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Characterizing the Interaction Between Candida albicans and Two Enterobacter Species

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Characterizing the Interaction Between *Candida albicans* and Two *Enterobacter* Species

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by

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ABSTRACT

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Candida albicans is the most common human fungal pathogen. The relationship between *C. albicans* and *Enterobacter* bacteria have yet to be explored. The hypothesis of this study is that *C. albicans* and both *E. aerogenes* and *E. cloacae* have a positive relationship and work together to infect the host. In this study, the physical cell-to-cell interaction, molecular components of said interaction, and the impact of the interaction on a live organism were explored. Results indicate that *Enterobacter* adheres to *C. albicans* and inhibits growth with unidentified secreted molecules. Als1p has potential involvement in the attachment of *E. cloacae* to *C. albicans*. Out of 480 *E. cloacae* mutants, 6 showed reductions in attachment to *C. albicans*. The presence of *C. albicans* in *C. elegans* may lead to less *Enterobacter* colonization. Future work involving this interaction should strive to identify the *Enterobacter* secreted molecules and genes necessary for their production.

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CHAPTER 1. INTRODUCTION

Polymicrobial Infections and Biofilms

The way in which pathogens are studied has evolved, from examining infections caused by a single species to research on polymicrobial interactions and their effect on infection outcomes. It is more readily accepted now that polymicrobial interactions can play a major role in infection. Pathogens, whether foreign or resident microbes, inevitably make contact with host cells and with the host's microbiota. The interactions between microbes can affect the outcome of an infection: leading to more severe illness in some cases and lessening the severity in others.

Koch's postulates are guidelines that are utilized in determining the microbial species responsible for a particular disease. The first postulate states that the microbe in question must be present in all diseased individuals but not in healthy individuals. A major issue to this postulate is the presence of opportunistic pathogens in the host microbiome. Opportunistic pathogens live commensally inside of healthy hosts, but when an opportunity arises, the microbes can become infectious. These opportunities can include immunosuppressive medications and treatments, dysbiosis in the microbiome due to antibiotics or diet, or an already present infection. Another problem with Koch's postulates is the assumption that one microbe causes one disease. This is not true for all diseases. Some diseases are caused by or exacerbated by dysbiosis of the microbiota, such as Inflammatory Bowel Disease (Singh et al. 2016). Because of this, it is important to not overlook how the interactions between microbes may play a role in infection and disease.

It has also been revealed over time that many pathogens can utilize, and may prefer, biofilm formation in order to infect, invade, and protect themselves. Unlike planktonic (free-

floating) cells, biofilms are communities of microorganisms that can attach to surfaces and are surrounded by an extracellular matrix that is composed of secreted proteins (Gulati and Nobile, 2016). These communities can consist of one species or many different species. The surfaces to which they attach themselves can be biotic, like human tissue, or abiotic, like catheters and pacemakers. In 2012, the NIH estimated that over eighty percent of infections are caused by biofilms (Fox and Nobile, 2012). Many times, biofilms offer pathogens increased protection from antimicrobials due to the thickness of the community and the presence of an extracellular matrix, making them difficult to combat.

Candida albicans

Candida albicans is a polymorphic, diploid fungus that can create biofilms and is the most common human fungal pathogen. *C. albicans* is an opportunistic pathogen that can be found living asymptomatically within the gastrointestinal tract, the genitourinary tract, and on skin of immunocompetent individuals. A study conducted from 1995-1998 over 49 hospitals across the United States found that *Candida* was the fourth most frequent cause of nosocomial (hospital-acquired) bloodstream infections, with a 40% crude mortality rate. In all regions of the United States, *C. albicans* was the most frequent of the *Candida* species (Edmond et al. 1999).

C. albicans utilizes morphogenesis to aid the switch from being a commensal organism to a pathogenic one and to form complex biofilm structures. *C. albicans* biofilm formation has four main stages: adherence, initiation, maturation, and dispersion. During the adherence stage, blastopores (yeast cells) adhere to a solid surface. This early stage lasts for roughly 6-11 hours (Hawser and Douglas, 1994; Gulati and Nobile, 2016; Uppuluri et al. 2018). In the initiation stage, yeast continue to grow, hyphae form, and an extracellular matrix of cell-wall

polysaccharides develops. This stage occurs during hours 12-24. The mature biofilm occurs approximately at 48 hours, consisting of a thick community of yeast, hyphae, pseudohyphae, and polysaccharides (Hawser and Douglas, 1994; Chandra et al 2001). The final stage, dispersion, occurs when lateral yeast cells form from hyphae and are released to seed new biofilm communities. Lateral yeast cells express many virulence genes associated with hyphae rather than planktonic yeast cells, allowing them to anticipate the need for upregulation of virulence genes without relying solely on external cues (Uppuluri et al. 2018).

Genes associated with the morphological shift from yeast to hyphae are *TUP1*, *EFG1*, and *CPH1*. *TUP1* is a transcriptional repressor of hyphal growth; deleting both copies leads to filamentation. *EFG1* and *CPH1* are both transcriptional activators of hyphal growth. As such, filamentation is decreased if both copies of either are deleted, and if *EFG1* and *CPH1* are deleted, *C. albicans* is completely impaired in its ability to transition from yeast to hyphal form, making it avirulent (Liu et al. 1994; Braun and Johnson, 1997; Lo et al. 1997; Stoldt et al. 1997; Braun and Johnson, 2000). Some of the *C. albicans* genes associated with adherence are agglutinin-like sequence genes (*ALS*) and the hyphal wall protein 1 gene (*HWP1*). These genes are important for biofilm formation, polymicrobial interactions, and virulence due to their effects on *C. albicans* adherence (Staab et al. 1999; Sundstrom, 2002; Hoyer and Cota, 2016).

Candida albicans-Bacterial Interactions

Various *C. albicans*-bacterial interactions have been studied in past research. Some of the interactions are synergistic, creating more severe infections than either species could alone. An example of such is the interaction between *C. albicans* and *Staphylococcus aureus.* The *C. albicans* Als3p aids in the attachment of *S. aureus* to hyphae, which *S. aureus* uses to enter

deeper into tissue, and together, the two cause infections of increased severity (Peters et al. 2012; Schlecht et al. 2015). It has been shown that *C. albicans* and its biofilm extracellular matrix, especially glucans, can protect *S. aureus* from vancomycin, an antibiotic commonly used to treat methicillin-resistant *S. aureus* (MRSA) (Garsin and Lorenz, 2013; Kong et al. 2016).

Interactions between *C. albicans* and bacteria can be antagonistic, such as the relationship with *Enterococcus faecalis*, which can utilize quorum sensing to inhibit hyphal formation. As a result, the interaction attenuates virulence of both *C. albicans* and *E. faecalis* in the model host organism *Caenorhabditis elegans*. Some interactions are complex and controversial, showing antagonism in vitro and synergy in vivo. *Pseudomonas aeruginosa* is capable of utilizing quorum sensing to inhibit hyphal morphogenesis and growth in vitro; however, in vivo, ventilator-associated pneumonia caused by *P. aeruginosa* is more common and more severe if the patient has *C. albicans* colonization. Due to its complexity and its importance to human health, *C. albicans*-bacterial interactions need to be explored further. This study is dedicated to understanding the relationship between *C. albicans* and two *Enterobacter* species, which has yet to be studied (Hogan, 2004; Azoubay et al. 2006; Cruz et al. 2013; Garsin and Lorenz, 2013; Hogan).

Enterobacter spp.

Enterobacter is a genus of Gram-negative, facultative anaerobic bacilli, and it is the sixth most common cause of bacterial nosocomial bloodstream infections in the US, with a crude mortality of 28% (Edmond et al. 1999). In a study of 372 candidemic patients from eight Veterans Affairs hospitals and a university hospital, it was found that *Enterobacter* was the third bacterial genus most commonly co-isolated with *Candida*, making up 12% of VA and 6.35% of

university *Candida*-bacterial co-isolations (Klotz et al. 2007). *E. aerogenes* and *E. cloacae* are opportunistic pathogens commonly found in the human gastrointestinal tract (Davin-Regli and Pages, 2015). Due to the lack of research covering the potential *Candida albicans-Enterobacter* relationship, *E. aerogenes* and *E. cloacae* are used in this study to identify what type of relationship might be present.

Goals and Hypothesis

The research goals for this project are: 1) characterize the interaction between *C. albicans* and *Enterobacter*, 2) identify genetic components involved in this interaction, and 3) determine outcomes of co-infection in-vivo. We hypothesize that *C. albicans* and *Enterobacter* have a commensal relationship and work synergistically to infect. To address the first goal, planktonic co-cultures were performed with wild-type, yeast-only, and hyphae-only *C. albicans*; biofilm co-cultures were grown with *C. albicans* either in the attachment phase or the mature phase; co-cultures and MTT assays with *Enterobacter* spent media were staged; and co-cultures to determine the effect of *Enterobacter* on *C. albicans* morphology were completed. To identify potential genetic components, ELISAs were developed involving *C. albicans ALS* mutants to determine if Als proteins are utilized in the cell-to-cell attachment. ELISAs were also utilized to screen *E. cloacae* mutants for reduction in adherence to *C. albicans.* RT-PCR analysis was used to determine if there is a change in expression of HWP1 during co-culture. To determine the outcome of co-infections, *Caenorhabditis elegans* was used as the host organism, and microbial burden assays were carried out to determine the effect of co-culture versus monoculture on *E. cloacae*.

CHAPTER 2. METHODS

Microbial Stains, Media, and Growth Conditions

Strains used in this study are found in Table 1. Unless otherwise noted, isolated colonies made from fresh freezer stocks were used to inoculate cultures. Bacterial strains were grown in LB (Luria-Bertani) broth and agar plates, Candida strains were grown in BHI (Brain Hearth Infusion) broth and agar plates or YPD (Yeast Peptone Dextrose) broth and agar. Co-cultures were performed using BHI media. When necessary, the antibiotic Kanamycin (50ug/ml) or the antifungal Amphotericin B (35ug/ml) was added to the media for selection. YENB (Yeast extract nutrient broth) was used to make electrocompetent *E. cloacae* cells. All culture were grown at 37^{o}C either statically or with shaking (160RPM). Specific growth conditions can be found in Table 2.

Table 1. Strains

Species	Strain	Description	Parent Strain	Source
C. albicans	SC5314	Wild type "CA"	Clinical Isolate	
	CAI4	Wild-type "CAI4"	CAF2-1	
	Efg1Δ/cph1Δ	*efg1/efg1;cph1/cph1*		
	BCa2-10	*tup1/tup1*		
	Δals1	*ura3/ura3 als1/als1-URA3*	CAI4	Zhao et al. 2007
	Δals2	*ura3/ura3 als2/als2-URA3*	CAI4	Zhao et al. 2007
	Δals3	*ura3/ura3 als3/als3-URA3*	CAI4	Zhao et al. 2007
	Δals4	*ura3/ura3 als4/als4-URA3*	CAI4	Zhao et al. 2007
	Δals5	*ura3/ura3 als5/als5-URA3*	CAI4	Zhao et al. 2007
	Δals6	*ura3/ura3 als6/als6-URA3*	CAI4	Zhao et al. 2007
	Δals7	*ura3/ura3 als7/als7-URA3*	CAI4	Zhao et al. 2007
E. aerogenes	ATCC 13048	Wild-type "EA"	Clinical Isolate	
E. cloacae	ATCC 23355	Wild-type "ECL"	Clinical Isolate	
E. coli	OP50	non-pathogenic strain, nematode food		
C. elegans	AU37	*glp-4; sek-1* temperature-sensitive reproduction		

Table 2. Growth Conditions

Strain	Growth Conditions (Unless Otherwise Stated)
CA	LB or BHI, 37ºC, 160 RPM
CAI4	LB or BHI, 37ºC, 160 RPM
Δ*efg1*/Δ*cph1*	LB or BHI, 37ºC, 160 RPM
Δ*tup1*	LB or BHI, 37ºC, 160 RPM
Δ*als1*-Δ*als7*	LB or BHI, 37ºC, 160 RPM
ATCC 13048	LB, 37ºC, 160 RPM
ATCC 23355	LB, 37ºC, 160 RPM
OP50	LB, 37ºC, 160 RPM
AU37	Room temp. on NGM plates seeded with OP50

Microscopy

Live/dead staining was performed on *C. albicans*, *E. aerogenes*, and *E. cloacae* using Syto9 and propidium iodide from the Invitrogen Live/Dead BacLight Bacterial Viability Kit. Mono- and co-cultures were created by growing each species separately in BHI broth overnight at 37ºC and 160RPM. Co-cultures were created by adding an equal amount of each species (OD_{600}=0.1) The mono- and co-cultures were allowed to incubate for either 4 hours or 24 hours in BHI before staining and imaging. A Leica TCS SP8 fluorescent microscope and the OLYMPUS cellSens software were used for live/dead imaging. Mono- and co-cultures were also stained using the Gram stain method and images were taken using a compound light microscope. For the Gram stain, co-cultures were made by adding *C. albicans* to a concentration of OD_{600} = 0.01 and *E. aerogenes/cloacae* to a concentration of OD_{600} = 0.1 from overnight cultures (cultured in BHI at 37ºC and 160RPM) to a fresh BHI tube. The co-cultures were allowed to incubate for 3 hours at 37ºC. Microscope slides with 10µl aliquots were Gram stained and examined.

Planktonic Co-cultures

C. albicans, *E. aerogenes*, and *E. cloacae* were each inoculated in LB and incubated overnight at 37ºC and 160 RPM. Fresh LB tubes were inoculated with: *C. albicans* alone, *E. aerogenes* alone, *E. cloacae* alone, *C. albicans* with *E. aerogenes*, and *C. albicans* with *E. cloacae*. The amount of each species added was determined using a final concentration of OD_{600} of 0.01. The tubes were incubated at 37ºC and 160 RPM for 24 hours. The samples were then serially diluted 1:10 with phosphate buffered saline (PBSx1, pH 7.4), and 100µL of the dilutions were plated on the following media to count colony-forming units: LB agar, YPD agar or LB agar with 50µg/mL Kanamycin (YPDK and LBK, respectively), or LB agar with 35µg/mL Amphotericin B (LBAB). The same procedure was used for the planktonic co-cultures with Δ*efg1*/Δ*cph1* and Δ*tup1*.

Cell-Free Spent Media Co-cultures

C. albicans, *E. aerogenes*, and *E. cloacae* were inoculated into separate LB broth tubes and incubated for 24 hours at 37ºC and 160 RPM. The *E. aerogenes* and *E. cloacae* cultures were placed into centrifuge tubes and spun for 5 minutes at 10,000-12,000 RPM. The supernatants were then filtered through a 0.22 µm syringe filter. The *C. albicans* was added to three tubes consisting of: LB broth, 1:1 LB/ *E. aerogenes* spent media, and 1:1 LB/ *E. cloacae* spent media. The amount of *C. albicans* added was determined using a final concentration of OD_{600} of 0.01. The samples were incubated for 24 hours at 37ºC and 160 RPM. The samples were serially diluted 1:10 with PBS and plated on either LB or YPDK.

MTT Assay

The same protocol was used in preparing the spent media as described previously. Using a 96-well plate, 100µL of sample and 10µL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were added to the wells. The wells were incubated for 30 minutes at 37ºC. To the samples, 100µL PBS and 50µL isopropanol were added. The absorbance was read using a plate reader set at a wavelength of 590nm.

Biofilm Co-cultures: Attachment Phase

C. albicans, *E. aerogenes*, and *E. cloacae* were each inoculated into BHI tubes and incubated for 24 hours at 37ºC and 160 RPM. To each well of a 6-well plate, 2mL of BHI broth was added along with each of the following samples: *C. albicans* alone, *E. aerogenes* alone, *E. cloacae* alone, *C. albicans* with *E. aerogenes*, or *C. albicans* with *E. cloacae*. The amount of each species added was determined using a final concentration of OD_{600} of 0.01. The plate was incubated at 37ºC for 24 hours. The liquid was gently removed without touching the sides of the wells. To each well, 1 mL of PBS was added by allowing it to run down the sides of the wells to keep from disturbing the attached cells. The PBS was then gently removed. Another 1mL of PBS was added, and the attached cells were meticulously scraped into the PBS. The liquid was then placed in a microcentrifuge tube, serially diluted 1:10 with PBS, and plated onto LB, YPDK, or LBAB plates.

Biofilm Co-cultures: Mature Phase

C. albicans was inoculated into a BHI tube and incubated overnight at 37ºC and 160 RPM. The *C. albicans* was added to 2mL of BHI broth in a 6-well plate, which was incubated

for 24 hours at 37ºC. The amount of *C. albicans* added was determined using a final concentration of OD_{600} of 0.01. *E. aerogenes* and *E. cloacae* were incubated in BHI tubes overnight at 37ºC and 160 RPM. OD_{600} absorbance readings of *E. aerogenes* and *E. cloacae* were taken to create subcultures of approximately 10^6 cells/ml (OD_{600}=0.01). The BHI in the 6-well plate was removed, and 2mL of fresh BHI were added. The *E. aerogenes* and *E. cloacae* were added to their respective wells already containing *C. albicans*. The plate was incubated again for 24 hours at 37ºC. The liquid was gently removed without touching the sides of the wells. To each well, 1 mL of PBS was added by allowing it to run down the sides of the wells to keep from disturbing the attached cells. The PBS was then gently removed. Another 1mL of PBS was added, and the attached cells were meticulously scraped into the PBS. The liquid was then placed in a microcentrifuge tube, serially diluted 1:10 with PBS, and plated onto LB, YPDK, or LBAB plates.

Yeast/Hyphae Ratio

C. albicans, *E. aerogenes*, and *E. cloacae* were each inoculated into YPD broth tubes and incubated at room temperature for 24 hours. The samples were used to inoculate fresh YPD tubes containing 20% horse serum and *C. albicans* alone, *C. albicans* with *E. aerogenes*, or *C. albicans* with *E. cloacae*. The amount of each species added was determined using an OD_{600} of 0.01. The tubes were then incubated at 37ºC and 160 RPM for 24 hours. At 4 hours and 24 hours, 10µL of each sample were placed on microscope slides. The number of yeast, pseudo hyphae, and hyphae were counted (either to 100 total if the number of *C. albicans* was low or by choosing 10 random positions if the number of *C. albicans* was too high to count them all) using a compound microscope and the Micron (USB2) software.

RT-PCR

C. albicans and *E. cloacae* were incubated separately overnight at 37ºC and 160 RPM. A monoculture of *C. albicans* and a co-culture of *C. albicans* and *E. cloacae* was created and allowed to incubate at 37ºC and 160 RPM for 2 hours. RNA isolation was completed using the Promega SV Total RNA Isolation kit. Equal amounts of isolated RNA (~500ng) from each sample were added to separate PCR tubes, along with other substrates shown in Table 4 to create the RT-PCR mix. The tubes were heated to 65ºC for 5 min and placed on ice for at least 1 min. The New England BioLabs Inc. One *Taq*® One-Step RT-PCR Kit used to perform reverse transcriptase polymerase chain reaction. The forward and reverse primers used in the RT-PCR reaction are listed in Table 2. The protocol for the reaction is as follows: 48°C for 5 min, 94°C for 1 min, [94°C for 15s, 55ºC for 30s, 68ºC for 1min]x40, and 68ºC for 5 min. A 1% agarose gel electrophoresis was performed on the PCR products and imaged using a Bio-Rad ChemiDoc™ Imaging System. Gel bands were measured using ImageJ.

Table 3. RT-PCR Primers and Sequences

Primer	Sequence
HWP1fRT (fwd)	5'-CTC CAG CTG GCT CAA GTG GT-3'
HWP1rRT (rev)	5'-TGG CAG ATG GTT GCA TGA GT-3'
18SrRNAfRT (fwd)	5'-GTG CCA GCA GCC GCG GTA-3'
18SrRNArRT (rev)	5'-TGG ACC GGC CAG CCA AGC-3'

Transposon Mutagenesis

The plasmid used in transposon mutagenesis was the pRL27 (CGSC 12551), a Kan^r, mini-Tn5 transposon (oriR6K) delivery vector (Larsen et al. 2002). *E. cloacae* was cultured in yeast extract nutrient broth (YENB) for 24 hours at 37ºC and 160 RPM. A 1:100 dilution was

made by adding ECL to fresh YENB, and Ethylenediamine tetra acetic acid (EDTA, pH 8.0) was added for a final concentration of 0.7mM EDTA. The subculture was incubated at 37ºC and 160 RPM for 24 hours (OD_{600}= 0.4-0.6). The subculture was centrifuged for 5 minutes at 5,000 x g, discarding the supernatant each time. The pellet was resuspended in 1mL of nuclease-free distilled water and centrifuged at 5,000 x g for 5 minutes twice, discarding the supernatant each time. The pellet was resuspended in 30µL of nuclease-free distilled water and 1µL of pRL27 plasmid was added. The sample was electroporated (set V: 2500, actual V: ~2410, time constant: ~5.0-5.2). Immediately after electroporation, 1 mL of 37ºC SOC was added. The sample was incubated at 37ºC and 160 RPM for 45 min to 1 hour. LBK plates were utilized to screen for Kanamycin resistance. Random mutants were selected to undergo PCR to make certain the transposon was present in the mutants (Table 4). The PCR reaction was performed as follows: 95ºC for 5 min, [95ºC for 30s, 52ºC for 30s, 72ºC for 1 min]x30, and 72ºC for 5 min. Each PCR tube contained: 12.5µl of Taq Master Mix 2x, 0.5 µl of forward primer, 0.5µl of reverse primer, 13.5µl of distilled H_2O, and a toothpick tip of bacteria. To ascertain if mutants were deficient in adherence to *C. albicans*, ELISAs were performed with *C. albicans* (SC5314), as described below.

Table 4. PCR Primers and Sequences

Primer	Sequence
tpnverifyF (fwd)	5'-AGG GCT TCT CAG TGC GTT AC-3'
tpnverifyR (rev)	5'-TCG CGA GCC CAT TTA TAC CC-3'

ELISA

CAI4 and Δ*als1*-Δ*als7* were grown overnight in BHI at 37°C and 160 RPM. The following day, OD_{600} readings were used to create 100µl subcultures of the *C. albicans* strains in

BHI in a 96 well plate (OD_{600}=0.1). The plate was incubated for 24h at 37°C and 160 RPM. *E. cloacae* was grown in BHI overnight and diluted to an OD_{600} of 0.1. The wells were washed gently with 100µl of wash solution (PBS, 0.05% Tween, 1% bovine serum albumin) and blocked for 1h at room temperature with 100µl of 1% bovine serum albumin (BSA). Block solution was removed, and wells were washed with 100µl PBS. PBS was removed and 100µl of *E. cloacae* (OD_{600}=0.05) was added to each well. The plate was incubated for 1 hour at room temperature. Liquid was removed, and the wells were washed with 100µl of the wash solution. Wash solution was removed and 100µl of 1:1000 (diluted in PBS) monoclonal mouse anti-*Enterobacter cloacae* IgG conjugated with horse radish peroxidase was added to each well. The plate was incubated at 4ºC overnight or 1-2h at room temperature. Antibodies were removed, and wells were washed with 100µl PBS. To each well, 100µl 3,3',5,5'-Tetramethylbenzidine (TMB) was and incubated for 5 minutes at room temperature. Immediately, 100µl of stop solution (1M H_2SO_4) was added to the wells. Absorbance was read at 550nm wavelength on the BioTek Synergy LX Multi-Mode Microplate Reader.

Crystal Violet Assay

C. albicans strains used in the ELISA were grown overnight in BHI at 37°C and 160 RPM. The following day, OD_{600} readings were used to create 100µl subcultures of the *C. albicans* strains in BHI in a 96 well plate (OD_{600}=0.1). The plate was incubated for 24h at 37°C and 160 RPM. The following day, liquid was removed, and the wells were washed with PBS. The PBS was removed, and 0.1% crystal violet was added to each well for 5 minutes. Crystal violet was removed, and the wells were washed with PBS. The wells were allowed to dry, and

30% glacial acetic acid was added to the plates before they were read at 590nm using a spectrophotometer.

Microbial Burden

Wild-type *C. albicans*, *E. aerogenes*, and *E. cloacae* were grown in BHI overnight at 37°C and 160 RPM. OD_{600} readings were taken to standardize (OD_{600}=0.01). In wells of a 6-well plate, 2ml solution of 80% M9 and 20% BHI was added. Nematodes were washed with M9 and a 40µm sterile cell strainer. In mono-culture controls, washed nematodes were added to the solution with either *C. albicans*, *E. aerogenes*, and *E. cloacae* and allowed to feed for 4 hours. In the co-cultures, nematodes were added to either *C. albicans*, *E. aerogenes*, and *E. cloacae* and allowed to feed for 2 hours. After which, the worms were washed, and nematodes that had been treated with CA were placed in wells containing either *E. aerogenes*, and *E. cloacae* for 2 additional hours. Half of the nematodes that had been treated with *E. aerogenes*, and *E. cloacae* were added to wells with *C. albicans* for 2 hours; the other half were washed and collected as *E. aerogenes*, and *E. cloacae* controls. From each well, an equal number of live nematodes were collected and added to a microcentrifuge tube, along with a solution of 1% Triton X and M9. The tubes were centrifuged at 400 x g for 2 min and vortexed for 2 min to lyse the nematodes. The lysate was then serially diluted with PBS and plated on LB plates with 30µg/ml Amphotericin B and 100µg/ml Ampicillin (LBABA plates).

Statistical Analyses

All statistical analyses were performed using GraphPad Prism 9.3.1. Data was first tested for outliers, parametric distribution, and significant difference in standard deviations. In cases involving multiple T tests or Mann-Whitney tests on the same data, the Bonferroni correction was utilized to correct for potential Type I Errors:

$$\alpha_{Bonferroni} = \frac{\alpha}{number\ of\ comparisons}$$

CHAPTER 3. RESULTS

Enterobacter Kills C. albicans

The interaction between *Enterobacter spp.* and *C. albicans* was first noticed in a Gram stain. Figure 1 is a replication of this discovery. Figure 1A and 1B shows wild-type *C. albicans* with *E. cloacae* and *E. aerogenes*, respectively, and Figure 1C and 1D shows Δ*tup1* with *E. cloacae* after 3 hours. *E. aerogenes* and *E. cloacae* appear to surround and adhere to *C. albicans* in both yeast and hyphal form. To further visualize the interaction between *C. albicans* and *Enterobacter spp.*, monocultures and co-cultures were stained with Syto9 and propidium iodide. Syto9 stains all cells green, and propidium iodide is a membrane impermeable DNA stain. Because of this, green cells are considered alive, and red cells are considered dead in overlapped images. Fluorescent images of the live/dead stain shown in Figure 2 shows that after 4h alone, *C. albicans* is completely alive and grows primarily in yeast form (Fig2A). When co-cultured with *E. aerogenes* for 4h, *C. albicans* showed both live and dead cells, and *C. albicans* and *E. aerogenes* formed large clumps (Fig2B). When co-cultured with *E. cloacae* for 4h, the same outcome occurred. *C. albicans* fluoresced red and green, and *C. albicans* formed clumps with *E. cloacae.*

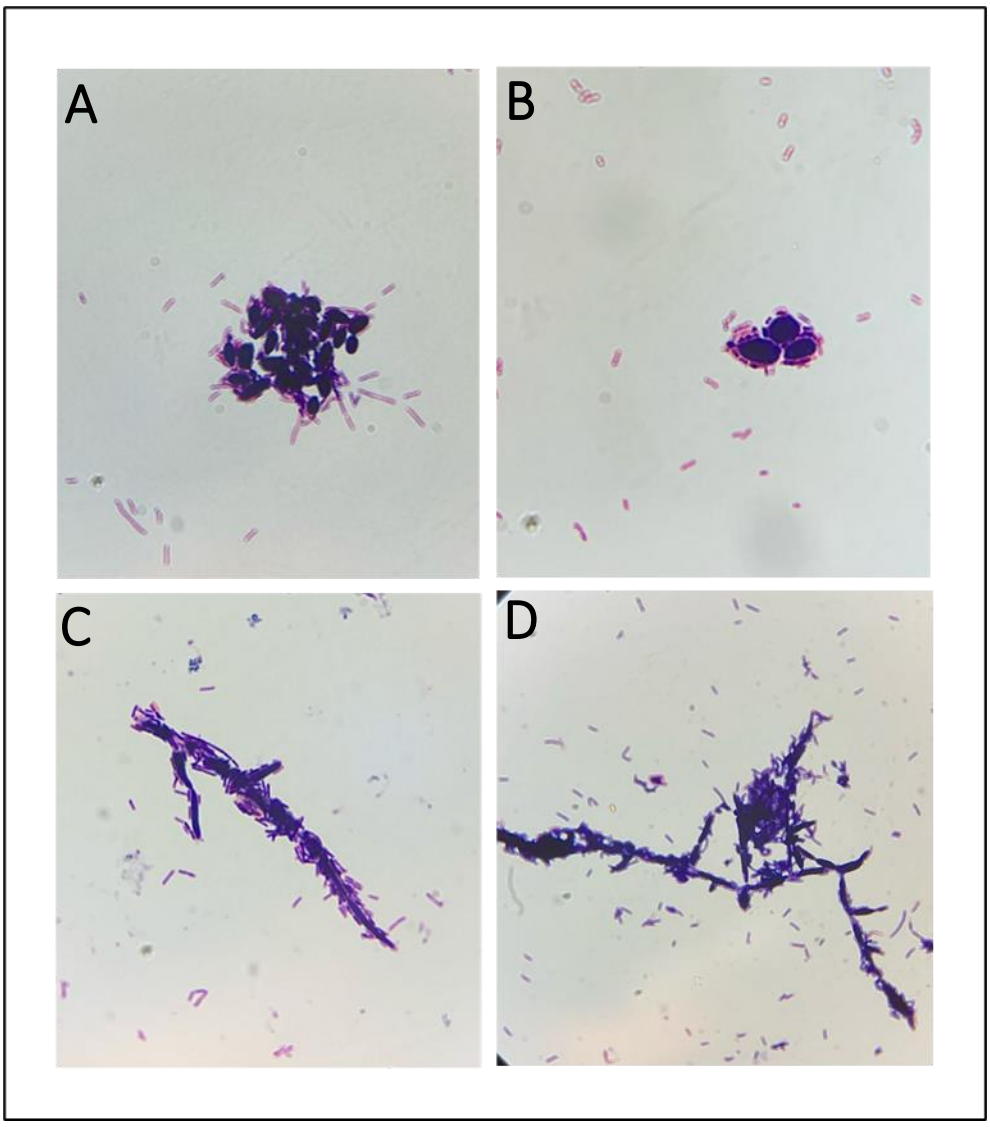

Figure 1. Gram Stain. *C. albicans* co-cultured with *E. cloacae* (A) and *E. aerogenes* (B) for 3h at 37ºC, Δ*tup1* co-cultured with *E. cloacae* (C-D) for 3h at 37°C. All samples were stained using the Gram stain method, and images were taken at a 100x objective lens on a compound microscope

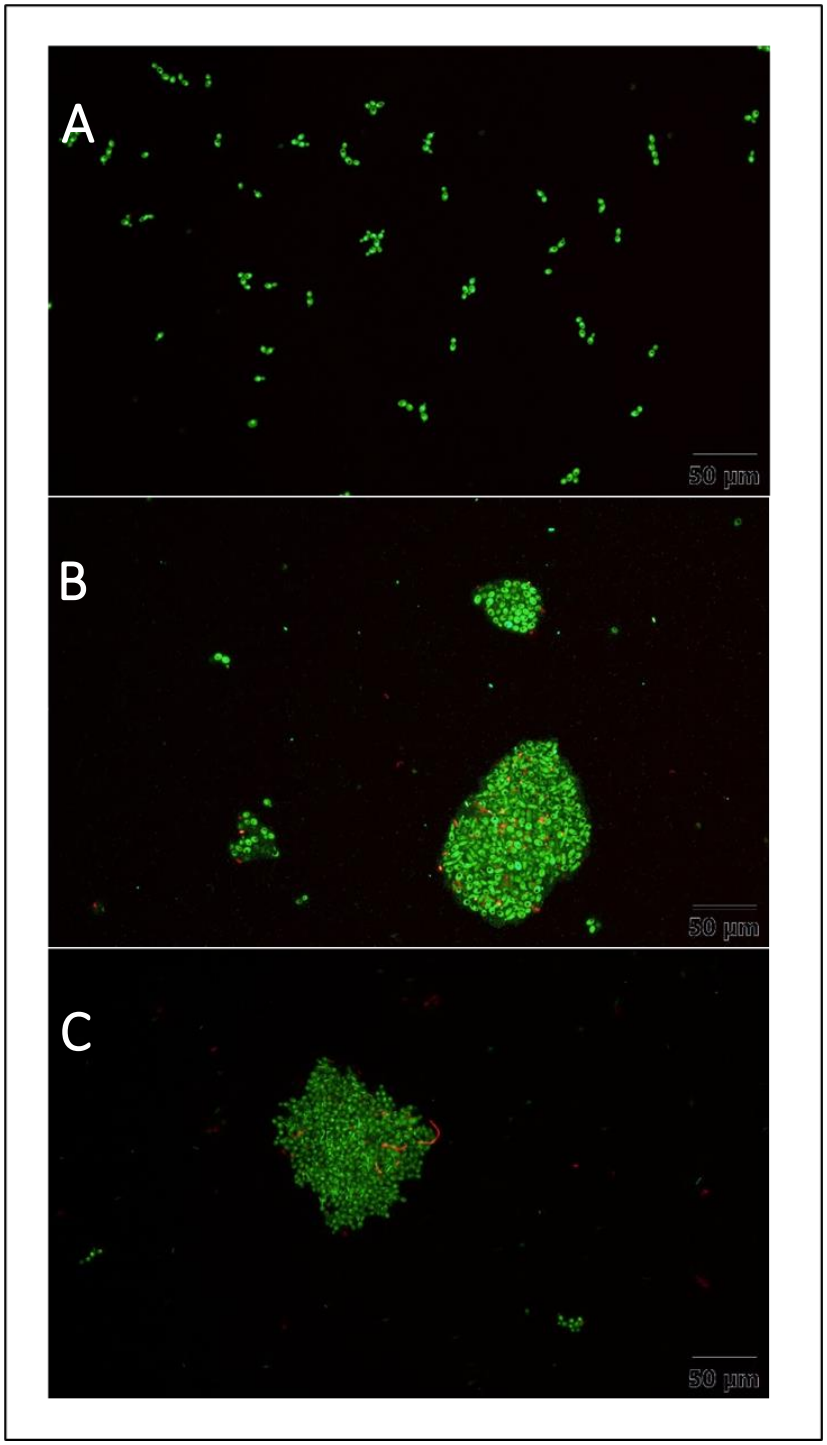

Figure 2. Live/Dead Staining. Syto9 stained all cells (green), and propidium iodide stained dead cells (red). (A) C. albicans cultured alone for 4h and imaged at 20x objective lens, (B) C. albicans co-cultured with E. aerogenes for 4 h and imaged at 20x objective lens, (C) C. albicans co-cultured with E. cloacae for 4h and imaged at 20x objective lens

Enterobacter Inhibits C. albicans Growth in All Morphologies

In order to measure the effect of *E. aerogenes* and *E. cloacae* on *C. albicans* (and vice versa) in a planktonic setting, monocultures and co-cultures were grown, diluted, and plated on selective media. Colony-forming units were counted, and calculations were performed to approximate the number of cells in the undiluted samples. Results from planktonic co-cultures of wildtype *C. albicans* and *E. aerogenes* (Fig3A) showed that growth of *C. albicans* had a 3.61 log reduction (p=0.002) when co-cultured compared to growth when cultured alone. When cultured with *E. cloacae*, wild-type *C. albicans* showed a 3.41 log reduction (p=0.008) in growth versus monoculture (Fig3B). When the Δ*efg1/*Δ*cph1* mutants, which can only produce yeast, were used in the experiment, they showed similar results. *C. albicans* had a 2.66 log reduction (p=0.0007) in growth when co-cultured with *E. aerogenes* (Fig3C) and a 3.36 log reduction (p=0.0016) in growth when co-cultured with *E. cloacae* (Fig3D). *tup1/tup1* mutants, which can only produce hyphae, showed significant decrease in growth when co-cultured, with a 1.94 (p=0.0087) and 0.94 (p=0.0152) log reductions when cultured with *E. aerogenes* and *E. cloacae*, respectively (Fig3E-F). This shows that when only yeast, only hyphae, or both are present, both *E. aerogenes* and *E. cloacae* are able to significantly decrease *C. albicans* growth. *E. aerogenes* and *E. cloacae* showed no significant difference in growth in any of the experiments. All of the planktonic co-cultures show that *C. albicans* produced significantly fewer CFUs, and therefore fewer cells in a 24h period when co-cultured with *Enterobacter spp.* versus monocultured, and the reduction in growth is not morphologically dependent.

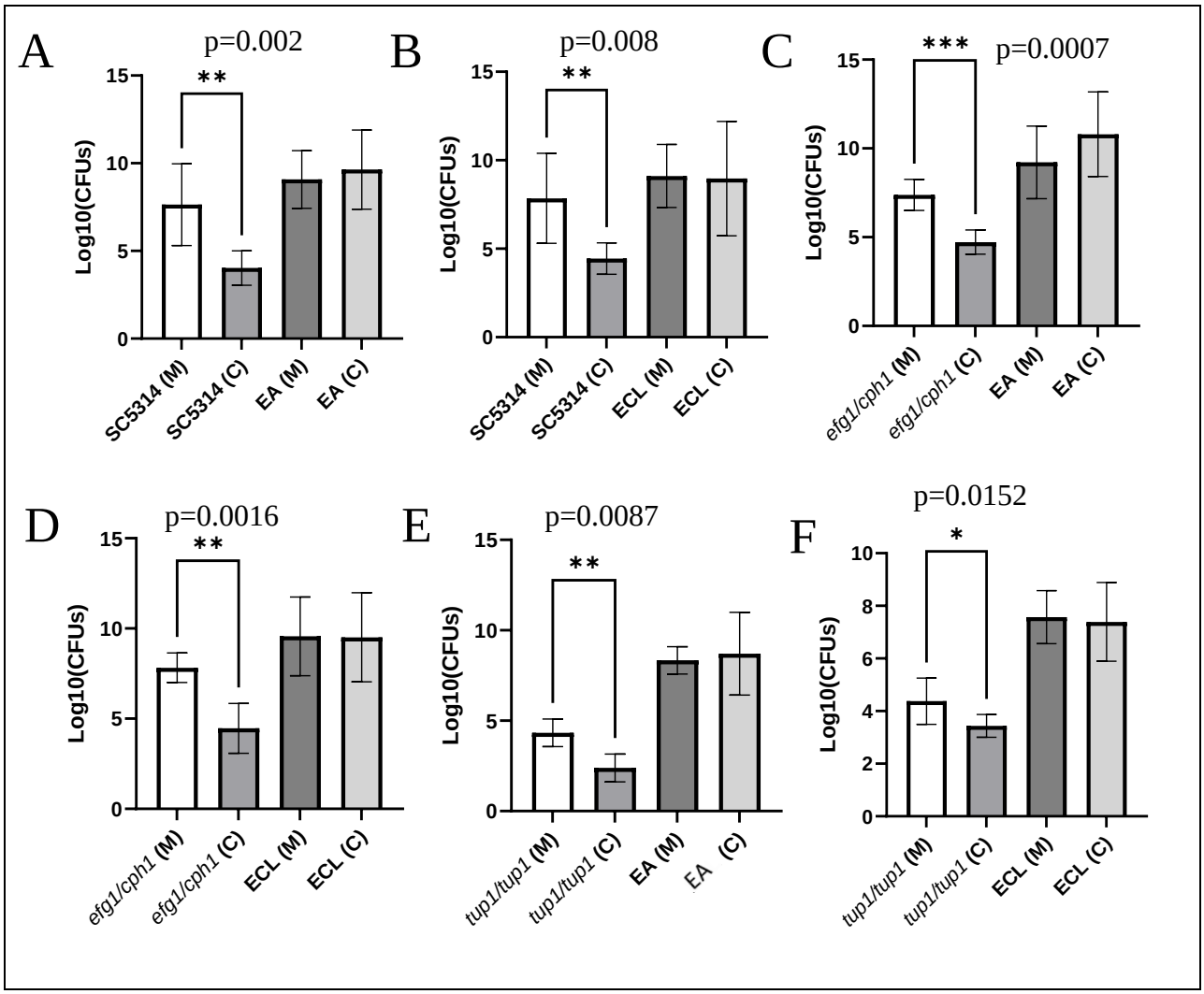

Figure 3. Planktonic Co-cultures. Average Log_{10}(CFUs) from monocultures and co-cultures of *C. albicans*, *efg1/cph1*, or *tup1/tup1* with either *E. aerogenes* (EA) or *E. cloacae* (ECL). (A) Unpaired Mann Whitney two-tailed test with Bonferroni correction, n = 6, α = 0.02, p=0.002 (B) Unpaired Mann-Whitney two-tailed test with Bonferroni correction, n = 5, α = 0.02, p=0.008 (C) Unpaired two-tailed T test with Bonferroni correction, n = 5, α = 0.02, p=0.0007 (D) Unpaired two-tailed T test with Bonferroni correction, n = 5, α = 0.02, p=0.0016 (E) Unpaired Mann-Whitney two-tailed test with Bonferroni correction, n = 6, α = 0.02, p=0.0087 (F) Unpaired Mann-Whitney two-tailed test with Bonferroni correction, n = 6, α = 0.02, p=0.0152. Error bars represent standard deviation.

Physical Contact is Not Necessary for Inhibition

To assess the ability of *Enterobacter spp.* to inhibit *C. albicans* growth without physical cell-to-cell contact, cell-free spent media of *Enterobacter spp.*, containing secreted molecules, were used in *C. albicans* co-cultures. The mono- and co-cultures were analyzed in a tetrazolium

bromide (MTT) assay. The MTT assay is colorimetric assay that measures metabolic activity. As the yellow MTT is metabolized by mitochondrial reductases, it turns into purple formazan. A spectrophotometer can then be used to measure absorbance at a wavelength suitable for detecting formazan. Co-cultures with *Enterobacter spp.* spent media showed a decrease in cell growth for *C. albicans* compared to monocultures (Fig4). When *C. albicans* was co-cultured with either *E. aerogenes* or *E. cloacae* spent media, its metabolic activity was significantly lowered, with a 74.32% decrease ($p=0.0003$) and 73.73% decrease ($p=0.0003$), respectively (Fig4A). The MTT assay shows that *Enterobacter spp.* spent media contains secreted molecules that can inhibit growth of *C. albicans*, leading to lower overall metabolic activity in the sample. CFU data was also collected (Fig4B), showing that in the presence of *E. aerogenes* spent media, *C. albicans* produced significantly less CFUs than when cultured alone ($p=0.0032$). *E. aerogenes* inhibited *C. albicans* growth by a log reduction of 1.43. When grown in the presence of *E. cloacae* spent media, *C. albicans* had a growth decrease of 1.53 log reduction ($p=0.0150$).

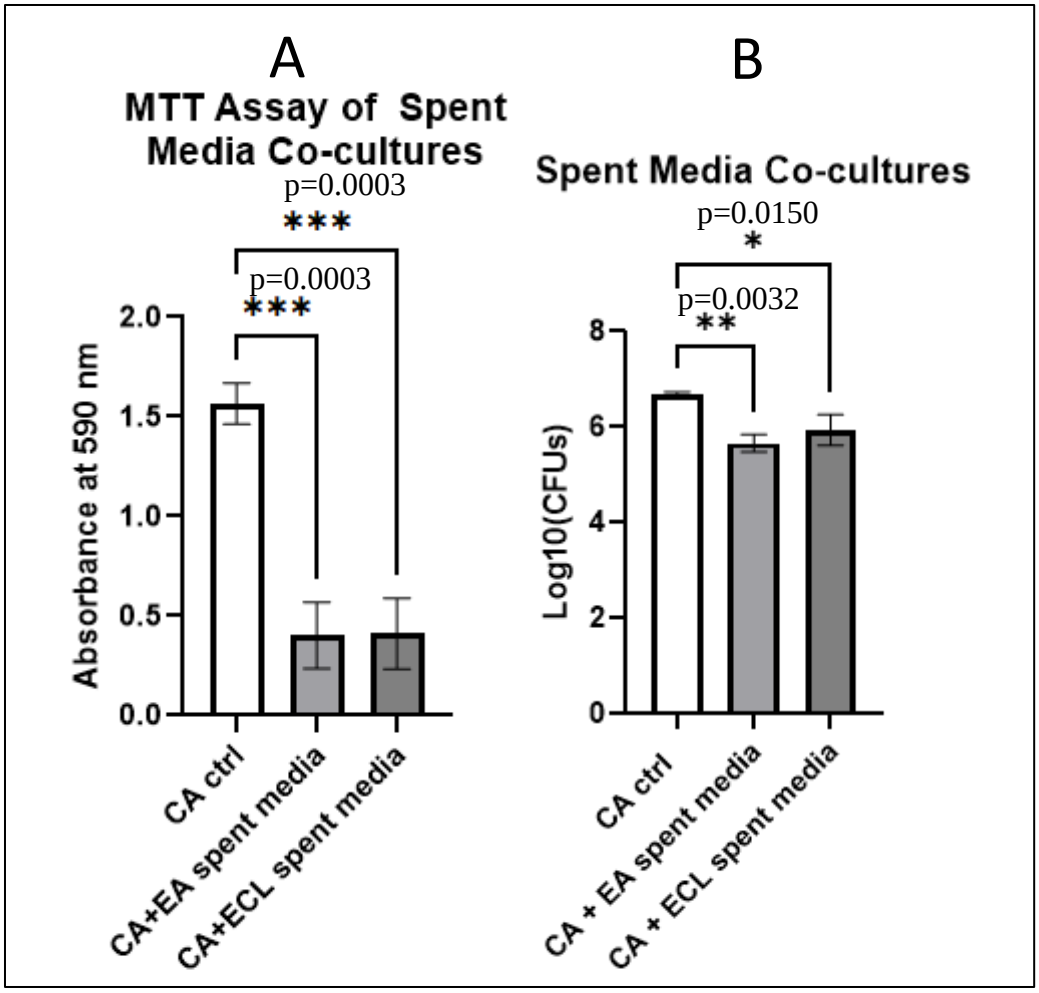

Figure 4. Spent Media Co-cultures. (A) MTT Assay performed on *C. albicans* alone and in the presence of either *E. aerogenes* or *E. cloacae* spent media with one-way ANOVA and post-hoc Šidák multiple comparison test, n = 3, $\alpha = 0.05$, p = 0.0003 for both comparisons. (B) Average Log_{10}(CFUs) of *C. albicans* cultured alone and in the presence of either *E. aerogenes* or *E. cloacae* spent media with One-way ANOVA and Šidák's multiple comparisons test, n = 3, $\alpha = 0.05$, p = 0.0032 and p = 0.0150. Error bars represent standard deviation.

Enterobacter Inhibits Growth of Early-Stage C. albicans Biofilms

To assess the ability of *Enterobacter spp.* to inhibit biofilm formation and inhibit growth of mature *C. albicans* biofilms, *Enterobacter spp.* was either added to the wells at the same time as *C. albicans* or 24h after *C. albicans* was allowed to form a biofilm. In the attachment-phase co-cultures, *C. albicans* experienced a significant decrease in biofilm growth compared to monoculture. When *E. aerogenes* was added, *C. albicans* experienced a log reduction of 2.93

(p=0.0002) in biofilm growth (Fig5A), and the addition of *E. cloacae* led to a log reduction of 2.75 (p=0.0003) in *C. albicans* growth (Fig5B). When *Enterobacter* was added after *C. albicans* biofilm formation, there was no significant difference in growth when co-culture was compared to monoculture (Fig5C). This shows that *E. aerogenes* and *E. cloacae* can be used to inhibit biofilm formation, however their inhibition is limited to early-stage biofilm production.

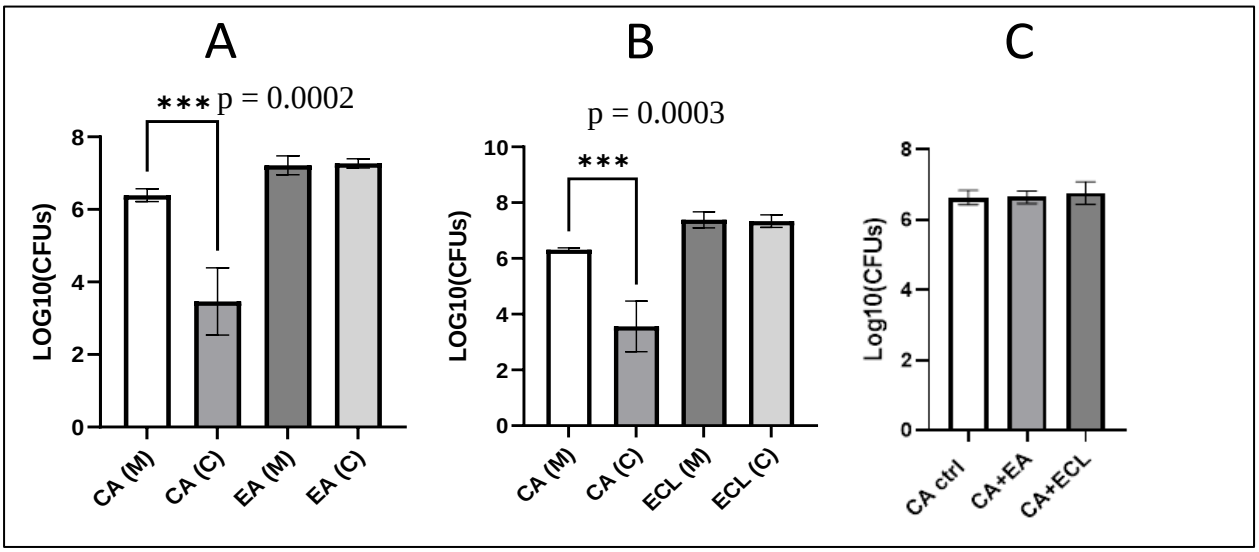

Figure 5. Biofilm Co-cultures. Average Log10(CFUs) from monocultures and co-cultures of attachment-phase (A-B) and mature-phase (C) *C. albicans* biofilms with *E. aerogenes/E. cloacae.* (A) One-way ANOVA with Šidák multiple comparison test, n = 3, α = 0.05, p = 0.0002 (B) One-way ANOVA with Šidák multiple comparison test, n = 3, α = 0.05, p=0.0003 (C) One-way ANOVA, n = 3, α = 0.05, p = ns. Error bars represent standard deviation.

E. aerogenes May Alter C. albicans Morphology

Even though *Enterobacter spp.* have been shown to inhibit *C. albicans* growth in all morphologies, we wanted to evaluate if *Enterobacter spp.* can alter *C. albicans* morphology. To do this, the percent of *C. albicans* that appeared as yeast or hyphal-like were recorded in the presence and absence of *Enterobacter spp.*, at 4h and 24h. Round *C. albicans* were considered yeast, and elongated *C. albicans w*ere considered hyphal-like. The percentages were used as

ratios, and due to very large differences in ratio, $\log_{10}$(yeast/hyphae) was used (Fig6). There were no significant differences at 4h between *C. albicans* alone and co-cultured. However, at 24h, the *E. aerogenes* co-culture produced a significantly lower yeast/hyphae ratio than *C. albicans* but was not significantly different from *E. cloacae. E. cloacae* and *C. albicans* showed no significant difference in morphology at 24h. These results show that *E. aerogenes* may alter *C. albicans* morphology and inhibit a conversion from hyphae to yeast at 24h.

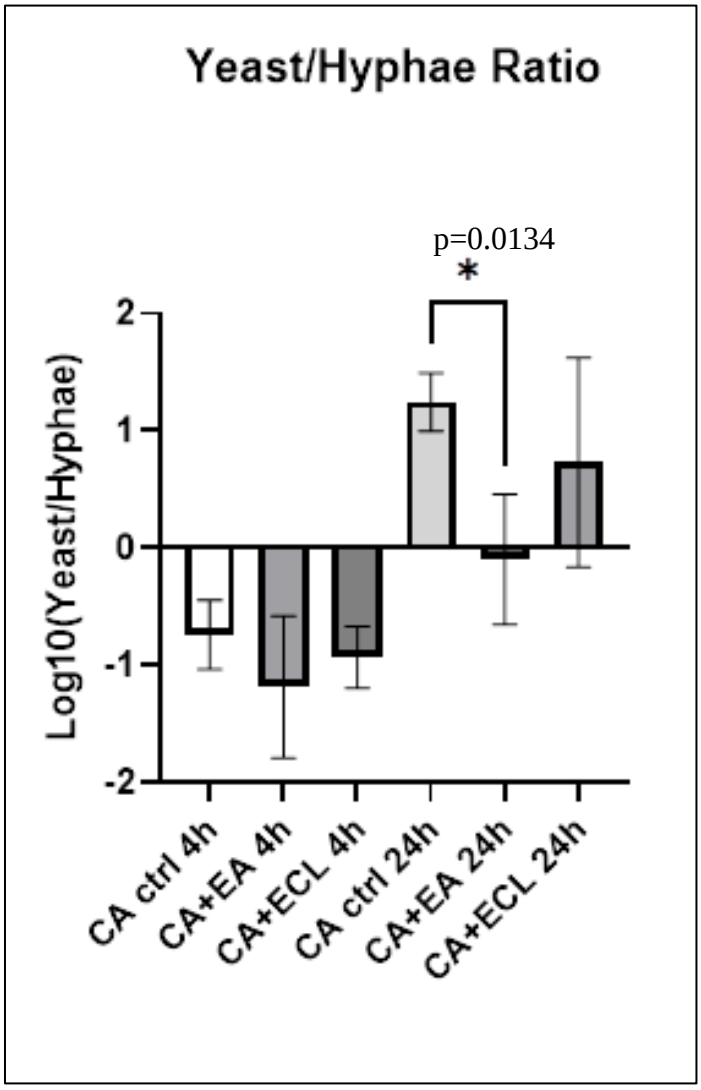

Figure 6. Yeast/Hyphae Ratio. Log_{10}(Yeast/Hyphae) of *C. albicans* cultured alone and co-cultured with *E. aerogenes* or *E. cloacae* at 4h and 24h in YPD broth with 20% horse serum. One-way ANOVA and Šidák's multiple comparisons test, α=0.05, n=4 for *C. albicans* ctrl and *C. albicans* + *E. aerogenes*, n=3 for *C. albicans C. albicans* + *E. cloacae*. Error bars represent standard deviation.

HWP1 is Not Involved in the Interaction

RT-PCR was performed on the *HWP1* gene of *C. albicans* when monocultured and co-cultured with *E. cloacae* (Fig7). The *18SrRNA* gene was also used as a control. Due to missing data in some trials, statistical analysis has yet to be performed on the RT-PCR experiments. Thus far, the *HWP1* gene does not appear to be involved in the interaction between *C. albicans* and *E. cloacae*, since the standard deviation for CA–HWP1 has a major overlap with the standard deviation for CAECL–HWP1.

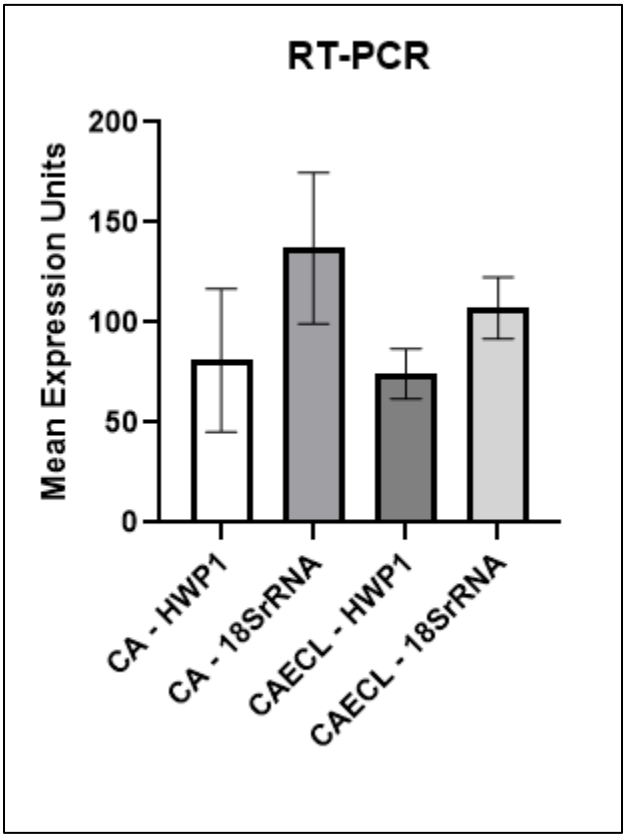

Figure 7. RT-PCR on *HWP1* gene. Mean expression units of the *HWP1* and *18SrRNA* genes of *C. albicans* when monocultured and co-cultured with *E. cloacae*; mean expression units were measured from bands on a 1% agarose gel using ImageJ, n=3. Error bars represent standard deviation.

Als Protein Involvement

The ELISAs performed on Δ*als1-7* (Figure8A) showed no significant difference between groups. However, this may be caused by a small number of trials (n=3) and large standard deviation in some groups. While there is no significant difference among the groups, the group with the most reduced attachment is Δ*als1*. To ascertain if any reduction in attachment seen in the ELISA is due to the mutants' inability to either bind to the wells or a decreased growth in mutants, a crystal violet assay was performed (Figure 8B). The crystal violet assay showed no significant difference between the groups, indicating that any attachment reductions that may be found in the Δ*als1-7* mutants are not due to factors other than attachment to *E. cloacae*.

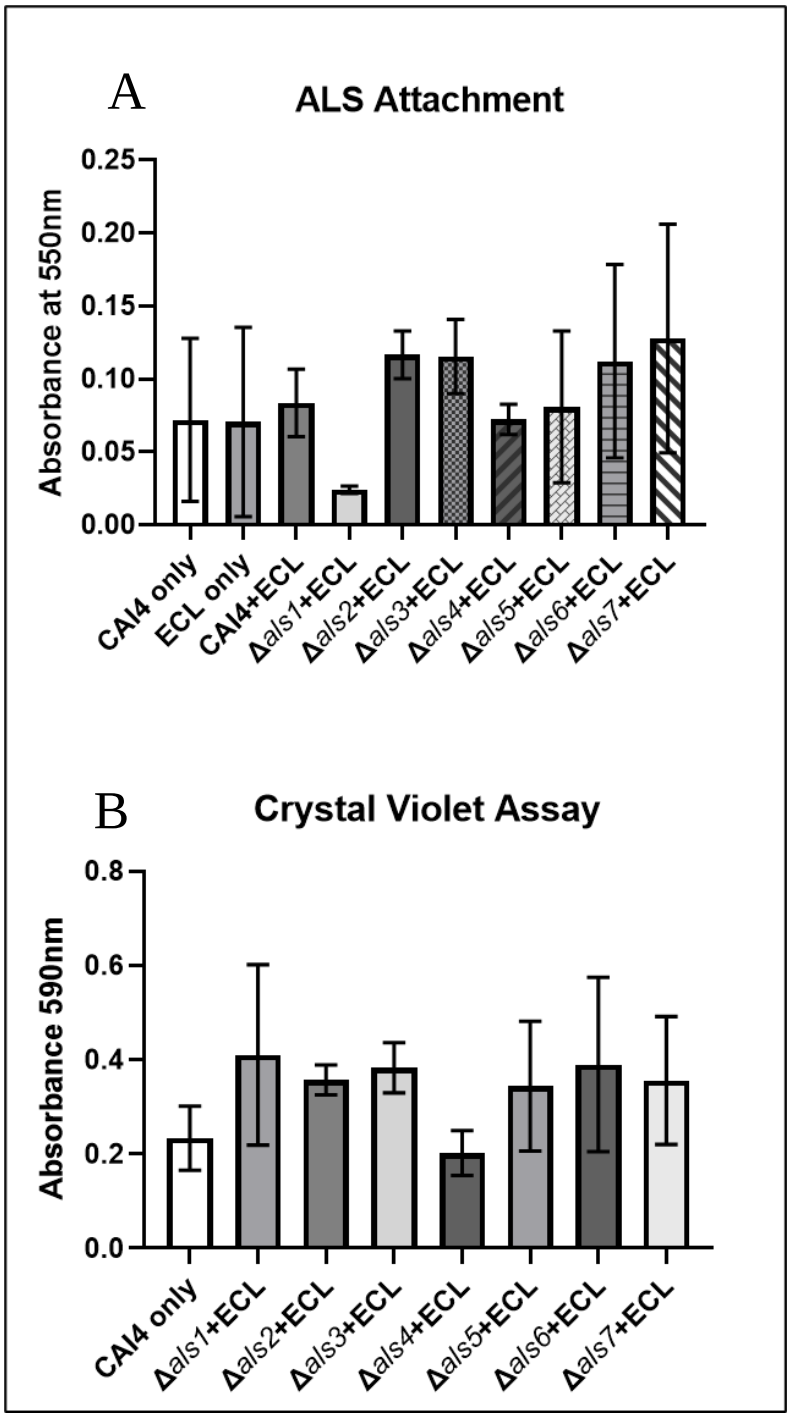

Figure 8. Als Protein Involvement. (A) ELISA results for Δ*als1-7* attachment to wild-type *E. cloacae* with a One-way ANOVA, n=3, α=0.05, p=0.3830 (B) Crystal violet assay results of Δ*als1-7* with One-way ANOVA, n=3, α=0.05, p=0.2908. Error bars represent standard deviation.

E. cloacae Transposon Mutagenesis

480 *E. cloacae* mutants were obtained through transposon mutagenesis. All mutants were screened for reduction in attachment to *C. albicans* (SC5314). The most notable reductions in attachment are shown below in Figure 9. A second ELISA with standardized amounts of each mutant (OD_{600}=0.05) will be performed on the notable mutants. The notable mutants will also undergo genomic isolation and sequencing to ascertain genes involved in attachment to *C. albicans*.

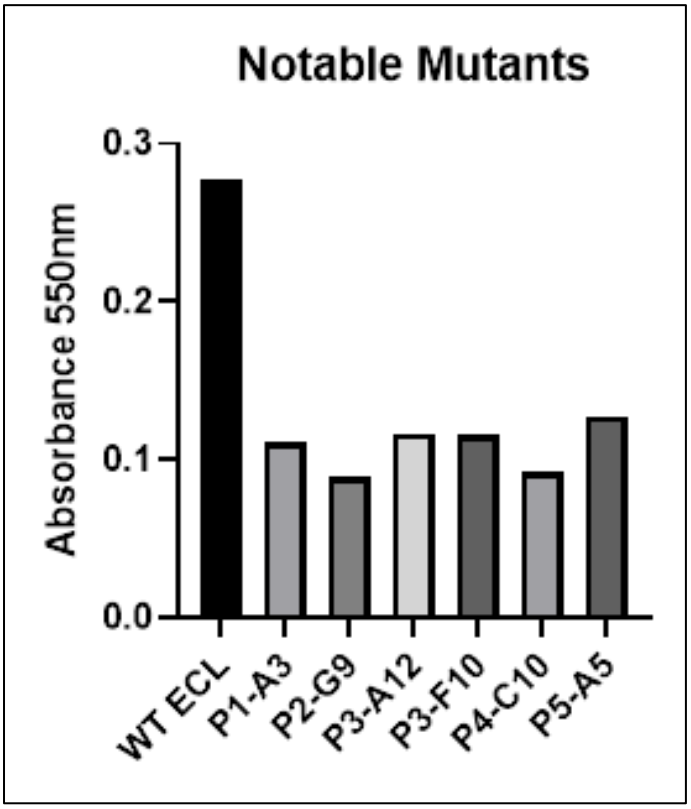

Figure 9. Notable Mutants. ELISA results from a screening of 480 *E. cloacae* mutants for reduction in attachment. The six most notable mutants from each plate are shown above. "WT ECL" represents wild-type *E. cloacae* as the control.

Microbial Burden

To determine if the relationship between *C. albicans* and *Enterobacter* alters the microbial burden that *E. aerogenes* and *E. cloacae* place on *C. elegans*, nematodes were allowed various feeding patterns (Fig10). After feeding, live nematodes were lysed, and microbial burden was measured as $\log_{10}$(*Enterobacter* cells). More trials are needed to determine significance; however, it appears that when *C. elegans* feeds on *C. albicans* then *E. cloacae*, fewer *E. cloacae* cells are present in the nematode (Fig10A). Similarly, when nematodes feed on *C. albicans* before *E. aerogenes*, fewer *E. aerogenes* are present in the nematodes (Fig10B).

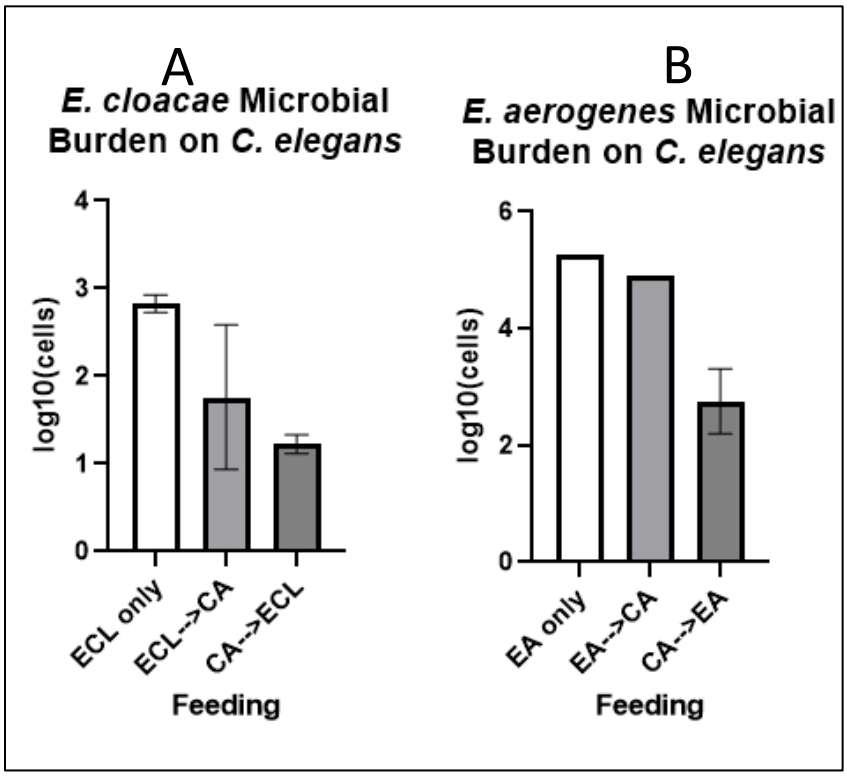

Figure 10. *Enterobacter* Burden on *C. elegans* Microbiome. Log_{10}(*E. cloacae* cells) from lysed *C. elegans* that fed on either *E. cloacae* only, *E. cloacae* then *C. albicans*, or *C. albicans* then *E. cloacae* (A); Log_{10}(*E. aerogenes* cells) from lysed *C. elegans* that fed on either EA only, EA then *C. albicans*, or *C. albicans* then *E. aerogenes* (B). For *E. aerogenes* only and *E. aerogenes* → *C. albicans*, n=1; n=2 for all other conditions. Error bars represent standard deviation.

CHAPTER 4. DISCUSSION

This project aimed to elucidate the polymicrobial relationship of two opportunistic pathogens: *Candida albicans* and *Enterobacter spp.* To do so, the physical cell-to-cell interaction, potential molecular components, and effects of the relationship on an in vivo model were analyzed. The results of this study show that the hypothesis, that *C. albicans* and *E. aerogenes/cloacae* have a commensal relationship, appears to be false. The results of the experiments in this study and their impact will be discussed below.

Cellular Interactions

Fluorescent images of the live/dead stain showed that when *C. albicans* was grown in the presence of *E. aerogenes* or *E. cloacae* for 4 hours, some *C. albicans* cells died. *C. albicans* grown alone for 4 hours showed no death. This implies that *Enterobacter* is capable of killing *C. albicans* in some manner. Future fluorescent images should compare wild type *C. albicans* to the morphological mutants, Δ*efg1*/Δ*cph1* and Δ*tup1*, to determine if morphology makes *C. albicans* more susceptible to the killing ability of *E. aerogenes* and *E. cloacae.* Even though all morphologies are affected by *Enterobacter*, a live/dead stain focused on morphological mutants would allow for comparisons on how greatly each morphology is affected by the interaction. It would be expected that yeast cells would have stronger protection from the effects of the antagonistic relationship with *E. aerogenes/cloacae.*

Planktonic co-cultures demonstrated that both *E. aerogenes* and *E. cloacae* are able to inhibit *C. albicans* growth. When *C. albicans* mutants, Δ*efg1*/Δ*cph1* and Δ*tup1*, were used, *E. aerogenes* and *E. cloacae* still showed inhibition. Many bacteria that form antagonistic relationships with *C. albicans* inhibit hyphal formation or utilize hyphae to attach and kill *C.*

albicans, whereas bacteria that form commensal relationships with *C. albicans* usually prefer the hyphal form for attachment and polymicrobial biofilm formation (Garsin and Lorenz, 2013). Typically, morphology is important in a *C. albicans*-bacterial relationship. However, both yeast- and hyphae-only *C. albicans* strains were negatively affected by *Enterobacter.* Co-cultures comparing the effect of *Enterobacter* on the different morphological strains were not conducted, however, they may provide insight on which morphology, if any, is more susceptible to the inhibitory ability of *Enterobacter* than the others.

E. aerogenes and *E. cloacae* cell-free spent media was utilized to determine if the inhibitory effect of *Enterobacter* on *C. albicans* can occur, not with cell-to-cell interactions, but with secretory molecules. Results of the spent media experiments showed that *E. aerogenes* and *E. cloacae* both have unidentified secretory molecules that can inhibit *C. albicans* growth and metabolic activity. Interestingly, *Salmonella enterica* Serovar Typhirium secretes molecules that can kill both yeast and hyphae and uses a type III secretion system to attach to and kill hyphal cells (Tampakakis et al. 2009; Garsin and Lorenz, 2013). Perhaps *Enterobacter spp.* have a similar mechanism, since *Enterobacter* appears to prefer attaching to *C. albicans*, and their spent media reduces *C. albicans* growth. In the future, fluorescent images using the live/dead stain can be used to determine if the unidentified molecules are just inhibitory or if they are also cytotoxic to *C. albicans*. Also, future work can focus on determining the gene(s) responsible for these secretions and determining the size and structure of the unidentified molecules.

Biofilm co-cultures in which *Enterobacter* was added at the same time as *C. albicans* permitted us to test the inhibitory ability of *E. aerogenes* and *E. cloacae* on adherence-stage biofilms. The results of the attachment-phase biofilm co-cultures showed that both *E. aerogenes* and *E. cloacae* are able to inhibit *C. albicans* growth during biofilm formation. Biofilm co-

cultures in which *Enterobacter* was added after *C. albicans* was allowed to grow a biofilm for 24 hours, showed that both *E. aerogenes* and *E. cloacae* cannot inhibit growth of *C. albicans* in a mature-phase biofilm. *C. albicans* extracellular matrix of cell wall polysaccharides may offer a buffer between *C. albicans* cells and *E. aerogenes/cloacae* cells and secreted molecules.

The effect of *Enterobacter* on morphology was demonstrated using a ratio of yeast to hyphae at 4 hours and 24 hours. At 4 hours, *E. aerogenes* and *E. cloacae* had no effect on *C. albicans* morphology. However, after 24 hours passed, *E. aerogenes* was able to keep *C. albicans* in a primarily hyphal state. Meanwhile, *E. cloacae* allowed *C. albicans* to convert into a predominantly yeast phase, similar to *C. albicans* grown alone.

Molecular Interactions

The RT-PCR for *HWP1* expression, though not completed, shows no difference in expression for *C. albicans* when monocultured versus co-cultured with *E. cloacae*. More trials should allow for statistical analysis to be conducted. Of the 480 *E. cloacae* mutants screened through the ELISA, 6 had reduction in attachment to *C. albicans* compared to wild-type *E. cloacae*. Those 6 mutants should be further studied to both corroborate this and to determine genes involved in the adherence of *E. cloacae* to *C. albicans*.

The ELISA performed on *ALS* mutants shows no significant difference between the strains. However, this may be due to the low trial number and high standard deviation for some groups, especially the controls. Because of this, *ALS1* cannot be deemed significant, however, *ALS1* may truly be important in the adherence interaction. More trials with lower standard deviation will be required to determine if *ALS1* truly plays a role in the adherence of *C. albicans* and *Enterobacter spp.* Als1p is structurally similar to Als3p, with 11 out of 14 of the amino acids

in the Als3p N-terminal domain that bind to peptides are also present in the Als1p N-terminal domain. Als1p and Als3p are both used in the interaction between *C. albicans* and *Streptococcus gordonii* which involves mixed-species biofilms. The Als1p is also used by *C. albicans* to adhere to endothelial and epithelial cells (Lin et al. 2014; Hoyer and Cota, 2016). While there is no statistically significant reduction in the attachment of *E. cloacae* to the Δ*als1* strain, it was the mutant strain with the least attachment and should be examined further to either eliminate or elucidate its potential role.

In Vivo Interactions

Though more trials are needed for the microbial burden experiment, the results are currently showing a potential reduction of the *E. aerogenes* and *E. cloacae* microbial burden on *C. elegans* when they feed on *C. albicans* prior to *Enterobacter.* This would indicate that the presence of *C. albicans* reduces the colonization of *Enterobacter.* That, however, does not align with what has been demonstrated in the in vitro experiments. In vitro, *Enterobacter* attaches to *C. albicans*, and though *Enterobacter* can harm *C. albicans*, the planktonic co-cultures showed no significant reduction in *Enterobacter* cells due to the interaction. Perhaps the in vitro relationship differs from the in vivo relationship. This has been shown before in the case of *C. albicans* and *Pseudomonas aeruginosa*, which is antagonistic towards *C. albicans* in vitro, but the two species work synergistically to infect in vivo (Garsin and Lorenz, 2013). In the future, experiments involving infection outcomes and measuring survival of nematodes in mono- versus co-cultured should be conducted to give insight into the overall impact that the complex interaction plays on an infected host.

Conclusions

The in vitro interaction between *C. albicans* and *E. aerogenes/cloacae* appears to be antagonistic, in which *Enterobacter* inhibits growth and kills *C. albicans*. The interaction can occur regardless of morphology and without cell-to-cell contact. Early-stage *C. albicans* are susceptible to the interaction, but mature biofilms are not. *E. aerogenes* can alter the morphology of *C. albicans* after 24h, keeping it in a predominantly hyphal state. The *HWP1* gene doesn't yet appear to be involved in the interaction, but the *ALS1* gene may be involved in adherence of *Enterobacter* to *C. albicans.* There appears to be a decrease in microbial burden of *Enterobacter* on *C. elegans* when they feed on *C. albicans* beforehand. Studying this relationship could aid in creating new antifungals by utilizing *Enterobacter* secreted molecules as their backbone. Therefore, future work should focus on identifying the secreted molecules and genes involved.

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APPENDIX: Media

Lysogeny Broth (LB)
12.5 LB
500 ml dH_2O
Autoclave
For agar plates, add 7.5g agar

Lysogeny Broth + Kanamycin (LBK)
12.5 LB
500 ml dH_2O
Autoclave
Add: Kanamycin for a final concentration of 50µg/ml
For agar plates, add 7.5g agar

Lysogeny Broth + Amphotericin B (LBAB)
12.5 LB
500 ml dH_2O
Autoclave
Add: Amphotericin B for a final concentration of 30µg/ml
For agar plates, add 7.5g agar

Lysogeny Broth + Amphotericin B + Ampicillin (LBABA)
12.5 LB
500 ml dH_2O
Autoclave
Add: 30µg/ml Amphotericin B and 100µg/ml Ampicillin
For agar plates, add 7.5g agar

Yeast Peptone Dextrose Broth + Kanamycin (YPDK)
5g yeast extract
10g peptone
10g dextrose
500 ml dH_2O
Autoclave
Add: 50µg/ml Kanamycin
For agar plates, add 7.5g agar

Yeast Nutrient Broth (YENB)
7.5g yeast extract
8g nutrient broth

1L dH_2O
Autoclave

Nematode Growth Media (NGM)
1.5g NaCl
1.25g peptone
500ml dH_2O
Autoclave
Add (pre-autoclaved): 5µg/ml cholesterol, 500µl of 1M $CaCl_2$, 500µl of 1M $MgSO_4$, 12.5ml of 1M KH_2PO_4
For agar plates, add 7g agar

M9 Buffer
0.3g KH_2PO_4
0.6g Na_2HPO_4
0.5g NaCl
100ml dH_2O
Autoclave

Brain Heart Infusion (BHI)
13g BHI broth
500 ml dH_2O
Autoclave
For agar plates, use BHI agar instead of BHI broth

1% Agarose Gel
1g Agarose
100 ml 50x TAE
Boil
Add: 7.5µl of 10mg.ml ethidium bromide

50x Tris Acetate EDTA Buffer (TAE 50x)
242g Tris-base
Dissolve in 700ml dH_2O
Add: 57.1ml 100% glacial/acetic acid, 100ml 0.5M EDTA (pH 8.0)
Volumize to 1L with dH_2O

0.5 M Ethylenediamine tetraacetic acid (EDTA, pH 8.0)
146.12g EDTA
1L dH_2O

Adjust pH to 8.0
Autoclave

Wash Solution (ELISA)
0.1g of Bovine serum albumin (BSA)
5µl Tween
9.995ml PBS 1x (pH 7.4)

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