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The Effect of *Alcaligenes faecalis* on *Candida albicans* Biofilm and Planktonic Growth Inhibition

by

Nausheen Siddiqui

An Undergraduate Thesis Submitted in Partial Fulfillment
of the Requirements for the
Honors In Discipline Health Sciences Program
Department of Health Sciences
College of Public Health
East Tennessee State University

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Abstract

Candida albicans is a fungal microorganism commonly found on the normal flora of the human body and the environment. An opportunistic pathogen causing local and systemic infection, this fungus is one of the leading causes of nosocomial infections through contamination of inserted medical devices. More alarming is Candida's growing multi drug resistance against an already limited antifungals for treatment of infections. *Alcaligenes faecalis* is a bacterium found in the soil and is a part of the normal flora of humans, but rarely causes disease. Currently, there is very little known about how microbes interact and there is increased interest in how we can exploit microbial relationships to better human health. The present study focused on the polymicrobial interactions between *C. albicans* and *Alcaligenes faecalis.* Previous research from our laboratory has shown that *A. faecalis* exerts an inhibitory effect on *C. albicans*. While considerable data suggests this inhibitory effect is significant in planktonic cultures, there is nothing known about how these interactions would work in biofilm growth. Therefore, we examined the interactions of *Alcaligenes* and *Candida* by attempting to inhibit Candida biofilm growth with Alcaligenes. This study may provide information on potential novel therapeutic methods and targets against *C. albicans.*

Acknowledgements

First and foremost, I would like to thank Dr. Sean Fox for his support and mentorship throughout my years at ETSU as a student and researcher. He allowed me to join his research team as a newcomer and guided me through the skills and knowledge required for this project. Dr. Fox has always been receptive to questions and concerns, and fostered a positive environment of curiosity, feedback, and advice. Working on this project has taught me valuable professional and personal skills which I will utilize throughout my academic and personal journeys. I will forever be grateful for this amazing opportunity from Dr. Fox. I would also like to thank my thesis readers Dr. Powers and Dr. King for reading and reviewing my thesis report. In addition, I want to thank all the members of Fox Lab, including Robin Grindstaff, for the friendships and the memories; these I will truly treasure. Last, but not least, I would like to send gratitude towards my family, friends, and other mentors without whom this project would not have been possible.

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Introduction

Candida albicans is an elliptical, dimorphic fungal species that commonly populates the mucous membranes of the intestinal tract, vaginal, and oral cavity of the human (and mammalians in general) body. The word "*candida"* has Latin origin from the word "candidus", which means white and "*albicans"* derived from the Latin word "albico", meaning to become white (1). It is a catalase-positive, opportunistic pathogen, and is known to cause mild to severe yeast infections called Candidiasis in people who are immunocompromised.

Polymorphic Properties

A defining characteristic of this microorganism (and other yeast species) is its polymorphic property. C *albicans* can transitionally express itself as yeast cells, psuedohyphaes, true hyphaes, or chlamydaspores, depending on the environmental/host conditions present (2). Under normal conditions (room temperature, anaerobic, pH lower than 6), its phenotype is the unicellular, elliptical yeast cell formed by asexual reproduction, or budding. These cells typically have diameters of 10-12 micrometers. Upon stimulation of a variety of environmental changes (such as temperatures of 37ºC, pH of 7, production of human serum and CO_2/O_2), *C. albicans* takes on a multicellular hyphal phenotype, forming mycelial growth (continuous cell replication without budding) (5). The pseudohyphal state occurs when a pinching ensues in a yeast cell, giving the appearance of a hyphae. Clamydospores can form in extreme and harsh conditions, such as during starvation. *C albicans* can readily switch/reverse between these phenotypes in order to increase its chance of growth and proliferation (3). *C albicans* ability to switch between its polymorphic phenotypes provides a great advantage in terms of its survival, pathogenicity, and prominence.

Biofilms

Another major virulence factor of C. *albicans* is its ability to form biofilms. Biofilms are groups of cells that are surrounded by a slimy, extracellular matrix that adheres to surfaces. A common example is the dental plaque biofilm layer formed by microorganisms that reside in the mouth (4). *C. albicans* can adhere to the surfaces of living and nonliving things, increasing its pathogenicity. Biofilm formation begins with the development of the basal layer. This layer adheres to the surface of an object or mucous membranes and is made up of joined yeast cells. The next phase in this process is the division and multiplication of the hyphal cells (4). Hyphal cells are extremely important components of biofilms because they provide organized structural support systems for the various cell types that make up this biofilm.

The cells are surrounded by an extracellular matrix made of glycoproteins, polysaccharides (glucose, mannose, rhamnose), lipids, and nucleic acids. Along with components created by the cells, the biofilm can incorporate molecules from host cells and the environment. These include neutrophils, erythrocytes, and epithelial cells from host cells and *C. albicans* cells that have lysed (13). Research suggests that along with protecting the biofilm from the host, extracellular matrix has digestive properties that breakdown and bring in nutrients to support the biofilm growth and sustenance. Cells that make up biofilms can also be individually spread to other areas of the host (4). These are typically the yeast form of C *albicans* and are likely to form more biofilms once they adhere to a surface. This perpetuates in a repeating cycle of biofilm growth and dispersion that can be harmful, even deadly, to the host.

Biofilms also play a role in virulence as they are able to resist the host immune response (6). The presence of high biofilm numbers initiates the innate immune response of the host organism; these include release of neutrophils, macrophages, dendritic cells, and epithelial cells.

In addition, cell receptors such as Toll-like receptors and lectin receptors can recognize and bind to sugars in the biofilm and initiate a signaling cascade response that attracts phagocytic cells to the area. The result can be engulfment and destruction of the fungal cells. Research suggests that C. *albicans* has properties that allow it to bypass and escape from this host immune response.

One way this can occur is through preventing neutrophil activation; a molecule called glucans, which is present in the extracellular matrix of C. *albicans* has the ability to inhibit the immune activation by neutrophils. Although these immune cells recognize and surround the fungus, they cannot directly cause any damage (4). In addition, the hyphal form of C. *albicans* can escape phagocytic cells even after engulfment by breaking though the membrane of the phagocytic cell. Additional research indicates that proteins in C. *albicans* have the ability to block signal cascade pathways, antimicrobial proteins, leukocytes, and other immune cells.

Infection/Disease

Controlling and regulating the growth of C *albicans* is of high interest to researchers due to the increasing levels of infections in clinical and nonclinical practices. Candidiasis is a mild to severe infection caused by the fungus. Serious forms of candidiasis (like invasive candidiasis) can be caused by surgery, antibiotics and immunosuppressive drugs, and burns (7). Many forms of this disease can be caused by intake of various antibiotics, which results in the destruction of the surrounding commensal bacteria, which would normally work to regulate overgrowth of C. *albicans*.

The infection in the mouth, throat, and esophageal areas is called thrush, or oropharyngeal candidiasis. This is more common in babies under the age of one, and those with diabetes, dentures, cancer, or take medications that alter the environment of the oral and pharyngeal cavities. The characteristics of this form are white patches on the tongue, mouth, and

throat, swelling, diminished sense of taste, soreness/redness, and dry skin and lips. Currently the treatments include antifungals like clotrimazole, miconazole, nystatin, and for severe cases, fluconazole (8).

In women, the form of infection in the vagina is vaginal candidiasis (commonly yeast infection). *Candida albicans* causes the second most number of cases of infections of the vagina after bacterial species; the number of hospital visits due to vaginal candidiasis is estimated to be about 1.4 million (9). This infection can occur as a result of unhygienic practices, consumption of hormonal contraceptives, diabetes, or compromised immune system. The symptoms include itchy, red, and sore areas in an around the vagina, pain during urination and sexual intercourse, and unusual discharge (9). Treatment is through antifungal medicine and include fluconazole, through ingestion or direct application on the infected area(s).

Along with this superficial infections, systemic infection can also occur when the fungus enters the blood or internal organs and can cause organ failure and death (11). "*C. albicans* is a leading cause of hospital-acquired infections, it accounts for 15% of all sepsis cases, and is the cause of 40% of bloodstream infections in clinical settings" (12). The fungus can colonize many medical devices such as catheters, implants, and prosthetics. This is way for the C. *albicans* to potentially enter sensitive areas in the body and blood and can lead to systemic infection and irreversible tissue damage (10).

Due to *C. albicans's* resistance to many antifungal medications and host immune responses, and its prevalence in clinical infections, it is of high interest to researchers to devise new methods of targeting C. *albicans*.

Alcaligenes faecalis

Alcaligenes faecalis, classified as a member of the Alcaligenaceae family, is known to be an aerobic, rod bacterium, motile by peritrichous flagella. It was named "*faecalis"* after its first discovery in feces (14). It is a Gram-negative bacterium, containing a cell wall consisting of a single layer of peptidoglycan surrounded by an outer membrane made of lipopolysaccharides, phospholipids, and surface proteins. It is further characterized as a strictly aerobic microbe which grows optimally at temperatures between 20-37 °C and tests positive for both the catalase and oxidase tests (15).

A. faecalis naturally resides in soil and water, as well as in the human gastrointestinal commensals, and rarely causes systematic infections. It can, however, in rare incidents, induce infections in individuals and animals that have an immunosuppressed immune system, making *A. faecalis*, an opportunistic bacterium in certain situations (16). Some of the infections it can induce are peritonitis, meningitis, appendicitis, endocarditis, hepatitis, among others.

Like other Gram-negative bacterium, *A. faecalis* has been known to be highly resistant to many classes of antibiotic medications and agents (17). Some of the antibiotics this bacterium is resistant to are tetracycline, chloramphenicol, aminoglycosides, polymyxin, streptomycin and many others. This resistance can be a factor in explaining why eradication of *A. faecalis* in infected individuals and animals can be especially difficult (18).

Current Project

This research project was conducted to test the effect on *A. faecalis* on *C. albicans* biofilm growth. Previous work in our laboratory has shown that *Alcaligenes* exerts a strong inhibitory effect on *Candida* growth in planktonic cultures. This inhibition is through a currently unknown physical mechanism where *Alcaligenes* cells must be present to inhibit the *Candida*

cells. As a commensal microorganism of the gastrointestinal tract, *A. faecalis* may play a role in preventing the overgrowth of C. *albicans*, potentially interfering with the formation of biofilms. We therefore wanted to focus our research on three main objectives: 1) can we create *Candida* biofilms in a laboratory environment; 2) does *Alcaligenes* induce an inhibitory effect on Candida biofilms as it does on planktonic *Candida* cultures; 3) what are the characteristics of the interactions between *Alcaligenes* and *Candida*. Understanding the interaction between these microorganisms may provide new therapeutic targets for treating both planktonic and biofilm growth inhibition of *C. albicans.*

Methods

Microorganisms and culture conditions. *C. albicans* SC5314 (clinical strain), *C. albicans* (ATCC#10295, a laboratory strain), *C. albicans* yeast-locked, and C. albicans hyphae-locked were routinely grown on Yeast Peptone Dextrose (YPD) agar and broth at 37ºC. The yeast-locked and hyphae locked *Candida* strains have a mutation that inhibits them from transitioning to their different morphologies and locks them into the single morphology. *A. faecalis* (ATCC#8750) was routinely grown on Luria Bertani (LB) agar and broth at 37ºC. For co-culture experiments, *Candida* and *Alcaligenes* were grown in Brain Heart Infusion (BHI) broth. When needed, YPD agar plates supplemented with 50µg/ml of Kanamycin were used to inhibit *Alcaligenes* growth while allowing *Candida* to grow.

Effect of Heat Killed, cell free supernatant, and wild type spot test on different forms of CA

Candida strains (yeast-locked, hyphae-locked, clinical strain, laboratory strain) were inoculated into YPD broth tubes, incubated at 37ºC for 24 hours, and used to make microbial lawns on BHI agar plates. *A. faecalis* was inoculated into LB broth, incubated at 37ºC for 24 hours, and 1mL was added to four microcentrifuge tubes. One tube was centrifuged for 5 minutes at 10,000rpm,

the resulting supernatant was passed through a 0.22µm filter, and 20µl spotted onto the *Candida* lawns. The second tube was placed in 70ºC water bath for 10 minutes and 20µl spotted onto the *Candida* lawns. The third tube contained the overnight culture and 20µl was spotted onto the *Candida* lawns. The *Candida* plates were incubated at 37ºC for 24 hours and observed for zones of inhibition (ZOI)

Microscope Observation of *Candida* Biofilms *C. albicans* (laboratory strain) and *A. faecalis* were inoculated into broth media and incubated at 37°C for 24 hours to use as seed cultures for biofilm growth. Coverslips were place in the bottom wells of a 6-well cell culture plate, To use a consistent amount of cells for inoculation into the 6-well plate, the OD600 value was found using a spectrophotometer, with *Candida* wells being inoculated at an OD600 = 0.01 (~$1x10^6$ cells/ml) and *Alcaligenes* wells being inoculated at an OD600= 0.1 (~$1x10^8$ cells/ml) into BHI broth. In a 6-well plate the following combinations were made: Well 1 (control) = *Candida* only; Well 2 = *Candida* + *Alcaligenes*; Well 3 = *Candida*; Well 4 = *Candida* + *Alcaligenes*; Well 5 = *Candida*; Well 6 = *Candida* + *Alcaligenes*. This plate was incubated for 24 hours at 37°C, coverslips washed with PBS, fixed to a microscope slide, Gram stained, and observed under a 100X objective with a Zeiss light microscope.

Quantitation of biofilm formation. *C. albicans* (laboratory strain) and *A. faecalis* were inoculated into broth media and incubated at 37°C for 24 hours to use as seed cultures for biofilm growth. Coverslips were place in the bottom wells of a 6-well cell culture plate, To use a consistent amount of cells for inoculation into the 6-well plate, the OD600 value was found using a spectrophotometer, with *Candida* wells being inoculated at an OD600 = 0.01 (~$1x10^6$ cells/ml)

and *Alcaligenes* wells being inoculated at an OD600= 0.1 (~$1x10^8$ cells/ml). In a 6-well plate the following combinations were made: Well 1 (control) = *Candida* only; Well 2 = *Candida* + *Alcaligenes* (1x); Well 3 = *Candida* + *Alcaligenes* (2x); Well 4 = *Candida* + *Alcaligenes* (3x). To increase the concentration of *Alcaligenes*, cultures were centrifuged to pellet cells, the liquid portion removed, and cells were resuspended in a decreased volume of PBS, but the same volume added to the appropriate well. This plate was incubated for 24 hours at 37°C. After incubation, wells were carefully washed with PBS to remove non-adherent cells, meticulously scraped with a pipet tip to dislodge the biofilm from the sides/bottoms of the well and added to a microcentrifuge tube. The tube was serially diluted and plated onto YPD agar plates containing 50µg/ml Kanamycin. These plates were incubated for 24 hours at 37°C and colony forming units (CFU) were enumerated.

Transposon Mutagenesis of *A. faecalis* and screening for loss-of-function mutants. To create random transposon insertion mutants of *A. faecalis*, we utilized bacterial conjugation with E. coli containing pRL27. The plasmid pRL27 is a pir-dependent Tn5 transposon system that contains a hyperactive transposase and Kanamycin resistance gene. To induce conjugation, a variety of conditions were tested. The first attempt, involved adding both *A. faecalis* and *E. coli* pRL27 to LB broth, incubating the tube at 37ºC, with shaking, for 24 hours. Aliquots were plated onto LB agar containing Kanamycin & Ampicillin and incubated for 24 hours at 37ºC and monitored for positive clones. Another method involved spotting both *A. faecalis* and *E. coli* pRL27 onto LB agar plates and incubating them for 24 hours at 37ºC. The resulting co-colonies that grew were meticulously scraped, added to fresh LB broth, serially diluted, plated onto LB agar containing Kanamycin & Ampicillin and incubated for 24 hours at 37ºC and monitored for positive clones.

From either method, positive clones were re-screened on Kanamycin & Ampicillin, then screened for loss-of-function on *Candida* lawns spread on LB agar plates. Those that were identified as not having the ZOI associated with their interaction with *Candida* were further examined genetically.

Mutant Genomic DNA Purification and confirmation of transposon insertion. Potential mutant *Alcaligenes* with the Tn5 transposon and wild-type *Alcaligenes* genomic DNA was purified using the Wizard Genomic DNA Purification Kit. The purity of this DNA was measured with a nanodrop spectrophotometer. Using primers directed toward the Tn5, polymerase chain reaction (PCR) was performed and visualized on a 1% agarose gel stained with Ethidium Bromide.

Results and Discussion

Analysis of *A. faecalis* inhibition on different strains of *C. albicans*.

Previous work in our laboratory has shown that *A. faecalis* induces a strong inhibitory effect on *C. albicans* in planktonic cultures. We therefore wanted to examine this interaction in a static biofilm form of growth. To confirm that this was a physical interaction, rather than a secreted molecule. *A. faecalis* cells, heat-killed *A. faecalis* cells, and cell-free *A. faecalis* supernatant were spotted on a variety of *Candida* strains grown as lawns on agar plates. A laboratory strain of *Candida* and clinical strain of *Candida* were used to determine if this inhibition was consistent among *Candida* strains. Additionally, *Candida* is dimorphic in morphology, existing transitionally between both a yeast phase and a hyphae phase of growth. Thus, we also used *Candida* strains that have been mutated to remain in either the yeast form (yeast-locked) or hyphae form (hyphal-locked) to determine if the morphological state of *Candida* plays a role in A. faecalis inhibition. When examining the *Candida* clinical and laboratory strains, there were

robust ZOI around the *A. faecalis* cells spotted onto the *C. albicans* clinical (Figure 1A) and the *C. albicans* laboratory (Figure 1B) strains. The heat-killed *A. faecalis* cells and the cell-free *A. faecalis* supernatant produced no ZOI for the *C. albicans* clinical (Figure 1E) and the *C. albicans* laboratory (Figure 1F) strains. When examining the morphology-locked *Candida* lawns, we found the same observations with ZOI around only the *A. faecalis* cells of yeast (Figure 1C) and hyphae-locked (Figure 1D), but not the heat-killed or cell free supernatant of *A. faecalis* on yeast (Figure 1G) or hyphae-locked (Figure1H) *Candida* lawns. This indicates that the inhibition *A. faecalis* exerts upon *C. albicans* is through physical contact between the cells. Also, the larger ZOI around the yeast and hyphae-locked *C. albicans* indicates that *Candida* locked into either form makes the fungi more susceptible to this inhibition.

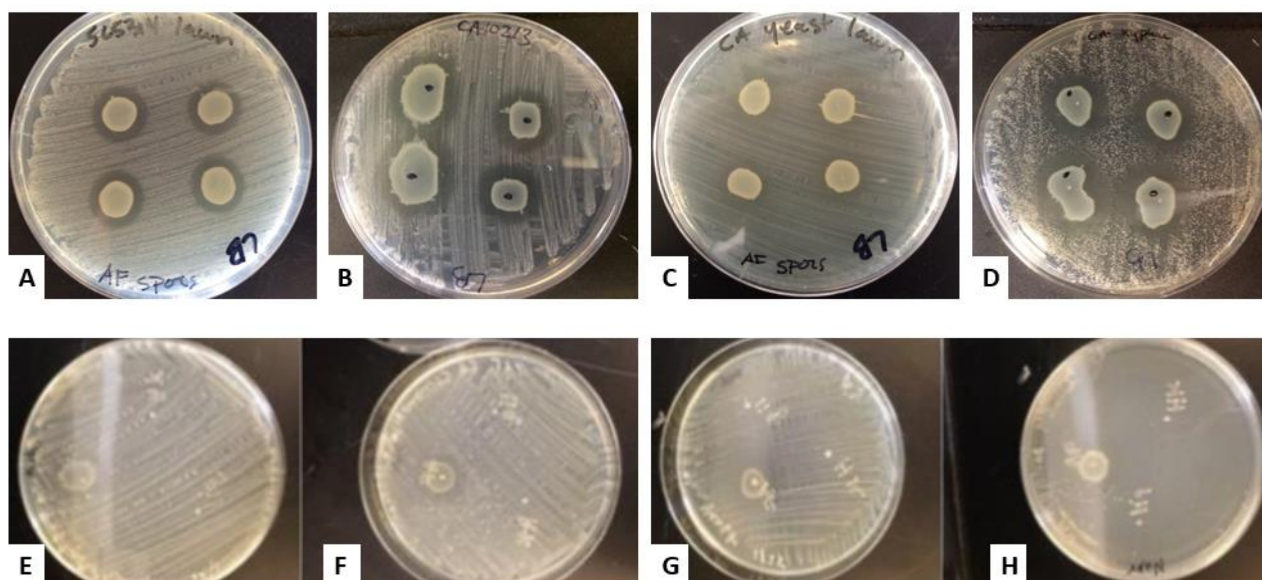
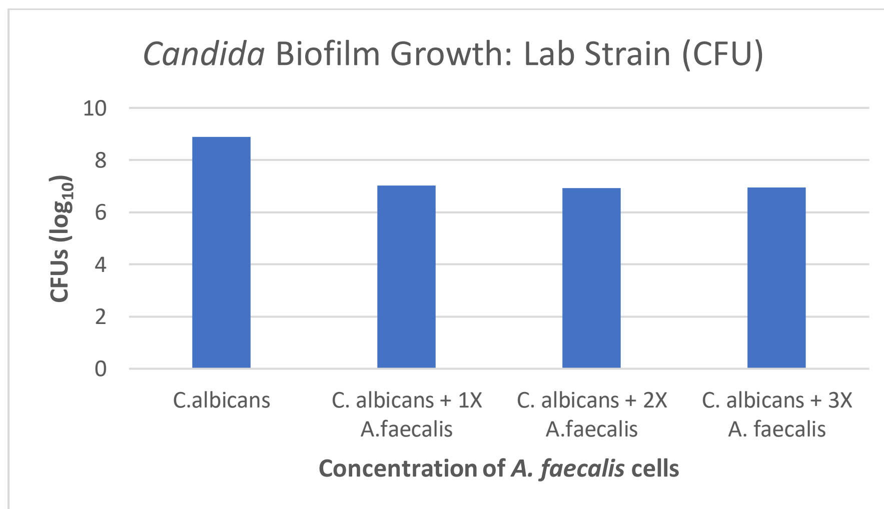


Figure 1: Zones of Inhibitions due to inhibition of *Candida* by *A. faecalis*. Observed zones of inhibition by AF onto lawns of **(A)** CA clinical **(B)** CA lab **(C)** CA yeast **(D)** CA hyphae and observed zones of inhibition by heat-killed AF and cell free AF supernatant onto lawns of **(E)** CA clinical **(F)** CA lab **(G)** CA yeast **(H)** CA hyphae

Inhibition by A. faecalis on *Candida* biofilms during the attachment phase.

Upon observing the inhibition of *A. faecalis* towards *C. albicans* on agar plates, we wanted to examine if this inhibition could also be observed in a much more complex biofilm form of

growth. Biofilms have several defined stages of growth with attachment being one of the earliest stages. By using 6-well cell culture plates, we inoculated either monocultures of *Candida* (control) or co-cultures of *Candida* with increasing amounts of *A. faecalis*, grown for 24 hours, and quantitated for the amount of *Candida* present by assessing colony forming units (CFUs). The laboratory strain of *Candida* had marked decrease in CFUs across the three concentrations of *A. faecalis* cells used (Figure 2). However, the decrease in *Candida* CFUs did not have a larger decrease as the number of *A. faecalis* cells increased. The inhibitory effect was equal among the *A. faecalis* concentrations used.



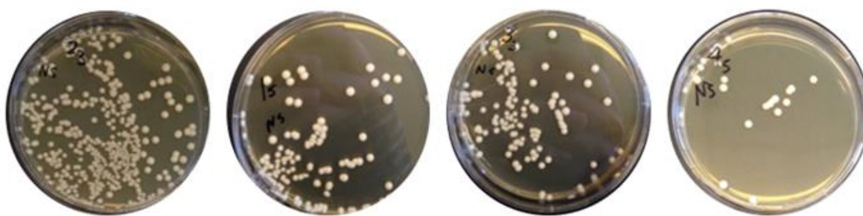
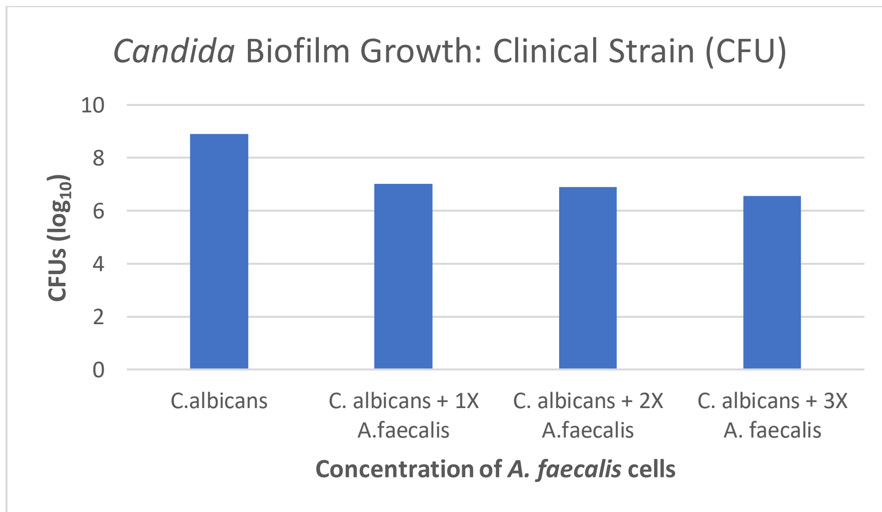


Figure 2: Concentration dependent inhibition of *C. albicans* (laboratory strain) biofilms by *A. faecalis*. Increases in the cell density of *A. faecalis* added to *C. albicans* biofilms at the attachment phase decrease the amount of Candida CFUs **(Top)** Representative photos of CFUs from serial dilutions of Candida biofilms **(Bottom).**

Taking the observations from the laboratory strain of *Candida*, we wanted to see how a clinical strain of *Candida* would respond to *A. faecalis* interactions. We wanted to try different source

strains of *Candida* because laboratory strains of microorganisms are rarely challenged which produces changes in the microorganism such as a reduction of virulence and reduction characteristics typically found in that microbe. On the other hand, clinical strains are challenged in the human body as they must combat the human body, antimicrobial treatments, and exposure to other microbes. Thus, clinical strains can have changes not only to their natural characteristics, but also changes at a genetic level. The clinical strain of *Candida* reinforced the observations found with the laboratory *Candida* strain and had decreases in CFUs across the three concentrations of *A. faecalis* cells used (Figure 3). Again, the decrease in *Candida* CFUs did not have a larger decrease as the number of *A. faecalis* cells increased and the inhibitory effect was equal among the *A. faecalis* concentrations used.



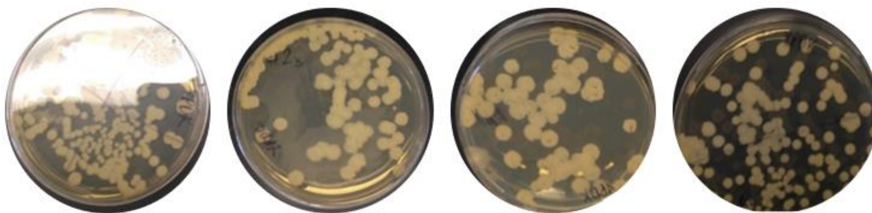
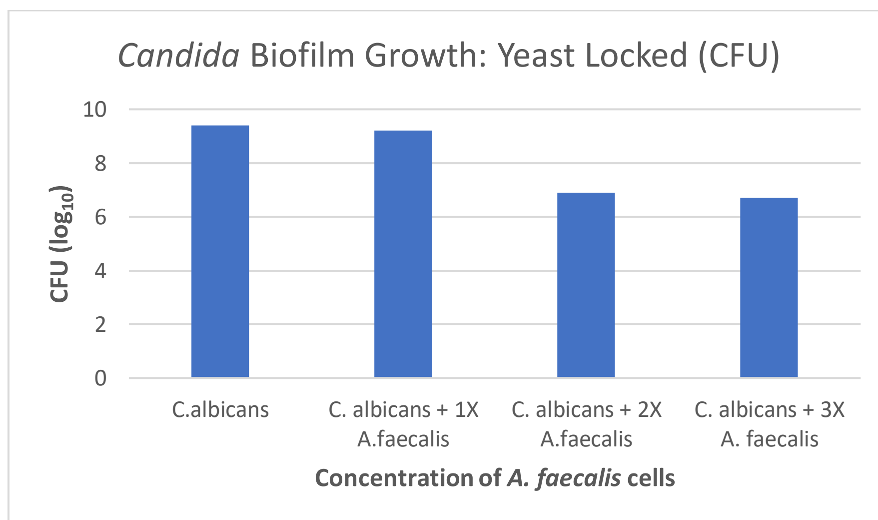


Figure 3: Concentration dependent inhibition of *C. albicans* (clinical strain) biofilms by *A. faecalis*. Increases in the cell density of *A. faecalis* added to *C. albicans* biofilms at the attachment phase decrease the amount of Candida CFUs **(Top)** Representative photos of CFUs from serial dilutions of Candida biofilms **(Bottom).**

The inhibition of both the laboratory and clinical strains of *Candida* indicate that *A. faecalis* does so ubiquitously among *Candida* strains. However, a unique and important virulence factor of *Candida* is the ability to change its morphology from a budding yeast, used for attachment and dispersion, to a filamentous hypha, used for penetrating and destruction of tissue. We therefor wanted to see if the inhibitory action of *A. faecalis* was evident on the attachment of the yeast-locked *Candida* strain which is the morphological form used for the early attachment phase of biofilm development. There is a concentration dependent inhibition of yeast-locked *Candida* by *A. faecalis*, but it required a higher concentration of *A. faecalis* to see the same inhibitory effects that we observed in the clinical and laboratory strains of *Candida* (Figure 4). This could be due to previous data in our laboratory that the yeast form of *Candida* is more of a "protected morphology" when compared to the hyphae form. Typically, different proteins are expressed in the hyphae form and the fungi is more susceptible to attack by other microbes, such as *Pseudomonas*. It could be a higher concentration of *A. faecalis* is needed to exert this inhibitory effect on the yeast morphology of *Candida*.



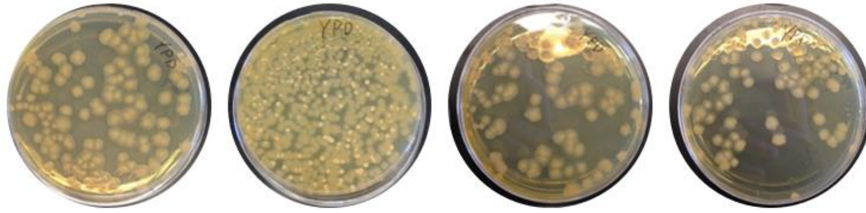


Figure 4: Concentration dependent inhibition of *C. albicans* (yeast locked) biofilms by *A. faecalis*. Increases in the cell density of *A. faecalis* added to *C. albicans* biofilms at the attachment phase decrease the amount of Candida CFUs **(Top)** Representative photos of CFUs from serial dilutions of Candida biofilms **(Bottom).**

Microscopic analysis of *A. faecalis* inhibition of *C. albicans* biofilm attachment.

The quantitative data obtained from the biofilm experiments gave us a good idea of the amount of inhibition *A. faecalis* has on the early stages of Candida biofilms. However, we wanted to visualize any of the phenotypic and morphological changes that may be occurring in the attachment phases. To this end, we added coverslips to the bottoms of 6-well cell culture plates and inoculated the wells as either monoculture (*Candida* alone or *Alcaligenes* alone) or as co-cultures. These coverslips then were Gram stained and observed for phenotypic differences. In this staining, *Candida* appear Gram-positive and *Alcaligenes* appear Gram-negative. As shown in Figure 5, *Candida* monocultures demonstrate an ability to attach to and create biofilms on coverslips (figure 5A & 5B). These biofilms have both yeast and hyphal morphologies, with extensive branching of the hyphae at this stage. *Alcaligenes* monoculture (figure 5c & 5D) also demonstrate an ability to attach to and create biofilms on coverslips. In the co-culture of *Candida* with *Alcaligenes*, we see fewer *Candida* cells (figure 5E & 5F). The *Candida* biofilms have limited, shorter hyphae that is not extensively branching. Most of the *Candida* cells appear to be in the yeast morphology. *Alcaligenes* appear to be clustered around the *Candida* cells in a physical interaction manner.

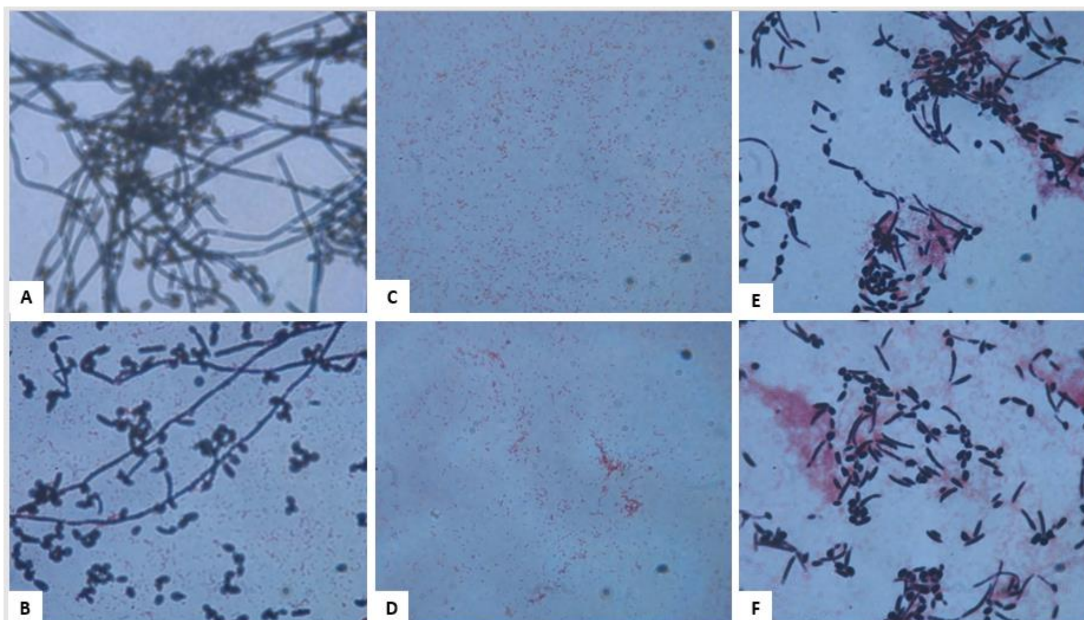


Figure 5: Inhibition of *C. albicans* biofilms by *A. faecalis*. Representative photos of monocultures of *Candida* **(A, B)**, monocultures of *Alcaligenes* **(C, D)** or Co-cultures of *Candida* with *Alcaligenes* **(E, F)** grown on coverslips.

Random transposon mutagenesis of *A. faecalis.*

The genetic elements that are responsible for the inhibitory effects of *A. faecalis* on *C. albicans* are, as of yet, unknown. To determine the possible genetic factors and pathways associated with this inhibition we utilized bacterial conjugation with *E. coli* containing the pRL27 plasmid to transfer a Tn5 transposon into the genome of *A. faecalis*. This insertion is random but can be identified by the transfer of the Kanamycin resistance gene with the transposon. Upon conjugation, cells are plated onto LB agar plates containing Kanamycin and Ampicillin. The Kanamycin will eliminate any of the *A. faecalis* that did not take up and incorporate the Tn5 transposon while Ampicillin will eliminate any of the *E. coli* in the culture. Positive potential *A. faecalis* mutants were confirmed again on LB Kanamycin/Ampicillin plates and then screened on *C. albicans* lawns. As seen in figure 6, a wild-type *A. faecalis* is placed on the lawn as a control, and potential mutants are patched onto the plate. The loss of function phenotype indicates that

the Tn5 transposon inserted into the genome somewhere vital, genetically, to the inhibition of *A. faecalis*. The genomes of these mutants were isolated, and PCR amplified, using primers directed toward the transposon, to verify the insert. Unfortunately, the potential mutants we generated were actually another bacterial species, probably a contaminant, obtained during the conjugation process.

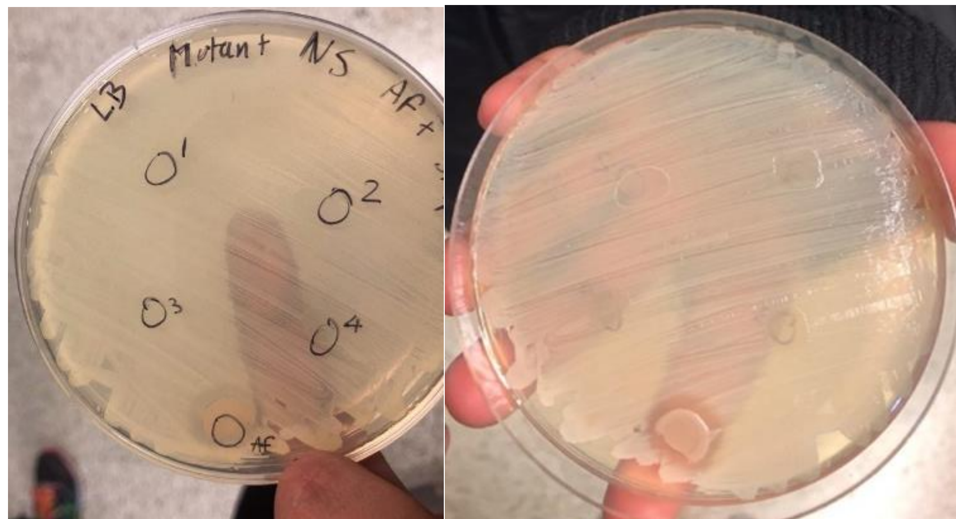


Figure 6: Generation of *A. faecalis* random transposon mutants. Potentially positive *A. faecalis* transposon mutants screened for loss of function on lawns of *C. albicans*.

Conclusion

This project focused on *C. albicans* growth inhibition with the bacteria *A. faecalis*. The growth control of *C. albicans* is of increased interest to researchers as well as clinicians due to the highly destructive nature of this nosocomial microorganism. *C. albicans* is one of the leading causes of death by blood infection in hospital settings as a result of contamination on surgical devices, catheters, and other objects. Additionally, the fungus can commonly cause various forms of candidiasis in humans and is becoming highly drug resistant. An essential virulence factor of *C. albicans* is biofilm growth. This property allows this fungal infection to attach,

persist, and rapidly spread to many parts of the body. In this study, the bacteria *A. faecalis*, through a form of physically dependent action, has shown significant inhibition of *C. albicans* biofilm growth. This inhibition initially appears to function in a cell-density dependent manner, but once saturated with cells, further inhibition is not detected. Additionally, when viewed from a microscopic level, this inhibition reduces the hyphal morphology of *Candida* cells, reduces the number of *Candida* cells present, and involves clusters of *Alcaligenes* growth around the *Candida* cells. In the future we plan to look at other forms of biofilm growth, particularly the maturation phase of *Candida* biofilms. Also of importance is the identification of *A. faecalis* mutants that cannot inhibit *Candida* to determine the mechanism of this physical interaction. In another study implementing the pRL27 Tn5 transposon system, the research group needed to screen 10,000 mutants to find the seven mutants that actually had the phenotype they were searching for. Further creation of *A. faecalis* mutants would need to be performed to saturate the *A. faecalis* genome to cover all of the genetic elements that could lead to this phenotype.

References

1. Ppdictionary.com. (2020). *Candida albicans » Fungi » Pathogen Profile Dictionary*. [online] Available at: https://ppdictionary.com/mycology/albicans.htm [Accessed 2 Mar. 2020].
2. Jones, T., Federspiel, N. A., Chibana, H., Dungan, J., Kalman, S., Magee, B. B., Newport, G., Thorstenson, Y. R., Agabian, N., Magee, P. T., Davis, R. W., & Scherer, S. (2004). The diploid genome sequence of *Candida* albicans. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(19), 7329–7334. https://doi.org/10.1073/pnas.0401648101
3. Sudbery, P. Growth of *Candida albicans* hyphae. *Nat Rev Microbiol* 9, 737–748 (2011). https://doi.org/10.1038/nrmicro2636
4. Gulati, M., & Nobile, C. J. (2016). *Candida* albicans biofilms: development, regulation, and molecular mechanisms. *Microbes and infection*, *18*(5), 310–321. https://doi.org/10.1016/j.micinf.2016.01.002
5. Sudbery, P. E. (2011, August 16). Growth of Candida albicans hyphae. Retrieved 27, 2020, from https://www.nature.com/articles/nrmicro2636?error=cookies_not_supported&code=6d076e62-f68a-4494-b4a8-2b77474670d5
6. Naglik, J. R., Gaffen, S. L., & Hube, B. (2019). Candidalysin: discovery and function in Candida albicans infections. *Current opinion in microbiology*, *52*, 100–109. https://doi.org/10.1016/j.mib.2019.06.002

7. Spampinato, C., & Leonardi, D. (2013). Candida infections, causes, targets, and resistance mechanisms: traditional and alternative antifungal agents. *BioMed research international*, *2013*, 204237. https://doi.org/10.1155/2013/204237
8. Cdc.gov. (2020). *Candida infections of the mouth, throat, and esophagus | Fungal Diseases | CDC*. [online] Available at: https://www.cdc.gov/fungal/diseases/candidiasis/thrush/index.html [Accessed 2 Mar. 2020].
9. Cdc.gov. (2020). *Vaginal Candidiasis | Fungal Diseases | CDC*. [online] Available at: https://www.cdc.gov/fungal/diseases/candidiasis/genital/index.html [Accessed 2 Mar. 2020].
10. Cheng, M. F., Yang, Y. L., Yao, T. J., Lin, C. Y., Liu, J. S., Tang, R. B., Yu, K. W., Fan, Y. H., Hsieh, K. S., Ho, M., & Lo, H. J. (2005). Risk factors for fatal candidemia caused by *Candida albicans*and non-albicans *Candida* species. *BMC infectious diseases*, *5*, 22. https://doi.org/10.1186/1471-2334-5-22
11. *Candida* Infections of Medical Devices. Erna M. Kojic, Rabih O. Darouiche
12. Delaloye, J., & Calandra, T. (2014). Invasive candidiasis as a cause of sepsis in the critically ill patient. *Virulence*, *5*(1), 161–169. https://doi.org/10.4161/viru.26187
13. Sciencedirect.com. (2020). *Biofilm - an overview | ScienceDirect Topics*. [online] Available at: https://www.sciencedirect.com/topics/immunology-and-microbiology/biofilm [Accessed 2 Mar. 2020].
14. Alcaligenes faecalis NEUF2011 - microbewiki. (n.d.). Retrieved March 3, 2020
15. ATCC ® 37159™. (n.d.). Retrieved March 3, 2020, from https://www.lgcstandards-atcc.org:443/products/all/37159.aspx?geo_country=nl

16. Center, B. S. (2015, June 30). Alcaligenes faecalis. Retrieved March 3, 2020, from https://www.bode-science-center.com/center/relevant-pathogens-from-a-z/alcaligenes-faecalis.html
17. Wisplinghoff, H. (2020). *Pseudomonas - an overview | ScienceDirect Topics*. [online] Sciencedirect.com. Available at: https://www.sciencedirect.com/topics/medicine-and-dentistry/pseudomonas [Accessed 2 Mar. 2020].
18. Clarke, M. R. G. K. R. (n.d.). An Alcaligenes Species with Distinctive Properties Isolated from Human Sources | Microbiology Society. Retrieved March 3, 2020, from https://www.microbiologyresearch.org/content/journal/micro/10.1099/00221287-40-3-343;jsessionid=oO7DKxACzdCSl4QDEFRlsxfb.mbslive-10-240-10-175?expires=1580692517&id=id&accname=guest&checksum=8FFB17BD8601CE9977CCABC68C330B53+
19. Clinical Microbiology Reviews Apr 2004, 17 (2) 255-267; DOI: 10.1128/CMR.17.2.255-267.2004
20. Nguyen, T. N., Yeh, C. W., Tsai, P. C., Lee, K., & Huang, S. L. (2016). Transposon Mutagenesis Identifies Genes Critical for Growth of Pseudomonas nitroreducens TX1 on Octylphenol Polyethoxylates. *Applied and environmental microbiology*, *82*(22), 6584–6592. https://doi.org/10.1128/AEM.01907-16
21. Seladi-Schulman, J. Candida albicans: Infections, symptoms, and treatments https://www.medicalnewstoday.com/articles/322722#other-infections (accessed Mar 2020).

22. Shapiro RS, Uppuluri P, Zaas AK, Collins C, Senn H, Perfect JR, et al. Hsp90 orchestrates temperature-dependent *Candida albicans* morphogenesis via Ras1-PKA signaling. Curr Biol. 2009;19:621–629

23. Site - pRL27. (n.d.). Retrieved March 3, 2020, from https://cgsc.biology.yale.edu/Site.php?ID=130131