Using endodontic instruments to mechanically debride dentinal walls is a principal step of the treatment. Activation of irrigant has also been shown to enhance the effects of the irrigants used. Incorporating a mechanical component into future analysis is required to better understand irrigant capabilities and the microbiological response. Finally, how *C. albicans* and *E. faecalis* react in the endodontic environment and how they modulate immune responses and react with the host are also important questions to explore.

6.2 Concluding remarks

This body of work represents a comprehensive investigation of *C. albicans* susceptibility to endodontic irrigants. It brings novelty to our understanding of inter-strain variability in tolerance to irrigants and the role interkingdom interactions play. It potentially reframes our understanding of how and why endodontic treatment failures occur. Historically, clinicians have highlighted the importance of the anatomical intricacies of the root canal system as a key factor in microbial persistence following treatment. It is clear from this work that even our “gold standard” irrigants fall short of the mark, with persister cells tolerant to treatment even in a model system devoid of anatomical niches. One could state that clinically we achieve a high degree of success, so does this really matter. However, bearing in mind the global burden of endodontic disease, even a 10% failure rate equates to huge morbidity and a colossal economic impact. This work paves the way for further work to elucidate intra-biofilm interactions and more specifically interkingdom interactions and the identification of novel targets for therapeutics to supplement or replace traditional clinical regimes.

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