ANALYSIS OF INTERACTIONS BETWEEN CANDIDA ALBICANS AND ENTEROCOCCUS FAECALIS IN AN IRON RICH ENVIRONMENT

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ABSTRACT

Objective: Failed root canals have been associated with a variety of microorganisms, such as *Entercoccus faecalis* (*E. faecalis*) and *Candida albicans* (*C. albicans)*. The goal of the present study is to explore the interactions between *E. faecalis* and *C. albicans*, specifically, in terms of growth, cellular morphology, cell aggregation and biofilm formation. Since much of *C. albicans'* adhesion mechanisms are regulated by high iron levels, this study also seeks to analyze the role of iron in the interactions between *C. albicans* and *E. faecalis*.

Methods: Individual cultures of *E. faecalis* and *C. albicans* as well as co-cultures of both were prepared in 1:1 mixture of brain heart infusion and RPMI media in high and low iron conditions. Growth curves were created for each culture. Biofilm formation of cultures was measured through crystal violet analysis. Sedimentation assay at OD600 was used to measure cellular aggregation. Cells were observed under microscope to view cellular interactions.

Results: Regardless of iron levels, growth of *C. albicans* was reduced when grown in co-culture with *E. faecalis* ($p < 0.0001$). Biofilm formation was reduced in cocultures of *E. faecalis* and *C. albicans* under high iron conditions, OD600=2.13, compared to low iron conditions, OD600=3.60 ($p < 0.0001$). Microscopy showed a lack of aggregation of *E. faecalis* along *C. albicans* hyphae.

Conclusion: *E. faecalis* interferes with growth of *C. albicans* in planktonic phase regardless of iron level, while limits biofilm formation only in high iron conditions.

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CHAPTER 1

INTRODUCTION

The goal of endodontic treatment is the elimination of microorganisms from the canal of the tooth. It is generally believed that failure in endodontic treatment is due to survival of microorganisms within the tooth (Baumgartner, 1991). Endodontic treatment is complicated by the fact that bacteria found within the pulpal space are present as a biofilm along the canal walls rather than as platonic cells. A biofilm is a microbial community characterized by cells that are firmly attached to a surface and each other that are embedded within a matrix (Donlan, 2002). The clinical implication of biofilms is that removal of necrotic pulp tissue is not enough to ensure treatment success. The biofilm itself must be physically removed from canal walls. Although modern instrumentation techniques have greatly enhanced one's ability to remove the biofilm, canal anatomy is irregular and files are unable to reach the biofilm in all locations of the canal. Fortunately, chemical disinfection with agents such as sodium hypochlorite give a better chance to eliminate microorganisms from inaccessible areas of the canal. However, the biofilm possesses more resistance to chemical agents than planktonic bacteria since the structure of the biofilm ensures that surficial cells are affected while underlying cells are relatively unaffected. This property makes biofilm bacteria 100 to 1000 times more resistant to antibacterial agents compared to planktonic cells (Ceri, 1999). Given the importance of biofilm to endodontic infections, understanding the interactions among the species present is of vital importance.

Given the importance of biofilms regarding failures in endodontics, great focus has been placed on understanding the biofilm in teeth undergoing endodontic retreatment. However, most studies on biofilms focus on bacteria only with relatively little emphasis on inter-kingdom interactions. Two important species found in the biofilms of failed root canals are *Enterococcus faecalis* and *Candida albicans*. *E. faecalis* is the most common bacterial species in failed root canals. Its prevalence in secondary root canal infections has been found to be as high as 77% (Rôças, 2004). *C. albicans* has been found to be the most common fungal organism found in root canal treated teeth, with a prevalence up to 18% (Egan, 2002). Both of these species are also well known for forming biofilms.

The prevalence of *E. faecalis* in failed root canals is nine times as high as its prevalence in primary infections (Rôças, 2004). This suggests that the other bacterial species present in primary infections are able to inhibit *E. faecalis* and that eliminating other species during treatment allows this species to flourish as an opportunistic pathogen. The prevalence of *E. faecalis* in secondary endodontic infections is also largely attributed to its ability to survive the harsh conditions of endodontic treatment. It is able to survive prolonged periods of nutrient deprivation within the root canal system and can use serine protease, gelatinase, and collagen-binding protein to order bind to dentin and metabolize the proteins within the organic matrix (Hubble, 2003). It is also small enough to reside within the dentinal tubules, making such cells unaffected by endodontic treatment (Love, 2001). It is also resistant to calcium hydroxide dressing (Distel, 2002). These properties make *E. faecalis* very difficult to treat.

C. albicans is rarely present in primary endodontic infections. Given the much higher prevalence in secondary infections, it is likely that *C. albicans* is introduced into

the canal system through contamination during endodontic treatment or through leakage of the coronal restoration (Waltimo, 2003). *C. albicans* is also highly resistant to the environmental conditions of root canals. When evaluating the size of hyphae, they have been found to be as small as 1.9 to 2.6 mm in diameter which gives it some ability to penetrate the dentinal tubules (Sevilla, 1986). In fact, *C. albicans* has been shown to directly penetrate the dentinal tubules (Sen, 1997). *C. albicans* is also highly resistant to calcium hydroxide, rendering disinfection difficult (Waltimo, 1999). In addition, calcium hydroxide dressing may actually display the calcium ions necessary for the growth and morphogenesis of *C. albicans* (Holmes, 1991). These characteristics help explain the prevalence of *C. ablicans* in endodontic treated teeth.

Much of *C. albicans*' growth properties are dependent on iron and current fungal research is focusing on the role of iron in *C. albicans* virulence. The importance of iron is so much so that when *C. albicans* is no longer able to utilize iron, it is rendered avirulent in systemic infections (Ramanan, 2000). Evidence linking iron to the virulence of *C. albicans* is limited, but aggravations of *Candida* spp. infections have also been reported in iron-overloaded thalassemia patients in Europe (Kontoghiorghes, 2010). Also, a positive correlation has been shown between *Candida* spp. infections and patients with iron overload (Alexander, 2006). In addition, the use of an iron chelator resulted in reduced adhesion of *C. albicans* (Puri, 2009). Therefore, it is possible that iron plays an important role in the formation of the endodontic biofilm for *C. albicans*.

Due to importance of *E. faecalis* and *C. albicans* in secondary endodontic infections, understanding the interactions between these organisms within the biofilm may yield clinically relevant findings. *C. albicans* has been shown to interact with other

bacteria. A notable example is the enhanced biofilm formation created by the interactions between *C. albicans* and *S. mutans* (Falsetta, 2014). In fact, this interaction has been shown to increase the cariogenic activity of the biofilm and can play an important role in early childhood caries (Du, 2020). Limited studies have been reported documenting the interactions between *E. faecalis* and *C. albicans*. Also, interaction studies with *C. albicans* do not tend to take iron into account.

The goal of this study is to evaluate the interaction between *E. faecalis* and *C. albicans*, specifically with respect to iron. Specifically, this study examined these interactions and the effect on growth as well as biofilm formation. In addition, cellular morphology was studied regarding cocultures of *E. faecalis* and *C. albicans*.

CHAPTER 2

MATERIALS AND METHODS

Microbial Growth Conditions

C. albicans with mCherry RFP and *E. faecalis* were streaked onto petri dishes with Yeast peptone dextrose (YPD) agar and Brain heart infusion (BHI, Sigma–Aldrich, Dorset, UK) media respectively, and then incubated at 30ºC/37ºC for 24 hr. For the growth of *C. albicans*, a single colony was incubated at 30ºC for 24 h on YNB or YPD medium with shaking at 220 rpm in an orbital shaker. *E. faecalis* strain was grown on BHI with incubation in 5% CO2 for 18h at 37ºC.

Growth Study

Two experimental sets, each with three groups, were used for this experiment. Groups consisted of *C. albicans*, *E. faecalis*, and coculture of *C. albicans* and *E. faecalis*. To achieve bacterial and fungal growth RPMI+BHI media was used in a 1:1 ratio (Alshanta, 2022). Each species type was grown in high and low iron conditions. Cells were grown overnight. A single colony of *C. albicans* or *E. faecalis* was inoculated in 10mL of 1:1 RPMI+BHI media. After growth overnight, the OD600 of the cells were determined by mixing cells in a 1:4 ratio with PBS. From those values, the appropriate volume of cells were calculated to obtain an OD600 of 0.05. Those volumes were then used when inoculating the media for the growth study. Six 10mL of RPMI+BHI media tubes were prepared, for each culture type. 500 µL of BPS was added to each solution

tube to eliminate the initial iron present. Iron was then added to the three high iron media tubes to obtain final iron concentration of 100 µM. The other tubes were used as low iron conditions. Cultures were incubated at 37ºC for 24 h with at 220 rpm in an orbital shaker.

Three 100 µL samples of each culture were taken at 0, 6, and 24 hours. Samples were serially diluted in PBS and plated onto agar plates to counting colony forming units (Thomas, 2015). Since some cultures included both *E. faecalis* and *C. albicans*, two types of plates were used to count for either bacteria or fungi. Plates to count for fungi were selective for fungal growth by using YPD agar that was prepared by adding 2.5 mL of penicillin/streptomycin per 500 mL of agar. Plates to count for bacteria were selective for bacterial growth by using BHI agar that was prepared by adding 0.5 mL of fluconazole per 500 mL of agar. Serial dilutions of each sample were plated and plates were incubated overnight. The next day, CFUs were counted on the concentration of cells to produce isolated CFUs. Total cells present in the culture were then calculated and student t test was used to assess statistical significance.

Microscopy Interaction Study

C. albicans and *E. faecalis* cells were grown under iron replete and limited conditions for 4h, as described above and observed microscopically using Zeiss Axio Imager Fluorescence Microscope at 40X magnification. Cellular morphology and interactions between *E. faecalis* and *C. albicans* were observed.

Sedimentation Analysis

For sedimentation analysis, both *C. albicans* and E. *faecalis* strains were grown under repleted and limited iron condition as described above. Next, both species were washed with PBS and resuspended in PBS to an OD600 of 0.8. 1000 µl of cells were transferred to cuvettes and absorbance was measured every 15 min over 2 hours. Sedimentation was analyzed as OD.Biofilm study

Cocultures of *C. albicans* and *E. faecalis* strains were grown under repleted and limited iron condition in RPMI+BHI media as described above. The biofilms were developed using 12-well plate and incubated for 24 hours in static condition. Three wells were used per growth conditions. Biofilms were quantified by crystal violet staining (Qian, 2020). After biofilm formation for 24 hours, the medium was pipetted out and washed with two times with 1x PBS. Further, 1 mL of 0.1% Crystal Violet was added to each well and then aside for 15 minutes. The dye was then pipetted out and thoroughly washed using 1x PBS. The well plate was air dried for 30 minutes and 2 mL 30% acetic acid was added for measurement of optical density. Mann Whitney test was used to assess statistical significance.

CHAPTER 3

RESULTS

E. faecalis growth was measured when grown in isolation and in coculture with *C. albicans* under high or low iron conditions (Figure 1). Cells grew until 6 hours, after which growth reached a stationary phase. No statistically significant differences were observed for the growth of *E. faecalis* between any growth conditions.

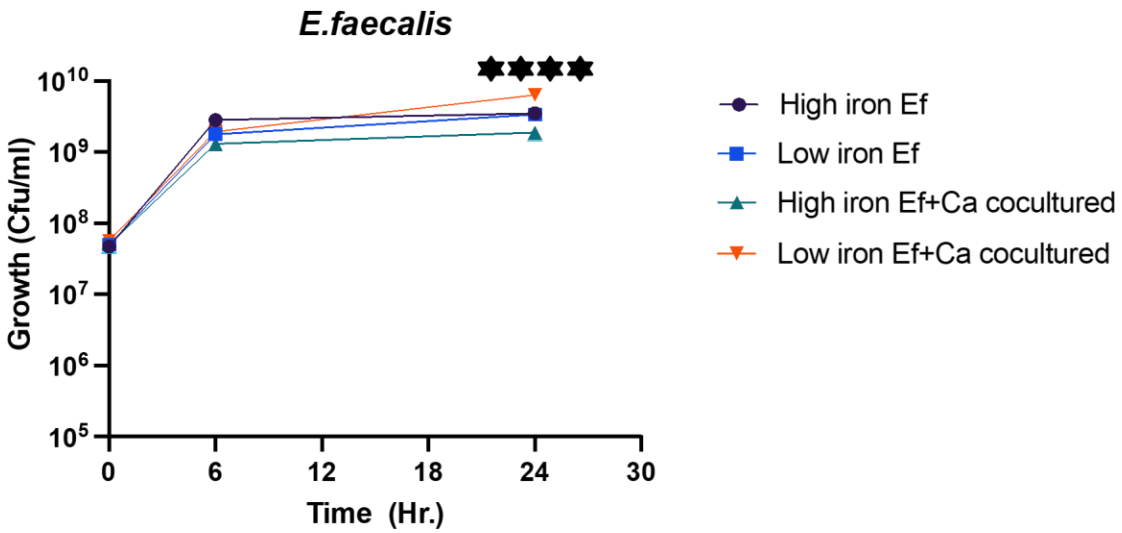

Figure 1. Growth of *E. faecalis* when grown alone or in coculture with *C. albicans* under high and low conditions over 24 hours.

C. albicans growth was measured when grown in isolation and in coculture with *E. faecalis* under high or low iron conditions (Figure 2). Growth was highest when *C. albicans* was grown alone under high iron conditions compared to low iron, close to one hundred times greater ($p < 0.001$). When analyzing growth of cocultures of *C. albicans* and *E. faecalis*, growth of *C. albicans* was much reduced compared to *C. albicans* cultures, even under high iron conditions. There was no statistically significant difference

between high iron and low iron conditions in regards to the growth of *C. albicans* when grown in coculture with *E. faecalis*.

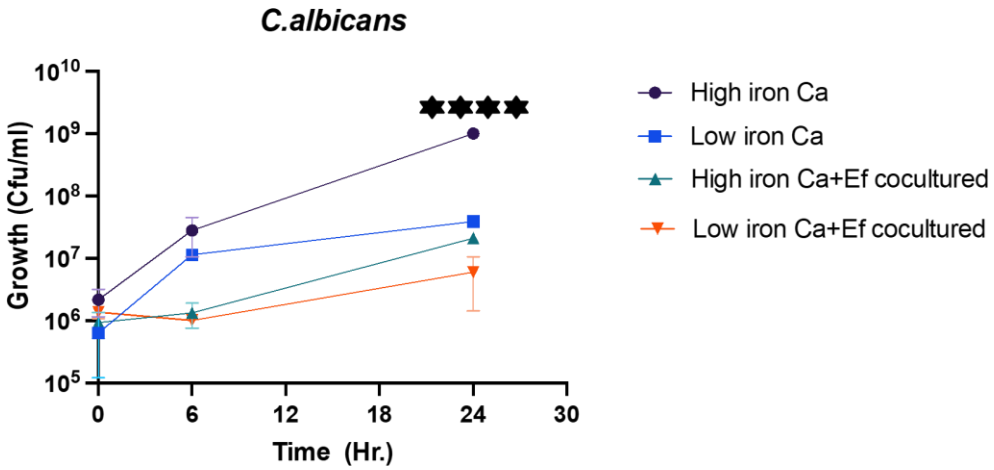

Figure 2. Growth of *C. albicans* when grown alone or in coculture with *E. faecalis* under high and low conditions over 24 hours.

Cocultures grown of *E. faecalis* and *C. albicans* in high and low iron conditions were quantified for biofilm formation. The OD_{600} for cocultures under high iron conditions was 2.12. The OD_{600} for cocultures under high iron conditions was 3.60. These results were statistically significant ($p < 0.001$).

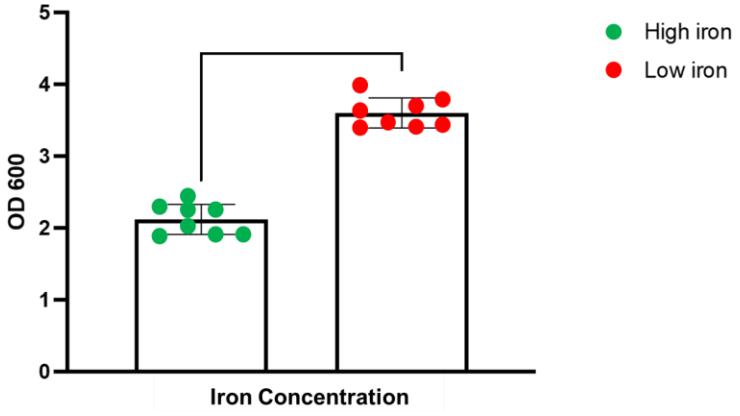

Figure 3. Biofilm quantification of cocultures of *C. albicans* and *E. faecalis* under high and low iron conditions.

Cultures of *E. faecalis*, *C. albicans*, and *E. faecalis* + *C. albicans* under high and low iron conditions were examined under fluorescent microscopy (Figure 4). *E. faecalis* consisted of cocci shaped cells. Little difference was seen between high and low iron conditions. *C. albicans* consisted of interjoining hyphae. Hyphae formation and networking was more abundant under high iron conditions. When grown in coculture, *E. faecalis* did not appear to be interacting with *C. albicans* hyphae and appeared to reside in its own space. Hyphae formation of *C. albicans* was not as prominent when grown with *E. faecalis* compared to its control.

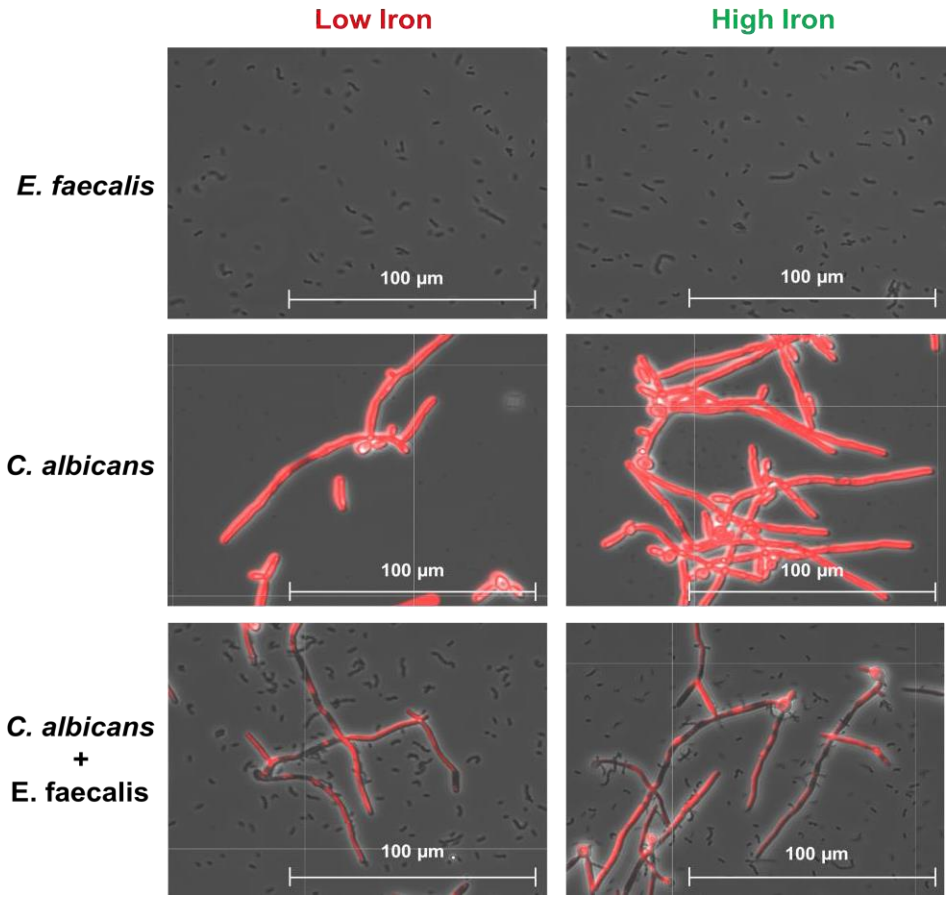

Figure 4. Morphology of cultures of *E. faecalis*, *C. albicans*, and *E. faecalis* + *C. albicans* under high and low iron conditions.

Sedimentation assay of *C. albicans* showed reduced optical density starting at 75 minutes. This suggests greater aggregation of cells under high iron conditions (Figure 5).

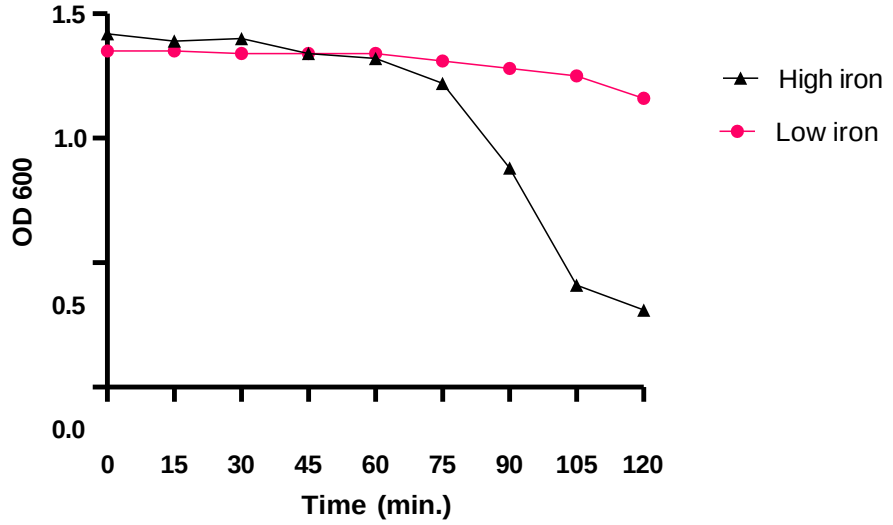

Figure 5. Sedimentation of *C. albicans* under high and low iron conditions.

Sedimentation of cocultures of *E. faecalis* + *C. albicans* was not as prominent as *C. albicans* cultures regardless of iron concentration. These results suggest that E. faecalis interfered with coaggregation of C. albicans (Figure 6).

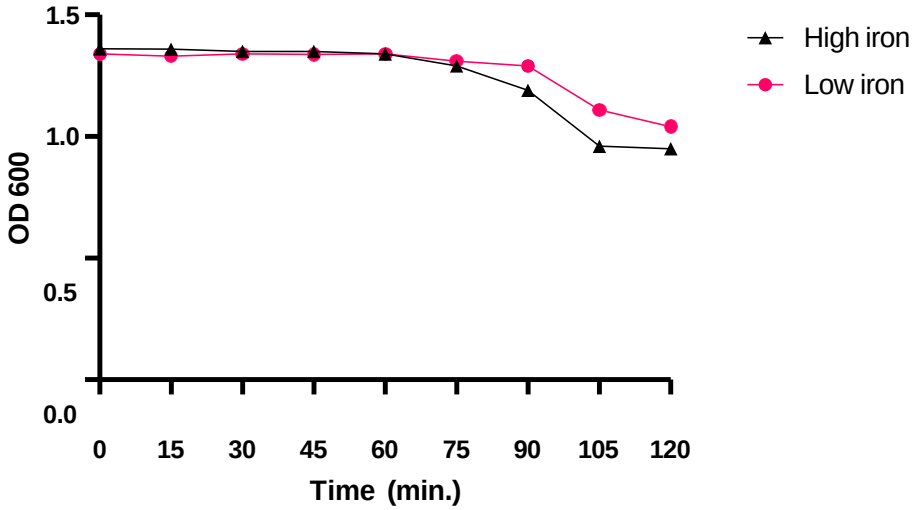

Figure 6. Sedimentation of *C. albicans* + *E. faecalis* under high and low iron conditions.

CHAPTER 4

DISCUSSION

This study found a statistically significant reduction in *C. albicans* growth when grown in coculture with *E. faecalis*. This suggests an antagonistic relationship between the two species. Moreover, this relationship appears to inhibit the capabilities of *C. albicans* rather than *E. faecalis* since growth of *E. faecalis* was unaffected throughout this study. Other studies have reported similar findings to this (Alshanta, 2022). However, other studies have not analyzed the effects of iron on this growth pattern. On the present study, reduction in growth occurred regardless of iron levels and was similar to having grown *C. albicans* in low iron conditions, meaning that high iron levels were not enough for cells to recover from this interaction. However, it is also possible that *E. faecalis* and *C. albicans* were competing for limited resources and that *E. faecalis* was able to out compete *C. albicans*, resulting in stifled growth. Further studies evaluating the mechanisms involved are required to better understand the true nature of this interaction.

Interestingly, biofilm formation was reduced in cocultures of *E. faecalis* + *C. albicans* under high iron conditions compared to low iron conditions. This is contrary to normal behavior of *C. albicans* which normally exhibits higher adhesion and biofilm formation under high iron conditions (Puri, 2019). Although not quantifiable, it was also noted during the procedure that the biofilms of cocultures under high irons conditions were more loosely attached to the wells compared to the low iron cocultures. This could be because *E. faecalis* has recently been shown to reduce the adhesion protein expression of *C. albicans* (Graham, 2017). Also noteworthy was that the hyphae formation of *C.*

albicans appeared to be reduced with grown in coculture with *E. faecalis* under high iron conditions.

In regards to endodontic infections, biofilm formation and hyphae formation are the most problematic virulence factors of *C. albicans*. Reduction in both of these when coculturing *E. faecalis* and *C. albicans* could implicate that secondary infections in which both species are present would be easier to treat than cases in which they present alone. Unfortunately, most studies on failed root canals have focused on identifying the prevalence of individual species rather than co-presentation of multiple species in failed root canals. But a future study to evaluate the prevalence of *E. faecalis* and *C. albicans* together in failed root canals could yield interesting information.

Noteworthy was that biofilm formation was specifically reduced in high iron conditions. Given the importance of the biofilm to treatment outcome, iron levels in the pulp canal space could impact the outcome of endodontic treatment. This could be relevant to patients with iron overload or iron deficiency anemia, as well as in cases of coronal leakage that could allow iron from the diet to leak into the root canal system. There are scant studies that measure the levels of iron in the pulp of vital or necrotic as well in previously treated teeth, let alone studies that link iron levels to endodontic outcome.

Although the results of this study show that there is reason to pursue future findings, some of the methodology can draw skepticism. For instance, it could be argued that the growth media that was implemented could favor bacterial growth over fungal growth, thus influencing the outcome of the study. However, other studies studying *C. albicans* in relation to *C. albicans* used the same media conditions (Alshanta, 2020).

Also, growth study of each individual species demonstrated normal growth behavior with the media conditions. Based on this reasoning, inadequate media conditions are unlikely the source of the results observed.

What cannot be ignored is that sedimentation study showed little room for interpretation since measuring optical density of cocultures did not identify whether *E. faecalis* or *C. albicans* was responsible for the increase in optical density when compared to *C. albicans* studied alone. For instance, perhaps the interaction reduced the aggregation of *C. albicans*, inhibiting cells from clumping and sinking within the tube and thereby increasing the optical density value. However, *E. faecalis* does not aggregate and because of this its very presence would cause an increase in optical density. Therefore, results of this portion of the study cannot be relied on. Results were included since it was one of the original goals of this study, but they do not contribute to the conclusions of this study.

This study shows promising implications regarding the interactions of *C. albicans* and *E. faecalis* with respect to iron. However, more studies are needed to better understand this relationship. The next step would be to analyze the mechanism responsible for this interaction and implicate specific genes. Such work would better show if these results are due to an antagonistic relationship or due to competition between the two species over limited resources.

CHAPTER 5

CONCLUSIONS

This study aimed to examine the interaction between *E. faecalis* and *C. albicans* under high and low iron conditions and the results suggest an antagonistic relationship. Growth of *C. albicans* was reduced when grown with *E. faecalis*. Biofilm formation of cocultures of *E. faecalis* and *C. albicans* was reduced under high iron conditions compared to low iron conditions. The morphology of *C. albicans* also showed less prominent hyphae formation when cultured with *E. faecalis*. Reduction of hyphae and biofilm formation may have clinical implications for secondary endodontic infection, but more studies are required to understand the precise nature of this interaction and the role of iron.

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