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INTERACTIONS BETWEEN STREPTOCOCCUS AGALACTIAE AND CANDIDA ALBICANS AFFECT PERSISTENCE AND VIRULENCE

By

Kathryn Patenaude

B.A. University of Maine at Presque Isle, 2016

A DISSERTATION

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Doctor of Philosophy

(in Biomedical Sciences)

The Graduate School

University of Maine

December 2023

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By Kathryn Patenaude

Dissertation Advisor: Dr. Melody N. Neely

An Abstract of the Dissertation Presented
in Partial Fulfillment of the Requirements for the
Degree of Doctor of Philosophy
(in Biomedical Sciences)
December 2023

Streptococcus agalactiae (Group B Streptococcus or GBS), a Gram-positive bacterium, and *Candida albicans*, a polymorphic fungus, are commensal microbes in most of the population they colonize but are also capable of causing severe and sometimes fatal infections in certain patient groups. Both organisms share similarities including the colonization the same tissue environments and causing infections in specific patient groups including those who are newborn, pregnant, suffering from chronic conditions like diabetes and HIV, as well as elderly patients. Previous research discovered that GBS and *C. albicans* can synergize to enhance the colonization of GBS in the bladders of mice, but besides this not much was known prior to the research in this dissertation about how interactions between these two infectious pathogens can alter infection or treatment effectiveness in co-infected hosts.

This dissertation aimed to determine if interactions between these two organisms can alter their viability, ability to withstand antimicrobial challenge, as well as influence their virulence in co-infections compared to solo infection. We discovered that interactions between the two opportunistic pathogens were influenced by media nutrient availability and pH, and that the presence of *C. albicans* in a culture reduces the effectiveness of certain antibiotics against GBS in vitro. We also utilized a larval zebrafish model to investigate differences in virulence in solo infection vs co-infections in vivo. Zebrafish co-infected with GBS and *C. albicans* had decreased survival rates compared to solo infections of either

pathogen depending on the initial infection route. Co-infection also led to an increased GBS burden compared to solo GBS infections. The antibiotic clindamycin was also less effective at reducing mortality rates in co-infected zebrafish compared to zebrafish infected with just GBS, indicating that *C. albicans* may make the antibiotic clindamycin less effective against GBS in vivo.

Overall, these findings highlight how interactions between GBS and *C. albicans* can influence treatment effectiveness and virulence. This is clinically relevant, as polymicrobial interactions are not often considered when choosing treatment options for patients infected with a microbe, and polymicrobial interactions like what we have identified in this dissertation may be influencing infection and treatment outcomes in patients.

DEDICATION

I dedicate this to my Mom, Dad, and Rachel, who have never doubted me and have provided unconditional love and support throughout my scientific career, and to the love of my life Noah, who has been steadfast in his support and love for me and has made earning this Ph.D. the most joyous time of my life.

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TABLE OF CONTENTS

DEDICATION……………………………………………………………………………………………ii

ACKNOWLEDGEMENTS………………………………………………………………………………iii

LIST OF TABLES……………………………………………………………………………………...ix

LIST OF FIGURES……………………………………………………………………………………x

INTRODUCTION……………………………………………………………………………………..1

1.1 Streptococcus agalactiae and Candida albicans interact *in vitro* and *in vivo*.………………………………1

1.2 Streptococcus agalactiae………………………………………………………………………………1

1.2.1 Clinical relevance of Group B Streptococcus……………………………………………………..1

1.2.2 Discovery and categorization of GBS……………………………………………………………2

1.2.2.1 Lancefield discovery and categorization of Group B Streptococcus………………………………2

1.2.2.2 Categorization of GBS strains by capsular polysaccharide serotype……………………………..3

1.2.3 Colonization and virulence factors of GBS……………………………………………………...4

1.2.3.1 Rates of colonization of GBS varies by geographical location……………………………………..4

1.2.3.2 Colonization of the vaginal tract by GBS…………………………………………………………..5

1.2.3.3 GBS surface-associated adhesion factors aid in colonization and invasive GBS disease……...6

1.2.3.4 Signal Transduction System Two-Component regulatory systems (TCSs)……………………10

1.2.3.5 GBS hemolytic pigment………………………………………………………………………...11

1.2.3.6 GBS virulence factor Hyaluronidase B……………………………………………………..11

1.2.3.7 D-alanylation of lipoteichoic acid (LTA) by GBS…………………………………………………...12

1.2.3.8 Utilization of host factors by GBS to enhance virulence…………………………………………12

1.2.4 Antibiotic resistance in GBS………………………………………………………………………13

1.2.4.1 Increasing rates of antibiotic resistance by GBS………………………………………………..13

1.2.4.2 Resistance of GBS to the antibiotic penicillin……………………………………………………14

1.2.4.3 Resistance of GBS to the antibiotics erythromycin and clindamycin……………………………14

1.2.5 Rates and characteristics of GBS invasive disease in different patient populations…………………15

1.2.5.1 Rates of GBS infection in neonates…………………………………………………………15

1.2.5.2 Early-Onset Disease (EOD)…………………………………………………………………16

1.2.5.3 Intrapartum prophylaxis and vaccine development against GBS………………………………..17

1.2.5.4 Late-Onset Disease (LOD)……………………………………………………………………..18

1.2.5.5 GBS infection in adults…………………………………………………………………………19

1.2.6 Polymicrobial interactions with GBS and other colonizing microbes…………………………………..21

1.2.6.1 Co-infection of GBS with other microbes in vivo…………………………………………………...22

1.2.6.2 Polymicrobial biofilm involving GBS……………………………………………………………..23

1.2.6.3 The impact of polymicrobial interaction on GBS colonization……………………………………23

1.3 Candida albicans……………………………………………………………………………………24

1.3.1 Discovery and categorization of *C. albicans*………………………………………………………..25

1.3.2 Cell morphology of *C. albicans* is influenced by environmental stressors……………………………25

1.3.2.1 Unicellular budding yeast cells…………………………………………………………………26

1.3.2.2 Filamentous *C. albicans*…………………………………………………………………………..26

1.3.2.3 Pseudohyphae…………………………………………………………………………………..27

1.3.2.4 Regulation of polymorphism by *C. albicans*………………………………………………………..28

1.3.3 Colonization of *C. albicans* in the human host……………………………………………………...28

1.3.4 Virulence factors of *C. albicans*…………………………………………………………………………29

1.3.4.1 Yeast-to-hyphal transition………………………………………………………………………...30

1.3.4.2 Adhesins……31

1.3.4.3 Invasins……32

1.3.4.4 Quorum sensing molecules……32

1.3.4.5 Secreted hydrolases……33

1.3.4.6 Secreted toxins……35

1.3.4.7 Environmental pH stress tolerance and heat shock proteins……35

1.3.4.8 Biofilm formation by *C. albicans*……36

1.3.5 Antifungal resistance by *C. albicans*……37

1.3.5.1 Pyrimidine analogue antifungal resistance by *C. albicans*……38

1.3.5.2 Polyene antifungal resistance by *C. albicans*……38

1.3.5.3 Azole antifungal resistance by *C. albicans*……39

1.3.5.4 Echinocandin antifungal resistance by *C. albicans*……40

1.3.5.5 Antifungal resistance due to biofilm formation……41

1.3.6 *C. albicans* infection in adults……42

1.3.6.1 Vulvovaginal Candidiasis……42

1.3.6.2 Oral Candidiasis……43

1.3.6.3 Invasive Candidiasis……44

1.3.7 *C. albicans* infection in neonates and young children……45

1.3.7.1 Superficial infections caused by *C. albicans*……46

1.3.7.2 Invasive Candidiasis……47

1.3.7.3 Treatment of invasive candidiasis in neonates……48

1.3.8 Polymicrobial interactions of *C. albicans* and oral streptococcal species……48

1.3.9 Known polymicrobial interactions of *C. albicans* and GBS……………………………………………..49

1.4 Zebrafish as an infectious disease model for GBS and *C. albicans* infections……………………………51

INTERACTIONS BETWEEN STREPTOCOCCUS AGALACTIAE AND CANDIDA ALBICANS AFFECT PERSISTENCE AND VIRULENCE………………………………………………………......52

2.1 Introduction……………………………………………………………………………………………..52

2.2 Results…………………………………………………………………………………………………..54

2.2.1 GBS and *C. albicans* synergize to enhance growth in vitro……………………………………………..54

2.2.2 *C. albicans* hyphal formation is not required for increased growth of GBS……………………………57

2.2.3 GBS antibiotic susceptibility is decreased when co-cultured with *C. albicans* in vitro………………..59

2.2.4 Route of infection plays a role in virulence in in vivo co-infections with GBS and *C. albicans*……….61

2.2.5 GBS is less susceptible to antibiotic treatment during systemic co-infections *in vivo*………………..65

2.3 Discussion……………………………………………………………………………………………… 65

2.4 Materials and Methods……………………………………………………………………………………72

2.4.1 Bacterial strains and growth conditions…………………………………………………………………..72

2.4.2 Fungal strains and growth conditions……………………………………………………………………72

2.4.3 Growth curve experiments………………………………………………………………………………72

2.4.4 Co-culture experiments with antimicrobials……………………………………………………………...73

2.4.5 Heat-killed experiments…………………………………………………………………………………74

2.4.6 Hyphal *C. albicans* strain vs. Yeast-locked strain assay………………………………………………..74

2.4.7 Zebrafish care and maintenance…………………………………………………………………………75

2.4.8 Zebrafish microinjections for infection for different infection routes…………………………………..76

2.4.9 Creation of GBS 515 Streptomycin resistant strain……………………………………………………..77

2.4.10 Bacterial and Fungal Burden Assay…………………………………………………………………..77

2.4.11 Statistical analysis……………………………………………………………………………...78

ENVIRONMENTAL PH DIRECTLY INFLUENCES INTERACTIONS BETWEEN STREPTOCOCCUS AGALACTIAE AND CANDIDA ALBICANS……………………………………….......79

3.1 Introduction…………………………………………………………………………………………...79

3.2 Results………………………………………………………………………………………………..81

3.2.1 *C. albicans* alters the pH of RPMI media when grown with our without GBS………………………81

3.2.2 *C. albicans* reduces the ability of GBS to acidify growth media in nutrient rich THY media with or without additional glucose supplementation…………………………………………………………83

3.2.3 *C. albicans* promotes the viability of GBS following increased acid stress caused by glucose supplementation in culture media……………………………………………………………………85

3.2.4 The morphology of GBS and *C. albicans* is altered in THY media supplemented with glucose compared to the media without glucose supplementation…………………………………………87

3.3 Discussion……………………………………………………………………………………………88

3.4 Materials and methods……………………………………………………………………………...93

3.4.1 Bacterial strains and growth conditions……………………………………………………………93

3.4.2 Fungal strains and growth conditions………………………………………………………………93

3.4.3 Creating different pH media types for Co-culturing experiments…………………………………94

3.4.4 Measuring pH following 24 hour Co-culture of GBS and *C. albicans*…………………………………..94

3.4.5 Images protocol………………………………………………………………………………………95

3.4.6 Statistical analysis……………………………………………………………………………………95

FUTURE DIRECTIONS AND CONCLUSIONS…………………………………………………………………97

APPENDICES…………………………………………………………………………………………..102

Appendix A...102

Appendix B...103

Appendix C...104

Appendix D...105

BIBLIOGRAPHY…………………………………………………………………………………………107

BIOGRAPHY OF THE AUTHOR……………………………………………………………………..166

LIST OF TABLES

Table 2.1 *S agalactiae* strains used in this study…………………………………………………………….78

Table 2.2 *C. albicans* strains used in this study……………………………………………………………….78

Table 3.1 *S. agalactiae* strains used in this study……………………………………………………………96

Table 3.2 *C. albicans* strains used in this study……………………………………………………………….96

LIST OF FIGURES

Figure 1.1 GBS and *Candida albicans* share colonization and infection traits……………………………24

Figure 2.1 The growth rate of GBS and *C. albicans* in solo and co-cultures……………………………...56

Figure 2.2 Interactions between GBS and *C. albicans* during co-culture in low nutrient media alters growth and viability……………………………………………………………………………………56

Figure 2.3 *C. albicans* must be viable to cause increase in GBS growth during co-culture………………..57

Figure 2.4 *C. albicans* hyphal formation is not required for GBS increased growth…………………………..58

Figure 2.5 Antibiotic effectiveness against GBS is reduced in the presence of *C. albicans*…………………60

Figure 2.6 Antifungal effectiveness against *C. albicans* is not altered in the presence of GBS……………...61

Figure 2.7 *C. albicans* and GBS synergize *in vivo* to increase virulence………………………………………63

Figure 2.8 Localized co-infection with *C. albicans* increases GBS burden *in vivo*……………………………64

Figure 2.9 The presence of *C. albicans* decreases the effectiveness of clindamycin against GBS *in vito*……..…………………………………………………………………………………………………..64

Figure 3.1 The ability of *C. albicans* to modulate media pH is altered in the presence of GBS………………82

Figure 3.2 *C. albicans* can reduce acid stress to GBS caused by glucose supplementation………………...85

Figure 3.3 *C. albicans* promotes the viability of GBS in glucose supplemented media………………………86

Figure 3.4 Changes in media pH following growth can alter the morphology of GBS and *C. albicans* in solo or co-cultures……………………………………………………………………………………......88

CHAPTER 1

INTRODUCTION

1.1 Streptococcus agalactiae and Candida albicans interact *in vitro* and *in vivo*

Recent research has discovered that *Streptococcus agalactiae* (also known as Group B Streptococcus or GBS) and *Candida albicans*, two pathogens commonly found to colonize the vaginal tract, may interact in that host environment to influence the ability of each pathogen to colonize and survive in the host. These interactions may be important in the context of infection, as these pathogens rarely cause severe infection in healthy patients but can cause morbidity and possibly mortality in patients who are immunocompromised, suffering from chronic illness, elderly, or neonatal. Both pathogens also cause infection more commonly in pregnant adults compared to non-pregnant adults, highlighting the similarities of these two infectious pathogens in both their colonizing environments and the patients they infect. This thesis further investigates the interactions between these two microbes utilizing *in vitro* culture-based experiments, and *in vivo* experiments using a larval zebrafish model. This research highlights how interactions between these two pathogens can influence the growth, antimicrobial treatment effectiveness, and infection outcomes for both pathogens *in vitro* and *in vivo*. The literature review below highlights the previous research and background knowledge necessary for this thesis project.

1.2 Streptococcus agalactiae

1.2.1 Clinical relevance of Group B Streptococcus

Streptococcus agalactiae, also known as GBS or Group B streptococcus, is a Gram-positive, non-motile bacterium that is an asymptomatic member of the host microbiota in most of the population it colonizes (1). However, in specific groups of people, such as those who are elderly, pregnant, newborn, or suffering from underlying disease, GBS can cause serious and sometimes fatal invasive infections (2). GBS is not only the leading cause of invasive infection and fatality in newborns, but invasive infections by GBS are also on the rise at a rapid rate in adult patients (3, 4). Treatment for GBS infections is also a concern, as no vaccine against GBS infection or colonization is currently available, and certain antibiotics previously used to treat GBS infection are less effective, as antibiotic resistance to erythromycin and clindamycin by GBS have risen dramatically in clinical samples (5–8). Understanding the factors that cause specific

patients to be more susceptible to GBS infection while most of the population colonized with this bacterium are unaffected is important not only for knowledge about the bacterium as a whole, but also for the development of more effective treatments in these populations.

1.2.2 Discovery and categorization of GBS

1.2.2.1 Lancefield discovery and categorization of Group B Streptococcus

Prior to its identification and classification by Rebecca Lancefield, GBS was originally considered to only be a concern for infection in veterinary animals, as it was originally isolated from dairy cows suffering from bovine mastitis (9). The species name "*agalactiae*" translates to "without milk", which refers to dairy cows suffering from bovine mastitis caused by GBS that are unable to produce milk (10). Prior to the discovery of penicillin, GBS was believed to be the cause of up to 90% of bovine mastitis in dairy cows, making the pathogen a major concern to dairy farmers who would lose profits due to the bacterium (11).

This bacterium was described and categorized by Rebecca Lancefield in her landmark publication categorizing *Streptococcal* strains by capsular carbohydrate antigen (9). While GBS is not the only streptococcal species to belong to the group b streptococcus category defined by Lancefield in the 1930s (other notable strains include *S. halichoeri* and *S. pseudoporcinus*), it is the most commonly isolated streptococcal species belonging to the group B streptococcus category, leading it to be referred to as "Group B Streptococcus" in many scientific and medical journals (12, 13). Soon after Lancefield's original categorization of GBS as an asymptomatic vaginal colonizer, reports of invasive GBS infections leading to fatal postpartum infection were published in 1938, establishing that GBS can cause infection in humans (14, 15). GBS today is the leading cause of neonatal sepsis and meningitis (1, 16, 17). Newborns who survive GBS invasive infection often suffer serious long-term morbidities, often including neurological development issues (18). GBS infections can also occur in nonpregnant adults, and infections in nonpregnant adults has risen rapidly in the last 30 years, as the case rate of GBS in the United States was 3.6 cases/100,00 people in the 1990, and had doubled by 2007 to 10.9/100,000 people (3, 4). The severity of disease in newborns as well as the increased rate of GBS infection in nonpregnant adults highlights the need for further research about infections in different patient groups caused by GBS.

1.2.2.2 Categorization of GBS strains by capsular polysaccharide serotype

GBS cocci are surrounded by capsular polysaccharide (CPS) that allows for the serotyping of different GBS strains to categorize these strains into different clusters based on their capsular polysaccharide layer structure. Strains of GBS have two types of carbohydrate antigens in their cell wall. First is the group B antigen as described by Rebecca Lancefield, which is present in all GBS strains (9). The second carbohydrate antigen in the cell wall of GBS used to categorize and differentiate GBS strains is their CPS antigen. Differences in CPS antigen structure and composition in different GBS strains has allowed for the classification of GBS into 10 different serotypes based on their CPS antigen structure (Ia, Ib, II, III, IV, V, VI, VII, VIII, and IX) (19, 20). The small number of distinct GBS serotypes is a unique trait to GBS that is uncommon in other pathogenic streptococcal species. For example, *S. pneumoniae* is known to have over 90 distinct serotypes among isolated strains, and *S. pyogenes* (also known as Group A Streptococcus) has over 100 serotypes related to M-protein alone (21). Serotype of GBS strains also appear to be involved with virulence, as specific serotypes have been implicated to be responsible for increased severity of GBS disease in specific patient groups, such as the prevalence of neonatal disease caused by serotype III strains, and the prevalence of adult GBS infections being caused by serotype V. Out of the ten known serotypes of GBS currently identified, it is thought that 6 specific serotypes (Ia, Ib, II-V) are responsible for roughly 98% of colonization of GBS worldwide, highlighting the relationship between serotype and colonization potential in GBS strains(22). CPS is also a major virulence factor for the bacterium. CPS Sialic acid is not only a major component of CPS, but is a component found on host cell surfaces, allowing for the bacterium to utilize sialic acid to evade immune response through molecular mimicry in multiple ways. Siglec (Sialic acid-binding immunoglobulin-type lectins) binding by CPS sialic acid in the host has been shown aid the bacterium in immune evasion through reduced neutrophil activation and inflammation of placental membranes, as well as reducing pro-inflammatory signaling … GBS strains that lack CPS have been shown to be less virulent in systemic infections compared to wild-type GBS strains, highlighting its importance for the bacterium during infection (23–25)

1.2.3 Colonization and virulence factors of GBS

1.2.3.1 Rates of colonization of GBS varies by geographical location

Colonization is a critical step for GBS to not only establish itself as part of the host microbiota asymptomatically but may also be the precursor to severe infection in certain patients following changes to the host environment. GBS, despite being a fastidious organism, can colonize a variety of locations in the human body, including the oral cavity, nasopharynx, gastrointestinal tract, rectum, genitourinary tract, as well as vaginal tract in women (26–32). A majority of the information we know about colonization by GBS relates to vaginal tract colonization, especially relating to the colonization of GBS in the vaginal tract of pregnant woman following universal screening for GBS at 35-37 weeks of pregnancy (1). The colonization of GBS in newborns can start at birth, as GBS can be transmitted vertically to newborns from their mothers during delivery (33). Colonization of GBS in the vaginal tract can vary over time due to changes in the host microbiota, and it is possible to be colonized with different GBS strains at different life stages or at the same time due to the rapidly changing vaginal microbiota (34, 35).

The global rates of GBS colonization in the vaginal tract is believed to be about 18% of women worldwide (22). However, the published rates of colonization of GBS in the vaginal tract varies greatly by geographical region. For example, one meta-analysis of colonization rates by GBS worldwide concluded that the highest rates of colonization were found in African countries, with an average of 22% of women colonized with GBS, and the lowest in Asian countries, with an average of 11% of women being colonized with GBS (36). The reported colonization rates of GBS in the vaginal tract of women in the United States has varied by study, ultimately ranging from 10-30% colonization (1, 31, 37–39). There are also regions where universal screening for GBS before delivery is not practiced and instead risk-based assessments are made to determine if pregnant mothers will be treated with antibiotics at the time of delivery to try to prevent GBS infection in newborns, potentially leading to underreported colonization data (40–42).

While some countries are proactive in reporting GBS colonization rates, other countries have infrequent or non-existing data for colonization rates of GBS, leading to uncertainty about the true global burden of GBS colonization (43). Multiple factors likely influence the lack of accurate reporting of GBS colonization. One major factor likely contributing to an underrepresentation of the full global burden of GBS

colonization is the fact that GBS colonizes asymptomatically in many patients, and therefore GBS remains undetected because there are no symptoms indicating the presence of GBS. In regions like the United Kingdom and New Zealand, risk-based assessment for mothers potentially colonized with GBS instead of universal screening is practiced, therefore colonization rate of GBS in these patient groups are also likely underreported due to a lack of testing for GBS in some patients, some of whom may be asymptomatic carriers of the bacterium (40–42). Also, GBS vaginal colonization rates in low-to-middle income countries are likely underreported due to poor funding, inconsistent isolation techniques, and a lack of resources in general (44). Overall, while reported GBS colonization rates globally are significant, the bacterium may be even more prevalent as a vaginal colonizer than previously reported due to limitations in GBS colonization surveillance.

1.2.3.2 Colonization of the vaginal tract by GBS

GBS is an effective colonizer of the vaginal tract, an environment many microbes struggle to colonize due to its low pH, lack of available nutrients, antimicrobial peptides, access to immune cells, mucus barrier, and resident microbiota comprised heavily of acid-producing lactobacilli species (43). Colonization of the vaginal tract by GBS can be transient, intermittent, or persistent, highlighting the importance of screening for the organism late in pregnancy. This is crucial to prevent GBS disease as pregnant women carry the risk of transmitting the bacterium to their offspring during delivery (45, 46). Vaginal tract colonization is believed to first start with colonization of the gastrointestinal tract, and later gets introduced to the vaginal tract (47). Vaginal colonization rates of GBS vary, with 10-30% of women being estimated to be colonized with GBS in the rectovaginal region (31, 38, 39). To colonize the vaginal tract successfully, GBS must be able to not only adhere to vaginal epithelial cells, but also do so without alerting the host immune system to successfully integrate itself into the vaginal microbiota. Interestingly, the factors that allow GBS to colonize the vaginal tract and maintain itself in that harsh environment asymptomatically are also often the same factors known to cause the dissemination of GBS throughout the body and contribute to virulence during infection, highlighting the ability of GBS to regulate its virulence genes (48). Because of this, pathogenicity in GBS strains is thought to be tightly regulated and influenced by both environmental and host factors. While there is still much that is unknown about how GBS can remain asymptomatic in

most of the population but cause severe infection in other patients, the ability of GBS to utilize its virulence genes to switch from a commensal pathogen to an invasive pathogen has been studied heavily.

1.2.3.3 GBS surface-associated adhesion factors aid in colonization and invasive GBS disease

GBS colonization starts with adherence to the extracellular matrix (ECM) and epithelial cells in the host environment it is trying to colonize. To do this, GBS utilizes surface-associated adhesion factors, which are expressed on the bacterial surface and allow for attachment to host cells. Some of the most well characterized adhesions that GBS uses to adhere to ECM and epithelial cells include fibrinogen binding proteins (Fbs), laminin-binding proteins (Lmb), C5a peptidase, hypervirulent GBS adhesion (HvgA), immunological bacterial adhesion (BibA), fibronectin binding protein (SfbA), plasminogen binding surface protein (PbsP), Antigen I/II family polypeptide adhesions (AgI/II), and pili (48–53). The ability to bind to tissue in a host is necessary for GBS to be able to cross host barriers in both colonization and infection, which highlights the importance of adhesions as a virulence factor for GBS (54). Following colonization, the expression of some of these adhesions, as well as other virulence factors, can be upregulated or downregulated by GBS depending on environmental and host factors.

Some of these adhesions have very specific functions beyond just binding to ECM and epithelial cells. Fibrinogen binding proteins such as FbsA, FbsB, and FbsC allow GBS to bind to human epithelial cells, which GBS uses to promote vaginal colonization (55, 56). FbsC specifically has been implicated to be a mediator of biofilm formation, which can be utilized with GBS in tandem with other commensal microbes in the vaginal tract to establish vaginal colonization, which is a major risk factor for neonatal disease in pregnant mothers(56). GBS serine-rich repeat (SRR) glycoproteins like Ssr1 and Ssr2 are also surface-associated fibrinogen binding proteins that are commonly found in GBS strains (57). Ssr1 is expressed by most GBS strains and is utilized by the bacterium to bind to fibrinogen on the surface of vaginal and cervical epithelial cells, which allows for persistent vaginal colonization (58–61).

Laminin binding protein in GBS (*lmb*) is known to mediate the binding of GBS to laminin, which is a major component of the basement membrane of tissues throughout the human body, allowing GBS to adhere to and/or infect multiple tissue environments (62, 63). The *lmb* gene has been found to be present in most human isolates of GBS, highlighting its importance in GBS for colonizing the human body (62). Lmb

adhesion binds to laminin in the human host through the use of a zinc-binding pocket, which also is important for zinc acquisition by GBS, which helps promote GBS survival and growth in multiple host niches (64–67).

C5a peptidase, a surface-associated serine protease, is a colonizing and virulence factor GBS utilizes in multiple ways (68). GBS can utilize C5a peptidase to evade the immune system by cleaving C5a, a chemotaxin that is used in neutrophil recruitment, as well as reduce the ability of macrophages to kill engulfed GBS cells (69, 70). There is also evidence that C5a peptidase can bind to fibronectin, which promotes cellular invasion and therefore enhances the ability of GBS to both colonize and infect (71, 72).

HvgA, a surface associated adhesion found only in hyper-virulent CC17 (clonal complex 17) GBS strains, can enhance the ability of GBS to adhere to multiple cell types, such as intestinal, choroid, and microvascular epithelial cells (73). The adherence of GBS to microvascular epithelial cells through the use of HvgA may help explain the prominence of serotype III ST-17 GBS strains in neonatal GBS meningitis infections in newborns, as microvascular epithelial cells are the cells which make up the blood brain barrier, a normally sterile site, that when GBS invades results in meningitis in newborns (73–80). HvgA expression in GBS cells isolated from the body fluids of infected mice is upregulated in comparison to the same GBS strains being cultured *in vitro*, highlighting the importance of the adhesion during infection for GBS (73). Due to the virulence of HvgA in neonates and its affinity for causing bacterial meningitis in LOD GBS cases, this adhesion has been proposed as a potential vaccine candidate to help prevent the development of LOD GBS (81).

BibA, a immunogenic bacterial adhesion cell-wall protein found in GBS, is highly conserved among GBS strains and has been shown to promote both colonization to mucosal surfaces as well as invasion into certain tissues (82, 83). This adhesion is able to adhere to multiple human cell types including cervical, lung, and intestinal epithelial cells, highlighting its importance for GBS adherence, an important step in colonization and invasion (82, 83). BibA has also been theorized to bind to the human complement regulator C4-binding protein, which allows GBS to disrupt host innate immune response (82, 83). The expression of BibA is regulated by pH in the host, as BibA has been shown to be upregulated in a neutral pH environment in comparison to an acidic environment (84). The upregulation of BibA in neutral pH environments may

indicate that this virulence factor is more important for GBS infection in neutral pH environments like the blood rather than acidic pH environments like the vaginal tract (48).

Streptococcal fibronectin binding protein (SfbA) has been found to help GBS with the invasion of multiple cell types, including vaginal, cervical, astrocytes, and brain microvascular endothelial cells (85, 86). SfbA is well conserved among GBS strains, highlighting its importance as a virulence factor (85, 86). In a murine model of GBS meningitis SfbA deficient GBS strains were not as effective at crossing the BBB and colonizing, implicating SfbA as important for the ability of GBS to adhere to the BBB (86). While this adhesion helps allow GBS to penetrate and cross the BBB, it has been shown that hosts use the ability of mast cells to secrete chymase, a protease capable of degrading fibronectin, to prevent the ability of GBS to bind to fibronectin with adhesions like SfbA (87). Mice lacking chymase are susceptible to GBS infection from wild-type strains, but not GBS strains lacking SfbA, indicating that the ability of mast cells to degrade fibronectin directly influences the ability of SfbA to aid in the dissemination of GBS in a host (87).

Antigen I/II family polypeptide adhesions (AgI/II) have been implicated as a virulence factor in multiple *Streptococcal* spp., and have been best described in oral streptococcal species (88). AgI/II adhesions have multiple functions, but most commonly facilitate the binding of streptococcal species to not only host cells but to other members of the microbiota as well (49, 89–94). Recently, AgI/II adhesions have also been identified in GBS, and have been theorized to contribute to virulence in the bacterium due to their role in binding to host cells and other microbes in the GBS colonizing environment (49). Currently, four distinct AgI/II polypeptides have been identified in GBS using *in silico* analysis, and the genes encoding these proteins have been named *bspA-D*, with the gene family names being named as an abbreviation of "group b *Streptococcus* surface proteins" (Bsp) (49). GBS strains carry different homologs of the Bsp genes, and some have more than one *bsp* gene (49). An adhesion target of AgI/II found readily in the host is glycoprotein-340 (gp340), an innate immunity pattern recognition receptor (PRR) which is a component of mucosal surfaces (95, 96). *BspA*, when cloned into a non-adhering *Lactococcus lactis* bacterial strain as a surrogate, enhanced the ability of the strain to adhere to gp340 compared to a wild-type *L. lactis* strain, indicating that BspA may increase the ability of GBS to adhere gp340 (49). This mutant *Bspa L.* lactis strain was also readily able to adhere with *Candida albicans*, which is an infectious fungal pathogen also found

to commonly colonize the vaginal tract, while the wild-type *L. lactis* does not adhere to *C. albicans*, indicating that bsp genes may have a role in polymicrobial adherence and interactions for GBS (49). Interactions involving other *Streptococcal* species and *C. albicans* has shown that these organisms can synergize to enhance colonization in a host, and it is possible that the ability of GBS to adhere to *C. albicans* may also enhance its virulence in a host (97, 98).

PbsP (Plasminogen binding surface Protein), a cell wall-associated surface protein that binds to plasminogen (an ECM component), is highly conserved in many GBS strains (99). Plasminogen (Plg), an inactive proenzyme found in plasma, is utilized by multiple bacterial species to promote the adherence to and invasion of host tissues (100, 101). The expression of PbsP on the surface of GBS cells varies depending on strain, and is tightly regulated due to its role in virulence (99). This adhesion is used by GBS to promote vaginal colonization, as GBS strains lacking PbsP are unable to colonize the vaginal tract of mice at the same rate as wild-type GBS (102). This adhesion also promotes invasive infection, as it can be utilized by GBS to adhere to brain endothelial cells and eventually cross the BBB during infection (99, 103). This adhesion can also bind to human vitronectin, a glycoprotein found in ECM and serum, which allows for the adherence to and invasion of epithelial cells by GBS (104). The ability of GBS to bind to plasminogen using PbsP is important for both vaginal colonization as well as the invasion of certain cell types, highlighting its potential as a vaccine candidate.

Pili, which are cell wall anchored appendages found in GBS, are a virulence factor of the bacterium due to their role in adhering to certain epithelial cell types, aiding in both colonization and biofilm formation. Pili in GBS have three distinct subunit proteins that have different functions for GBS in its virulence; The tip of the pilus (PilA), the pilus shaft backbone protein (PilB), as well as the base of the pilus (PilC) (105–108). PilA is important for cellular adherence, as *in vitro* studies have found that when GBS strains lack PilA, it results in reduced adherence by the bacterium to human pulmonary, brain microvascular, vaginal, and cervical epithelial cells (59, 105, 109). PilB subunits make up a majority of the backbone of the pilus structure in GBS and has its own unique role in virulence (105, 106, 109, 110). PilB seems to have a role in immune evasion for GBS strains, as GBS strains lacking PilB are more susceptible to macrophage and neutrophil killing compared to a wild-type GBS strain (111). Mutant GBS strains lacking PilB have also less

effective at forming biofilms and are more susceptible to antimicrobial peptides in comparison to wild-type GBS (110, 112). PilB is important for infection *in vivo*, as systemic infections caused by GBS strains without PilB are less lethal than wild-type GBS infections and results in increased bacterial clearance in infected mice (110). Less is known about the role of PilC in GBS *in vitro* and *in vivo*, but it has been theorized that PilC may also have a role in the initial attachment and adherence to host cells by GBS alongside PilA, but this has not been proven experimentally (109). Overall, pili are important for multiple virulence traits in GBS, and variation in pili subunit protein composition likely have an influence on virulence traits in specific GBS strains, as these different pili subunit proteins have distinct roles in certain virulence traits.

1.2.3.4 Signal Transduction System Two-Component regulatory systems (TCSs)

The regulation of adhesions and other virulence factors are very important, as reduced expression of these factors may prevent GBS from being able to invade and integrate itself into the host niche. Overexpression of virulence genes can complicate the ability of GBS to establish itself as part of the host niche due to triggering the immune response or causing harm to the host that prevents the survival of GBS. One of the most common ways that GBS regulates its virulence factors in a host is by using signal transduction systems like two-component regulatory systems (TCSs). The ability of bacteria to use TCSs is widely conserved among many bacterial species, but the specific structure of these systems varies not only by bacterial genus, but within bacterial species as well. This is true for GBS, as 21 different distinct TCSs have been identified to date, and most GBS strains are thought to contain 17-21 TCSs within their genomes (113, 114). Typically, TCSs are made up of two major components; a sensor histidine kinase protein (HK) located in the cellular membrane, as well as a response regulator (RR) protein, which works alongside HK to sense environmental changes and regulate the genes within the system according to those cues (115). The most well-studied TCSs found within GBS strains is the CovR/S TCS, which is conserved among many *Streptococcus spp* (116). CovR/S is responsible for regulating the expression of over 100 genes, with many of those being linked to virulence in GBS strains (116–118). Host factor stimuli, including nutrient availability, pH, and osmolarity can activate signal transduction systems like CovR/S, which then regulate virulence factors to promote the survival of GBS in stressful environments (117).

1.2.3.5 GBS hemolytic pigment

One GBS virulence factor regulated heavily by the CovR/S TCS is hemolytic pigment. Hemolytic pigment is theorized to be responsible for hemolytic activity in GBS due to ornithine rhamnolipid pigment produced by the *cyl* operon (119). CovR/S negatively regulates the transcription of *cyl* genes in GBS, indicating that the repression of the *cyl* operon and therefore reduced hemolytic pigment production may help GBS remain as a commensal colonizer in the vaginal tract (117). When TCS function is lost, *cyl* operon overexpression occurs, resulting in hyperpigmented and hyper-haemolytic GBS strains (116, 117). Hyperpigmented GBS strains have previously been linked to severe invasive GBS infections in otherwise healthy humans, indicating that a loss of regulation of pigment formation by CovR/S in GBS may lead to worse infection outcomes in patients (119, 120). Hemolytic pigment is able to help GBS infect in multiple ways, as it not only reduces the effectiveness of reactive oxygen species (ROS) against GBS, but is a cytotoxin in general, allowing GBS to survive host phagocyte challenges (119, 121, 122). Interestingly, in some murine models of colonization of GBS, hyper-pigmented GBS strains are not as efficient at colonizing the murine vagina, likely due to the triggering of an immune response in these strains compared to wild-type GBS strains (123, 124). The regulation of pigment formation in GBS strains is important for the ability of the bacterium to successfully colonize its host, and an overexpression of hemolytic pigment in these strains can lead to severe infection.

1.2.3.6 GBS virulence factor Hyaluronidase B

GBS can utilize the endoglycosidase HylB (hyaluronidase B) as a virulence factor in hosts. HylB in GBS helps the bacterium evade host immune factors through the reduction of hyaluronic acid (HA) to disaccharides (125). HA is ubiquitously expressed in the ECM of many tissue types, and is necessary for maintaining the integrity and structure of the ECM (126, 127). HA has a major role in immune surveillance, as the breakdown of HA by ROS and other hyaluronidases reduces HA in to small fragments which in turn stimulates immune activity (126, 128). GBS, through the use of HylB, can inhibit TLR2 and TLR4 inflammatory pathways by preventing the binding of HA and other pathogen-associated molecular patterns (PAMPs) to these TLRS, resulting in a reduced immune response (128). Mutant GBS strains lacking HylB generate a larger pro-inflammatory response both *in vitro* and *in vivo*, further highlighting the role of HylB

in the immune evasion of GBS (129). It has been theorized that immune suppression by HylB in GBS strains aid the bacterium in vaginal colonization, which has been proven in a non-pregnant mouse model, where a GBS strain lacking HylB was not as effective as a vaginal colonizer compared to a wild-type GBS strain containing HylB (128). Kolar et al. also discovered that pregnant mice, when infected with a GBS strain lacking HylB, had increased uterine inflammation, reduced rates of ascending infection into the uterus, and reduced fetal demise compared to mice infected with wild-type GBS (128). The role of HylB in immune evasion likely aids GBS in not only vaginal colonization, but invasive infection as well, highlighting its role as a virulence factor in the bacterium.

1.2.3.7 D-alanylation of lipoteichoic acid (LTA) by GBS

The ability of GBS to utilize cell wall components to evade antimicrobial killing is a virulence factor for the pathogen. For example, lipoteichoic acid (LTA), which is a polymer containing components like phosphate, glycosyl, and D-alanine ester substituents, is a major cell wall component of GBS and can be modified by the pathogen through D-alanylation (48). When the pathogen undergoes D-alanylation of LTA in its cell wall, the result is a reduced negative charge on the bacterial cell wall surface, which ultimately grants the pathogen increased resistance of antimicrobial compounds that have a cationic charge (130). This process directly influences the virulence of the pathogen, as a mutant strain of GBS lacking the ability to undergo D-alanylation has been shown to have attenuated virulence compared to wild-type GBS (131). Another cell wall component of GBS that has a direct influence on virulence is capsular polysaccharide (CPS), which is rich in sialic acid and can utilize that acid to help evade innate immune recognition by mimicking the terminal glycan structures present on most host cell surfaces (48). Strains of GBS lacking sufficient CPS production have reduced virulence in systemic infection models, showing a directly link to CPS composition and virulence by the pathogen (23–25).

1.2.3.8 Utilization of host factors by GBS to enhance virulence

While GBS has many cell-wall based and secreted factors it utilizes to colonize and/or infect a host, it also can rely on host factors to influence its virulence in a host. For example, acquired maternal CPS-antibodies are important for newborns, as newborns who acquire CPS-antibodies have a reduced risk of early-onset-disease caused by GBS (132). However, GBS has a large diversity of CPS composition/types

and can undergo capsular switching through recombination events, allowing the organism to evade previously acquired antibodies (133, 134). Another virulence factor utilized by GBS that is directly linked to the host is vaginal epithelial exfoliation, which is a process where vaginal epithelial cells detach from the membrane and are shed by the host, which aids in the clearance of infected cells by unwanted microbes by the host (135–137). This process, while normally detrimental to infecting microorganisms, is actually beneficial to GBS, which can survive vaginal epithelial exfoliation and studies have shown that this process actually promotes ascending GBS infection in mice (138). GBS can also stimulate vaginal epithelial exfoliation utilizing β-catenin signaling, indicating the pathogen likely utilizes this process to aid in its ability to colonize and infect the vaginal epithelium (138). Overall, GBS is a highly adaptable microorganism capable of using various cell-wall bound, secreted, and human host factors to establish colonization and subsequent infection in certain patient groups.

1.2.4 Antibiotic resistance in GBS

1.2.4.1 Increasing rates of antibiotic resistance by GBS

The rise of antibiotic resistance by infectious bacteria is a prominent global public health threat. Bacterial infections are a major cause of death among patients in the United States as the CDC estimates that antibiotic resistant infections contribute to over 2.8 million infections annually, with roughly 35,000 of those infections leading to death (8). While the use of antibiotics was revolutionary in treating previously untreatable bacterial infections, the rise of antibiotic resistance in bacterial strains has been driven by the widespread use, overprescription, and misuse of antibiotics (139, 140). While some antibiotics remain effective against a large proportion of bacterial strains, it is likely that further widespread use of antibiotics will continue to drive the increased rate of antibiotic resistant bacterial strains, which would further complicate effective treatment of bacterial infections and may eventually make previously susceptible bacteria untreatable during infection.

Monitoring the antibiotic resistance of GBS isolates collected from infected patients is important, as antibiotics against GBS are used as both a preventative measure against GBS infection (as seen with the use of intravenous antibiotic treatment during delivery for pregnant women colonized with GBS) as well as being the only treatment available against GBS infections in both neonatal and adult patients. While

potential vaccine candidates against GBS have been identified (some of which are in active clinical trials), they are not commercially available yet, leading antibiotics to be the only current option against GBS infection (141–143).

1.2.4.2 Resistance of GBS to the antibiotic penicillin

Penicillin, a β-lactam antibiotic, is the front line antibiotic used against GBS in patients infected with GBS or for expectant mothers receiving IAP treatment due to GBS colonization prior to delivery (1, 40). GBS growth is inhibited by penicillin preventing bacterial peptidoglycan formation in GBS cell walls through the use of penicillin-binding proteins (PBPs), which work by preventing peptidoglycan cross-links from forming in cell walls, which leads to cell wall damage and eventual rapid cell death (144). Penicillin is still considered to be highly effective against the organism despite reports of reduced susceptibility of GBS to the antibiotic in some clinical isolates (145–148). Reduced susceptibility to penicillin in GBS isolates is driven by mutations in PBPs, which reduces the ability of penicillin to bind to the cell wall of GBS (145, 146). Reduced susceptibility to penicillin in GBS strains is a major concern, as reduced susceptibility to penicillin was the precursor to complete penicillin resistance in other *streptococcal* strains that are no longer susceptible to the antibiotic (145, 149). Notably, the minimal inhibitory concentration (MIC) of penicillin against GBS is frequently 4 to 8-fold higher than the MIC seen for penicillin against *S. pyogenes* (150, 151). The increased MIC of penicillin for GBS compared to other streptococcal species may indicate that future effectiveness of this antibiotic against GBS following further widespread use of the antibiotic may be reduced over time.

1.2.4.3 Resistance of GBS to the antibiotics erythromycin and clindamycin

When a patient has an β-lactam allergy and is unable to be treated by the front-line antibiotic Penicillin, second line antibiotics are recommended for the patient. Common second-line antibiotics for GBS treatment include the antibiotics erythromycin and clindamycin, belonging to the macrolide and lincosamide families of antibiotics respectively (145). While erythromycin and clindamycin belong to different antibiotic families, they have a similar mode of action, and this similarity often leads to GBS being cross-resistant to both antibiotics (152). Both erythromycin and clindamycin bind to the 50S subunit of the bacterial ribosome, but in different ways, with erythromycin binding directly to the 50s subunit, and clindamycin binding to the

23S rRNA in the GBS 50S ribosomal subunit, both of which ultimately leads to the inability to complete protein synthesis by GBS (152, 153). Cross-resistance between the two antibiotics may be due to lincosamide antibiotics being able to bind to the 50s subunit in sites close to or overlapping the macrolide binding sites on the GBS cell wall (154). GBS strains have shown a staggering increase of resistance to erythromycin in the last few decades, with erythromycin resistance rates in the early 2000s being estimated to be between 10%-38%, with estimates in the 2020s now ranging to be as high as 40%-76% depending on the study (7, 155–157). Similar trends have been seen with the antibiotic clindamycin, with rates of resistance being estimated to be 10%-21% in the early 2000s, to roughly 42%-58% in the early 2020s (7, 155–157). Increases of clindamycin resistant GBS clinical isolates have led the CDC to designate these strains as a "concerning threat" in their *Antibiotic Resistance Threats in the United States, 2019* report, where they estimate that these GBS strains were responsible for roughly 13,000 infections and 720 deaths in the United States in 2016 (8). In the same report, the CDC estimated that roughly 42% of the GBS strains causing infection were strains found to have clindamycin resistance, and 58% of the GBS infectious strains carried erythromycin resistance (8). These trends of erythromycin and clindamycin resistance in GBS strains are causing challenges for physicians looking for alternative antibiotics against GBS in patients allergic to β-lactams, and in some regions like the United Kingdom, clindamycin is no longer being recommended as a second-line antibiotic against GBS (40). The rapid rise of resistance to these antibiotics may be fueled by the numerous methods GBS can use to avoid antimicrobial activity by these antibiotics. For example, GBS can become resistant to these antibiotics using efflux pumps, drug inactivation, and ribosomal modification (158). The rise of antibiotic resistance to erythromycin and clindamycin by GBS further highlights the importance of closely monitoring the use of antibiotics against GBS, as well as the development of novel treatments against GBS no longer involving antibiotics.

1.2.5 Rates and characteristics of GBS invasive disease in different patient populations

1.2.5.1 Rates of GBS infection in neonates

GBS is a major concern for pregnant women, as the bacterium is the leading cause of infection during pregnancy and can cause harm to unborn fetuses, newborn children, and infants. GBS is also responsible for the highest rates of neonatal infection and preterm birth (43, 159–161). GBS infection is the

leading cause of morbidity and mortality for infants in the United States currently, highlighting the importance of prevention of the infection in these patients (1). Children who survive infections caused by GBS often suffer long-term health complications, including neurodevelopment impairment, epilepsy, cerebral palsy, hearing loss, and vision impairment (18, 162–164). Despite advancements in research for alternative treatment/prevention methods such as bacteriophage therapy, probiotic therapy, and vaccine development, the only current option for treatments of GBS infection are antibiotics (141–143, 165–172). Invasive GBS infections in newborns are categorized into two separate categories: early-onset disease (EOD) and late-onset disease (LOD). EOD is any GBS infection occurring in the first week of life, and LOD GBS infections can occur anywhere from the second week of life to 3 months after delivery (1).

1.2.5.2 Early-Onset Disease (EOD)

EOD occurs in neonates who have acquired GBS by vertical transmission, as pregnant mothers colonized with GBS can pass the bacterium to their offspring in utero through ascending infection into the amniotic fluid of the fetus or during birth through the vaginal tract colonized with GBS (1, 173). There is also evidence that heavy maternal GBS colonization increases the risk of EOD in neonates, establishing the importance of monitoring GBS carriage in mothers and actively working to reduce the bacterial burden of the bacterium in pregnant women prior to delivery (32, 174). While GBS maternal colonization is considered to be the biggest risk factor for EOD development in newborns, other risk factors include being prematurely born (<37 weeks gestation), membrane rupture in the mother, intra-amniotic infection, low levels of GBS-specific anticapsular antibody in the mother, young maternal age, and black race (33, 175–179). EOD should be monitored in newborns whose mothers who have previously had infants with GBS invasive disease, as newborns who have had siblings with EOD are at an increased risk to also develop EOD (180–183). EOD can also be acquired in neonates following aspiration of GBS into fetal lungs during delivery or during GBS infection of their mother's amniotic fluid, which can lead to bacteremia (184). In some cases, ascending GBS infection can cause premature birth or stillbirths (185, 186). The most common EOD GBS disease manifestations in newborns are sepsis and pneumonia (1). Meningitis is also a potential severe GBS disease manifestation of EOD in newborns, but this is less common than pneumonia or sepsis (1). Newborns suffering from EOD will typically first present with respiratory distress, apnea, as well as signs of

sepsis within the first 48 hours of life, highlighting the ability of GBS to cause swift infection in EOD cases (1, 187, 188). The case-fatality ratio for EOD GBS disease has declined rapidly since the 1970s, where newborns faced an almost 50% fatality rate due to EOD GBS disease, dropping to 4-6% fatality currently due to advances in neonatal care and the use of Intrapartum Antibiotic Prophylaxis (IAP) in at-risk mothers(189–192). The case rate of EOD GBS has also declined since the introduction of antibiotics, with the CDC estimating case rates of EOD GBS in 2020 to be 0.2/1000 live births (193).

1.2.5.3 Intrapartum prophylaxis and vaccine development against GBS

There is no current vaccine for expecting mothers against GBS, but instead mothers who are believed to be at risk of passing GBS to their newborns are recommended IAP treatment, which has been effective at both reducing the vertical transmission of GBS as well as preventing EOD (1, 194–200). The most recommended IAP treatment for at-risk pregnant women is receiving the antibiotic penicillin G every 4 hours during labor until delivery (201). Shorter exposure times to antibiotics before delivery are not as effective as the recommended guideline for IAP, but may still offer some protection for neonates born to GBS colonized mothers (202). Mothers colonized with GBS who do not receive IAP treatment can pass the bacterium to their offspring, as roughly 50% of newborns exposed to GBS will become colonized by the bacterium, and 1-2% of those newborns will develop EOD caused by GBS (33, 202–204). IAP has been shown to be very effective at preventing EOD, with an estimated 80% reduction of EOD GBS disease being linked to the treatment (205). Providers may determine whether a mother is at risk of passing GBS to their offspring and need IAP treatment using different screening methods depending on the region. In the United States, the CDC recommends universal culture based screening for GBS, where the rectum and vaginal tract of expecting mothers are swabbed at 35-37 weeks gestation and are tested for evidence of GBS colonization (1, 201). Pregnant women found to be colonized with GBS using universal culture-based screening will then be recommended to have IAP at the time of delivery (1, 201). In some other regions (including the UK and New Zealand), risk-based assessment is practiced, where GBS colonization is not tested using a culture-based method but instead IAP is administered if the pregnant mother exhibits risk factors for GBS disease, which include maternal fever, a prolonged time between the rupture of membranes and delivery, a prior child with EOD GBS, and bacteriuria (40–42, 182). GBS universal culture-based

screening has been shown to lead to lower incidences of EOD GBS compared to risk-based policies (206). There is also evidence that nations practicing risk-based assessments have actually seen a slight increase of the rate of EOD GBS cases in the last 20 years, while this increase has not been seen in regions practicing GBS universal culture-based screening (74, 206, 207). It has been shown that up to 50% of EOD GBS cases occur in newborns whose mothers did not exhibit any known risk factors for EOD GBS, highlighting the limitations of the risk-based assessment approach to monitoring GBS carriage in pregnant women (208–210).

While universal culture-based screening for GBS and subsequently IAP treatment has been highly effective at reducing the case rate of EOD GBS in newborns, there are still weaknesses to the approach. For example, approximately 60% of cases of EOD is estimated to occur in neonates born to mothers with negative GBS cultures at 35-37% weeks (1). This subset of cases in children born to mothers with negative GBS cultures following universal culture-based screening could be due to a false-negative GBS culture following screening, temporary GBS colonization, or new GBS colonization occurring after the initial GBS universal culture screening (1, 45, 46). To help reduce these factors, rapid detection of GBS using PCR is actively being developed to help detect the presence of GBS at the time of delivery, but these methods are not commercially available yet (211–213). There is also some concern that the overuse of IAP may contribute to increased rates of antibiotic resistance by GBS to certain antibiotics used in IAP, and that the widespread use of IAP could lead to antibiotic resistance in non-GBS strains also exposed to IAP (214, 215). Until there is an effective, affordable, and commercially available vaccine against GBS for expecting mothers, IAP will continue to be the most effective treatment to prevent EOD GBS in newborns.

1.2.5.4 Late-Onset Disease (LOD)

While IAP has been shown to be effective at preventing EOD GBS cases, no known reduction of LOD GBS cases has been seen with the use of IAP, highlighting the need for developing alternative preventative measures (216). Most LOD GBS infections in infants present as meningitis, but can occasionally also manifest as bone/joint infection, pneumonia, bacteremia, urinary tract infection, or soft tissue infections (216, 217). In the United States, LOD GBS case rates are estimated to be 0.35 per 1000 live births, and has remained steady for years despite the increased use of IAP administration, highlighting

the importance of developing alternative prevention methods for the disease (218). While case rates of LOD GBS infection remain low, fatality rates for LOD GBS cases are concerning, as LOD carries a fatality rate of 7% worldwide (217). While a majority of EOD GBS infections are acquired vertically from GBS colonized mother to neonate, LOD GBS infections can be transmitted to infants by multiple routes, including vertically from colonized mothers, from GBS contaminated breastmilk, from environmental exposure, or by nosocomial transmission (219–221, 221–231). Infants who were born premature are at an increased risk of LOD GBS diagnosis than full term infants despite not showing signs of EOD following birth (216). The role of maternal GBS colonization as a risk factor for LOD GBS cases is not as well understood as in EOD GBS cases, as some studies have shown there to be less correlation between maternal colonization of GBS and neonatal GBS infection in LOD GBS compared to EOD GBS, while others have estimated that almost half of infants with LOD were colonized at birth by the same GBS strains carried by their mothers (232, 233). Some studies have even shown that mothers of infants who contracted LOD GBS were more likely to be colonized with GBS at the time of LOD diagnosis than at the time of GBS universal culture-based screening, further complicating the role of maternal colonization in the development of LOD GBS in infants (216). The mortality and morbidity caused by LOD GBS is a major health concern, and more research investigating how LOD occurs and how to prevent the infection is necessary to reduce the burden of LOD GBS infections worldwide.

1.2.5.5 GBS infection in adults

Most of the adult population colonized with GBS will never experience any symptoms or infections caused by the bacterium. While patients who are considered “healthy” can become invasively infected with GBS, a majority of the invasive GBS cases occurs in specific patients with underlying health conditions (2, 37, 234, 235). Patients who are at an increased risk of infection by GBS include patients who may be pregnant, elderly, immunocompromised, or suffering from chronic illnesses such as diabetes, HIV, and cancer (236). GBS can be transmitted to adults in multiple ways, including fecal-oral, sexual, and vertical transmission (237). Infections caused by GBS in adults are typically categorized by patient type; pregnant women or nonpregnant adults. This categorization happens due to differences in disease manifestation and rates in pregnant vs. nonpregnant adults.

Pregnancy has been identified as a risk factor for invasive GBS disease. One study in particular found that in 2007-2009, the incidence of GBS invasive disease caused by GBS was twice as common in pregnant women (0.04/1000 women) than in non-pregnant women (0.02/1000 women), highlighting the increased risk pregnancy poses for women being susceptible to GBS infection (238). Chorioamnionitis, which is inflammation of the intrauterine structures (including the placenta, chorion, and amnion), can be caused by microbial infection, with GBS being the most commonly isolated bacteria from patients suffering from chorioamnionitis (239–242). GBS causes chorioamnionitis by first adhering to and invading chorionic and amniotic epithelial cells, followed by the infection of placental membranes (119, 243). GBS infection of placental membranes can lead to an inflammatory response, possibly leading to chorioamnionitis and preterm birth (119, 244, 245). Pregnant women are also more susceptible to urinary tract infections (UTIs), and GBS is a bacterium capable of causing UTIs in both pregnant and non-pregnant adults (246–248). Interestingly, patients who are pregnant and also diabetic are more likely to have a UTI caused by GBS than non-diabetic pregnant women (249). Another possible but rare invasive GBS infection that can occur in expecting mothers is maternal sepsis, which has been linked to an increased risk of both premature delivery and sepsis in newborns (250). Pregnant women colonized with GBS, even if they were not actively infected by GBS, were also found to be at a higher risk for preterm birth compared to women without GBS colonization in the vaginal tract (251). While most GBS infections in pregnant women are discovered during labor and delivery, it is also not uncommon for women who are postpartum to also have an increased risk of GBS invasive disease (238, 250). Endometritis, a condition where the uterine lining is inflamed, can be caused by GBS infection in postpartum women (252, 253). Patients colonized with GBS who undergo C-sections are at an increased risk of endometritis following the procedure compared to women who were not colonized by the bacterium (254). Overall, women who are pregnant are not only at a higher risk of invasive infection by GBS, but also are at a higher risk of complications during pregnancy that can lead to negative health outcomes for their children.

GBS infection in nonpregnant adults differs from infection in pregnant women not only in the frequency of GBS infection but in the manifestations of the disease as well. In non-pregnant adults, GBS infection is increasing at a rapid rate, as the case rate of GBS in the United States was 3.6 cases/100,00 people in 1990, and had nearly tripled by 2016 to 10.9/100,000 people (3, 4). While the cause of increased

GBS infection rates in adults is not fully understood, it is thought that the increased prevalence of patients with chronical medical conditions, as well as patients with comorbidities living longer lives may be contributing to the rise of invasive GBS infections in adults (255–259). The most common types of GBS infection in this patient group are bacteremia, soft and skin tissue infections, pneumonia, urinary tract infections, joint infection, endocarditis, meningitis, and urosepsis (236, 260, 261). Many cases of adult GBS infections are found to develop more than 48 hours following hospitalization, highlighting the risk of GBS infection as a nosocomial infection in hospital patients (2, 236, 256, 262). Patients who are diagnosed with GBS disease following hospital admission have likely either acquired the pathogen from the hospital itself, or have become infected with GBS due to GBS transmission from previous skin or mucosal colonization (257). It is not uncommon for adults who have had GBS infections to have recurrent infections caused by the same GBS strain, with the most common recurrent GBS infection type being skin and/or soft tissue infection (3, 261). Patients who are elderly (those 65 years and older) are at a higher risk of death following invasive GBS infection than younger adults, with risk factors for GBS infection in these patients including being bedridden and long-term care facility admissions (248, 263, 264). The first line treatment for adults infected with GBS is penicillin G, as GBS is still considered to be effective against GBS despite rising reports of reduced susceptibility of GBS to penicillin and other β-lactam antibiotics (1, 40). In nonpregnant adults, certain serotypes of GBS are more prevalent than others as the cause of GBS invasive disease. The most common serotypes found to cause infection in nonpregnant adults are serotype Ia and V (256). Other serotypes of GBS such as serotypes Ib, II, III, and IV have also been shown to cause invasive GBS disease, with serotype IV being recently implicated as a GBS serotype capable of causing invasive GBS infections in adults (192, 256, 265–276). Some studies have theorized that erythromycin and clindamycin resistance may be higher in serotype V GBS isolates (277).

1.2.6 Polymicrobial interactions with GBS and other colonizing microbes

Typically, when research is conducted investigating a particular microbe, experiments are performed involving that microbe alone. However, this may not always be the best representation of the behaviors of the pathogen during infection, as many human tissue environments are polymicrobial in nature. Polymicrobial interactions between GBS and other microbes likely alter the behaviors of the organism due

to factors like possible synergy between the organisms or competition of the organisms due to limited nutrient resources, secreted antimicrobial peptides, and stress factors. There has been limited research investigating the polymicrobial interactions of GBS with other pathogens, and most of what is known about these interactions are in the context of polymicrobial infection, biofilm formation, and colonization of specific tissue environments.

1.2.6.1 Co-Infection of GBS with other microbes *in vivo*

Polymicrobial infections of GBS and other infectious pathogens can occur in patients with bacteremia, causing adverse outcomes for the patients. Polymicrobial bacteremia (PMB), a condition where more than one microorganism has been isolated from the bloodstream of a patient at least twice in a 24-hour period, is not an uncommon infection type involving GBS. In some population studies it has been estimated that as many as 41% of bacteremia cases with GBS also involved another infectious microbe (2). Some of the most common bacteria that have been co-isolated with GBS in bacteremia patients include *Staphylococcus aureus*, *coagulase-negative staphylococcus*, and *Escherichia coli* (3, 236, 278). Urinary tract infections (UTI) caused by GBS can also be polymicrobial, often with other Gram-positive bacteria, leading to challenges in treatment for patients. Interactions between GBS and other microbes during polymicrobial UTI infections may not only influence the effectiveness of antimicrobial treatment during these infections but may also directly influence the behaviors of these pathogens themselves. For example, in a murine model of co-infection with GBS and uropathogenic *Escherichia coli* (UPEC), GBS directly influences the pathogenicity of UPEC by enhancing the ability of UPEC to survive during early infection despite GBS being cleared quickly during infection, likely due to GBS mediated reduction of the host innate immune response (279). Ascending infection of GBS in placental tissues of pregnant women can cause serious health complications for not only mothers, but for their unborn offspring, and it has been theorized that polymicrobial interactions may enhance the ability of GBS to colonize and infect this tissue environment. For example, in a murine *in vivo* model it has been shown that mice co-infected with GBS and *Gardnerella vaginalis* (which is another member of the vaginal microbiota associated with bacterial vaginosis when lactobacilli are depleted) led to ascending GBS infection into the placenta and uterine tissues (280).

Ascending GBS infection in these tissues was not seen in mice infected with GBS alone, indicating that interactions between the two pathogens promote GBS virulence (280).

1.2.6.2 Polymicrobial biofilm involving GBS

Biofilms, which are layers of bacteria that grow and stick to surfaces, are well known for being difficult to treat with antibiotics, as well as often being polymicrobial in nature depending on the host niche. Biofilms can form on a variety of surface types including natural surfaces like the teeth, as well as on artificial medical devices like joint replacements and catheters. Most of what is known about GBS biofilms is in mono-species biofilms. However, *in vitro* GBS can attach to a biofilm formed by *Streptococcus mutans*, integrating itself into the mono-species biofilm and ultimately forming a polymicrobial biofilm (281). The ability of these two streptococcal species to form polymicrobial biofilms likely have an influence on oropharyngeal colonization, which is important to monitor, as it has been theorized that asymptomatic oropharyngeal colonization of GBS in healthy individuals may be a transmission source of GBS to neonates who later develop LOD (282). Polymicrobial interactions have also been shown to be responsible for a reduction of GBS biofilm formation, as the supernatant of multiple *Lactobacilli* spp. cultures have been shown to reduce GBS biofilm mass *in vitro* (283).

1.2.6.3 The impact of polymicrobial interactions on GBS colonization

Polymicrobial interactions between GBS and other microbes have been implicated in effectively hindering or enhancing the ability of GBS to colonize certain host niche environments. For example, GBS must overcome challenges caused by Lactobacilli in the vaginal microbiota to colonized that tissue environment, as it is typically dominated by a combination of different *Lactobacilli* species, These organisms help reduce microbial competition by secreting antimicrobial compounds, acidifying the vaginal tissue environment through the production of lactic acid, and utilizing adhesion sites needed by other microbes to survive the harsh environment of the vaginal tract (284). Studies have shown that a reduction of *Lactobacilli* in the vaginal tract is correlated with increased GBS colonization of that tissue environment, (285). Conversely, the use of lactobacilli bacteria has been proposed as an alternative treatment to IAP in mothers colonized with GBS prior to delivery, as there is evidence that lactobacilli can reduce GBS populations *in vitro*, indicating that interactions between these two organisms may directly influence their ability to

effectively colonize the vaginal tract (168, 172, 283, 286) . While the ability of GBS to colonize the vaginal tract is influenced by polymicrobial interactions, it is also likely that GBS colonization of other tissue environments are also influenced by other microbes in the same environment. More recently, research investigating interactions between GBS and the opportunistic fungal pathogen *Candida albicans* have highlighted how interactions between these organisms can influence the colonization of certain host tissues (287–289)

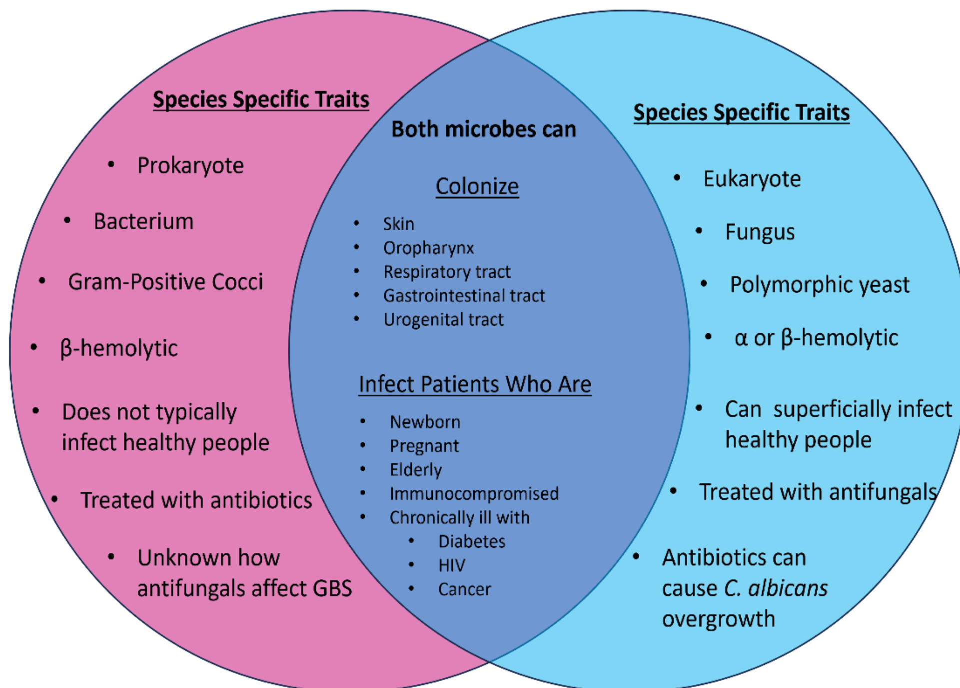


Figure 1.1: GBS and *Candida albicans* share colonization and infection traits. While the bacterium GBS and the fungus *C. albicans* have many notable differences, their shared ability to colonize difficult tissue environments and their ability to be commensals in healthy people but cause serious infections in the same specific patient groups indicates that these microbes may interact in a human host or share similarities when infecting these patients.

1.3 *Candida albicans*

Candida albicans is a eukaryotic polymorphic yeast considered to be a harmless commensal organism in most of the population. However, *C. albicans* can occasionally cause mild or moderate infections in healthy people, especially in women in the form of Vulvo Vaginal Candidiasis (VVC). The organism is also an opportunistic pathogen, as it can severe invasive infections in certain patient groups, like those with medical conditions that make them immunocompromised, neonates, and elderly patients.

Pregnant adults are also considered to be at a higher risk of superficial infection (especially VVC) by *C. albicans* than non-pregnant adults, highlighting the opportunistic nature of the microbe. Considered to be the most common human fungal pathogen, the ability of *C. albicans* to adapt to survive in many different host tissues and stress conditions, as well as the wide range of disease manifestations it can cause, highlights the role of this organism as a major infectious pathogen of concern to many people.

1.3.1 Discovery and categorization of *C. albicans*

Human infections caused by *C. albicans* have been theorized to have been happening as early as 400 BC, as Hippocrates recorded oral thrush as a microbial infection seen in patients that caused symptoms described as "mouths affected with aphthous ulcerations" (290). However, it would take many years to identify the causative etiological agent of these infections, and oral thrush (oral candidiasis) would finally be attributed to a mold-like fungal microbe in 1840 following the cooperative work of David Gruby and Fredrik Theodor Berg at the Paris Foundling Hospital, which was an environment where thrush was commonly found in children due to poor living conditions (291). Charles Philippe Robin, a French mycologist, first classified the fungus as *Oidium albicans,* with "*albicans"* meaning "to whiten" (292). *C. albicans* was finally given its current species name in 1923, coined after the white robe (*toga candida*) worn by Roman senators by Dutch mycologist Christine Berkout during her doctoral work for the University of Utrech (293). While infections caused by *C. albicans* have been reported and researched for many years, interest in investigating the pathogen as an infectious agent is just as relevant today due to its global impact on health as a common cause of nosocomial infection, as well as a common cause of severe and invasive infection in the growing population of immunocompromised patients.

1.3.2 Cell morphology of *C. albicans* is influenced by environmental stressors

C. albicans is a polymorphic yeast that has the ability to grow as a yeast (also known as white) cell, a pseudo-hyphae, or as a hyphae depending on certain host conditions (294–296). The ability of *C. albicans* to alter its morphology due to environmental conditions is a major advantage for the microbe, allowing it to adapt and survive in challenging environments. The morphology of *C. albicans* is reversible, and the pathogen will proliferate in the ideal cell morphology based on its current environment. There are many environmental factors that influence the morphological shape of *C. albicans* cells in a host, some of which

include cell density, nutrient availability, carbon dioxide concentration, pH, host temperature, stress, etc (297–303). The ability of *C. albicans* to change its morphological cell shape is also tightly regulated, highlighting the importance of cell type to the organism. The cell morphology of *C. albicans* is also very important to note in the context of the behaviors of the organism, as each morphology has its own role in virulence.

1.3.2.1 Unicellular budding yeast cells

Unicellular budding yeast cells are considered to be the cell morphology for *C. albicans* found in most *in vitro* situations, and these cells have traits that are specific to this morphology type (304, 305). Yeast cells are typically round or oval in shape and range from 5-6μm in size (306). On agar, these cells typically form white, smooth, and domed colonies (304, 305, 307). Yeast cells undergo budding to reproduce, and nuclear division happens at the junction where mother and daughter cells are connected (308–310). The cell shape of *C. albicans* is tightly regulated, and the ability of the organism to remain in its unicellular yeast morphology is dependent on certain environmental factors. For example, *C. albicans* remains mainly in its yeast form in an acidic (pH < 6) environment (294). Quorum sensing molecules also influence *C. albicans* morphology as high cell densities ($>10^7$ cells per mL^{-1}) promote *C. albicans* cells proliferating in its yeast morphology (311, 312). In general, the morphological state of *C. albicans* strongly influences infection, as yeast cells are believed to be prominent in the dissemination of infection, while the hyphal morphology of *C. albicans* has been noted as being the invasive morphological form of the pathogen, important for tissue penetration, damage, and infection progression (313–316).

1.3.2.2 Filamentous *C. albicans*

Filaments formed by *C. albicans* are important not only in invasive infection, but also in the survival of the pathogen in stressful environmental situations independent of infection. Hyphal filaments form directly from yeast cells in specific environmental conditions and the morphology can be reversed back to yeast form, also dependent on environmental conditions. Filamentous morphology in *C. albicans* is distinctly different than yeast and pseudo-hyphae morphology, as hyphae are long, tube-like filaments that do not have constrictions at the neck of the mother cell and have completely parallel sides (307). The pH of the host environment can influence filamentous hyphal growth of *C. albicans*, as environmental pH that is more

alkaline (>7) stimulates the morphological change to filamentous hyphae from yeast cells (294). The presence of serum in a culture can also induce hyphal formation, and growing the organism with serum at 37°C is often used to identify *C. albicans* in medical microbiology diagnostic tests due to the hyphal formation that occurs readily in these conditions (297). The temperature of a host directly effects the morphology of the organism, as *C. albicans* cells are more likely to be in yeast morphology at temperatures below 30°C, while hyphal morphology is stimulated at 37°C, which is significant as 37°C is also human body temperature, highlighting adaptations by the organism to a human host (294, 307). Hyphal development can be stimulated by the presence of peptidoglycan, a bacterial cell wall component, and this process likely plays a role in interactions between *C. albicans* and other microbes in host tissue environments (317).

1.3.2.3 Pseudohyphae

The role of pseudohyphae for *C. albicans* is less well known than for yeast or hyphal morphology, but pseudohyphae are typically considered to be a transition state between yeast cells and hyphal *C. albicans* cells. This transition state can be seen readily in certain *in vitro* conditions, including an environmental pH of 6.0 and temperature of 35°C, both of which are directly between the ideal temperature and environmental pH for yeast and hyphal morphologies (294, 307). While there are some morphological similarities between pseudohyphal and hyphal *C. albicans* cells that include elongation from the mother (yeast) cell, pseudohyphae have distinct morphological features that include a constriction at the neck of the mother cell, as well as buds along subsequent septal junctions (318). Another way to distinguish pseudohyphae from filamentous hyphal *C. albicans* cells is to measure the width of the segments of the pseudohyphae, as they are typically wider than hyphae, and the width of the filaments are not always constant, with them being wider in the center compared to the outer portions of the filament (295). Despite sharing a similar cell appearance with hyphae, its believed that pseudohyphae are more similar to yeast than hyphae due to its mechanism of polarized growth and cell cycle organization (319).

1.3.2.4 Regulation of polymorphism by C. albicans

The polymorphism of *C. albicans* cells is tightly regulated due to its involvement in both colonization and virulence. Multiple signal transduction pathways can be involved in hyphal development depending on the environmental conditions. For example, mitogen-activated protein kinase (MAPK) pathway in *C. albicans* can be induced by the embedded matrix environment, cell wall damage, and low nitrogen, with the induction of this pathway stimulating filamentation (320–325). Cyclic adenosine monophosphate-protein kinase A (cAMP-PKA) pathway is also very important for the regulation of hyphal growth in *C. albicans*, and it can be induced by factors like the exposure of *C. albicans* to serum or bacterial peptidoglycan (317, 326, 327). Hyphal formation in response to specific pH conditions is regulated by the Rim101-pH sensing pathway, and the deletion of *RIM101* disables the ability of *C. albicans* to hyphae in alkaline pH environments (328). Some notable transcriptional factors heavily involved in hyphal development that are activated by these pathways include Efg1 (which is downstream of the cAMP-PKA pathway), Cph1 (which is downstream of the MAPK pathway), and Ras1 (which is able to stimulate both the cAMP and MAPK pathways) (329–332). Overall, the morphology of *C. albicans* is tightly regulated, and environmental factors are responsible for the morphological state of *C. albicans* in different tissue environments.

1.3.3 Colonization of *C. albicans* in the human host

C. albicans is a very common asymptomatic member of the human microbiota and is thought to colonize approximately 25-40% of the human population in at least one tissue environment at any given time (333, 334). It is not uncommon for humans to first acquire *C. albicans* at birth through vertical transmission for their mothers, which can lead to early colonization by the organism (335). Transmission of the fungal pathogen can occur at any time in life, however, and it is especially common to acquire the pathogen in a hospital setting (subsequently putting the patient at risk of nosocomial infection) (336, 337). Colonization by *C. albicans* in healthy individuals can occur on the skin and in mucosal environments, some of which include the mouth, vaginal tract, respiratory tract, urinary tract and the gastrointestinal (GI) tract (294, 338, 339). Like GBS, colonization by *C. albicans* is often asymptomatic, but is also an important factor in causing infection following changes to the host environment, which includes antibiotic use, disrupting the microbiota, changes in diet, and new immunocompromising factors including immunosuppressive drugs or

disease development (340). Immunocompromised patients exhibit higher rates of *C. albicans* colonization in certain host tissues depending on their medical condition. For example, some studies have shown that Human immunodeficiency virus (HIV) patients, as well as diabetic patients, are commonly colonized with *C. albicans* in the oral cavity or the vaginal tract, with those patients also at an increased risk of infection by the opportunistic pathogen (341–349).

To colonize, *C. albicans* must adhere to the epithelial cells of the tissue environment it is attempting to colonize (350). During adhesion, it is vital that the immune system is not alerted to the presence of the organism to avoid clearance by immune cells. The recognition and subsequent clearance of *C. albicans* can occur in a host by utilizing a variety of both innate and adaptive immune cells, including macrophages, neutrophils, dendric cells, monocytes, T cells, and B cells (351–356). *C. albicans* contains multiple pathogen-associated molecular patterns (PAMPs), which are either expressed on the cell wall or secreted and are recognized by pattern-recognition receptors (PRRs) found on the cell wall of immune cells, with the binding of PAMPs and PRRs leading to phagocytosis by immune cells or the secretion of inflammatory factors. To avoid immune system-mediated clearance, *C. albicans* can mask cell surface PAMPs like β-glucan depending on environmental and morphological conditions, as well as secrete proteases that degrade the immune factors responsible for complement opsonization, disrupting the immune response (357–362). Maintaining cell density homeostasis is also important for *C. albicans* to maintain asymptomatic colonization by avoiding overgrowth, with the organism utilizing quorum sensing molecules like farnesol to slow growth and prevent the yeast-to-hyphal transition when cell density is high in the colonizing tissue environment (363, 364). Colonization can lead to infection in immunocompromised people following overgrowth, with specific virulence factors of *C. albicans* allowing the pathogen to transition from an asymptomatic to invasive state in the host.

1.3.4 Virulence factors of *C. albicans*

Typically, it is more advantageous for a microbe to asymptomatically colonize a host to establish itself as a member of the host microbiota and avoid immune response. However, like GBS, *C. albicans* as an opportunistic pathogen utilizes a wide range of virulence factors when colonization is no longer ideal for the organism and instead must infect the host to survive host and environmental challenges. *C. albicans*

can colonize and infect a wide range of host tissue environments due to its large number of virulence factors, some of which include polymorphism, adhesions, invasins, quorum sensing, secreting hydrolytic enzymes and toxins, withstanding pH challenges, and forming antifungal resistant biofilms.

1.3.4.1 Yeast-to-hyphal transition

While the morphological state of *C. albicans* itself is important to note for its ability to survive stressful environmental conditions, the ability of *C. albicans* to be polymorphic is a critical virulence factor for the organism. For example, *C. albicans* are able to undergo a phenotypic transition from "white" yeast cells to "opaque" cells both spontaneously as well as due to environmental conditions (365–368). These two morphological states, while both being able to cause infection, exhibit different virulence traits depending on environmental conditions. In a zebrafish infection model it was shown that while both white and opaque cells can cause infection, temperature can influence the pathogenicity of these cells, as warmer body temperatures enhanced the virulence of white cells, but not opaque cells *in vivo* (369). The response of macrophages and neutrophils to both white and opaque cells differ *in vitro*, with white cells being phagocytosed more efficiently than opaque cells (369). Beyond white and opaque cells, the ability of *C. albicans* to be polymorphic and transition from yeast-to-hyphae is also an important virulence factor for the organism, with each morphological state having their own distinct roles in virulence. Yeast cells are involved in virulence in multiple ways, including dissemination of the organism into tissues and the bloodstream, in the colonization of the organism into different host niches, biofilm formation, and in the adhesion of the organism to host cell surfaces (294, 313–316, 370–373). Hyphal filaments have their own unique role in adhesion and biofilm formation, and are also critical for invasion into tissues, damaging host epithelial cells, and damaging immune cells like neutrophils and macrophages (372–383). Previous work has shown that strains of *C. albicans* unable to filament, or strains growing in a constitutively filamentous state (unable to switch to yeast cells from mature hyphal filaments), have reduced virulence compared to strains capable of switching from yeast-to-hyphae *in vivo* (315, 384–387). This is likely due to the ways in which *C. albicans infects*, which often starts with yeast cells adhering to host cells, then triggering the yeast-to-hyphae transition in order to form hyphae that are then able to penetrate, damage, and invade the host cells using invasins (380, 388–393).

1.3.4.2 Adhesins

C. albicans utilizes adhesins, which are specialized proteins that mediate the adherence of *C. albicans* not only to host cells but also to other microorganisms or abiotic surfaces, to establish itself as a member of the host microbiota (94, 392, 394–400). Agglutinin-like sequence (ALS) proteins are one of the most well-studied families of adhesins, with eight ALS cell surface glycoproteins proteins being currently known to exist in the genome of *C. albicans* (ALS 1-7, 9) (401). While the role of most of the ALS family proteins in adherence and virulence has been studied in some capacity, the most well-studied ALS family protein is Als3 (401–405). *In vitro C. albicans* binds to N- cadherin found on the endothelial cell membrane and E-cadherin in oral epithelial cells to induce the endocytosis of *C. albicans* into these cells, and the loss of *ALS3* prevents this process from occurring (383, 406). Als3 has been implicated as not only an important adhesin for *C. albicans* in binding to host-cell surfaces, but is also involved in the adherence of *C. albicans* to other microbes, allowing for the formation of polymicrobial biofilms (383, 400). Research investigating the role of Als3 in active infection has shown conflicting results, as some groups have found *Δals3* strains to have attenuated virulence in murine models, while others have seen no difference in virulence between *Δals3* and wild-type strains (407, 408). Differences in virulence of *Δals3* mutant strains *in vivo* has been hypothesized to possibly be due to differences in infection route and immunocompetency in the murine models used, and/or due to the conservation of other adhesion genes such as *HWP1* (407, 409). Hyphal wall protein (HWP) family adhesins are expressed solely on germ tubes and true hyphae, and included the genes *HWP1, HWP2,* and *RBT1* (382, 410–413). The most well studied adhesin in the HWP family hyphal wall protein 1 (Hwp1), which is a GPI-linked adhesin protein (382, 410). The adherence of Hwp1 to host cells is theorized to be due to the structural similarities of the amino acid sequence of the NH_2-terminal domain of Hwp1 to the mammalian proteins responsible for binding to mammalian transglutaminases, providing a substrate for adherence to host cells by *C. albicans* (382). Multiple mutant strains of *C. albicans* lacking functioning *HWP1* have shown deficiencies in both binding to buccal epithelial cells, as well as in virulence in murine mouse models of systemic *C. albicans* infection (373, 382, 414–416). Hwp1 is also important for biofilm formation both *in vitro* and *in vivo*, as biofilms formed by mutant strains lacking functional *HWP1* were found to be defective (417, 418). Adhesins belonging to different families can work together to form biofilm, as Als3 and Hwp1 are complementary adhesions during biofilm formation *in vitro*

(419). Other notable proteins involved with adherence in *C. albicans* to host cells and abiotic surfaces include integrin-like surface protein (Int1), secreted aspartic surfaces proteases (Sap6 and Sap9), enhanced adherence to polysterine protein (Eap1), and the cell-surface GPI-linked protein Mp65 (420–424).

1.3.4.3 Invasins

Following the adherence of *C. albicans* to host cells such as epithelial and endothelial cells, *C. albicans* must invade these cells to cause damage and subsequently cause infection in the host (379, 380, 425). One way that the pathogen invades following adherence to host cells is to undergo a yeast to hyphae transition, allowing for active penetration of tissues and damage to occur in the host cells (426, 427). An alternate way *C. albicans* can penetrate epithelial and endothelial cells is to utilize invasions, which are expressed on the fungal surface and allows for endocytosis to occur following binding to host ligands, which triggers the engulfment of *C. albicans* by the attached cells (406, 427). Interestingly, Als3, a well-known adhesin protein discussed above, is also known to function as an invasin by *C albicans* (383). *C. albicans Δals3* strains have shown a reduced ability to adhere to and invade epithelial cells, highlighting its important role in virulence during active infection (383). Another invasin protein identified in *C. albicans* is Ssa1, which is member of the heat shock protein 70 (Hsp70) family that is expressed on the cell-surface of the pathogen (428). Knock-out mutant strains of Ssa1 are not only unable to effectively invade epithelial and endothelial *in vitro*, but also exhibit reduced virulence in an murine oropharyngeal candidiasis infection model despite forming filaments similar in size to wild-type *C. albicans,* highlighting the role of Ssa1 in active penetration (428). Overall, both adhesins and invasins are very important to the initial stages and pathogenesis of infection caused by *C. albicans*.

1.3.4.4 Quorum sensing molecules

C. albicans can also utilize secreted factors like quorum sensing molecules (QSM) to both enhance its survival in microbially competitive tissue environments as well as alter host immune response (429). Quorum sensing (QS) is a virulence tool that both bacteria and fungi utilize that involves secreting hormone-like molecules, QSM, in response to microbial cell densities, which in turn can regulate gene expression (312, 430). QSM molecules in *C.albicans* are a major regulator of polymorphism for the organism, as cell

densities less than 10^6 allows for the yeast-to-hyphae transition to still occur depending on environmental conditions, while cell densities larger than 10^6 promotes the secretion of farnesol, a major QSM for the pathogen, as well as the growth of *C. albicans* as budding yeasts *in vitro* (312). Farnesol, the best characterized QSM known to be secreted by *C. albicans*, is responsible for additional physiological events beyond the inhibition of filamentation by the microbe. Biofilm formation, another virulence factor of *C. albicans*, can be directly influenced by farnesol, as the secretion of farnesol has been shown to inhibit biofilm formation as well as downregulate the expression of *HWP1 in vitro* (364). Beyond reducing filamentation of *C. albicans*, farnesol can also act as an antimicrobial compound, reducing the growth of multiple species of bacteria and fungi *in vitro*, likely allowing the organism to outcompete in the microbiota of some tissue environments (431–435). Farnesol can also directly influence virulence through host cell damage, as the QSM has been theorized to be involved in damaging the epithelial cell layer of a host, which is important in the initial invasion stages of infection (436). Immune responses can also be altered in human immune cells in *in vitro* assays, as the introduction of farnesol to these cells stimulated low-grade activation of neutrophils and monocytes but did not alter the ability of these cells to uptake or kill *C. albicans* (429). In a systemic *C. albicans* murine infection model exogenous administration of farnesol to infected mice led to increased mortality as well as fungal burden in the kidneys, indicating that the QSM may have a direct role in disease pathogenesis during *C. albicans* infection (437). Tyrosol, another recently discovered QSM of *C. albicans*, has the opposite effect on filamentation compared to farnesol, with the secretion of the QSM leading to a reduced lag time in growth, as well as stimulating both filamentation and biofilm formation *in vitro* (438, 439). The effects of tyrosol can be reduced by the introduction of farnesol to growth medium *in vitro*, indicating that the regulation of the secretion of QSM is likely tightly regulated in *C. albicans* and is directly influenced by environmental factors (438, 439).

1.3.4.5 Secreted hydrolases

QSM are not the only *C. albicans* secreted factors heavily involved in virulence. The fungus can secrete a variety of different hydrolase compounds, including proteases, lipases, and phospholipases, and these hydrolases have been hypothesized to have a role in virulence for the pathogen. Secreted aspartic proteases (Saps) are the most investigated family of secreted proteases, with 10 distinct genes (*SAP*1-10)

belonging to the *SAP* family (440–442). The expression of *SAP* family genes are often associated with other virulence factors for the organism, including hyphal formation, adhesion, and phenotypic switching (443). In HIV-positive patients, *SAP* expression was significantly higher in *C. albicans* strains isolated compared to strains in HIV negative patients following oral candidiasis or vaginal candidiasis, highlighting the possible role of *SAP* expression in the ability of the pathogen to infect certain patient groups (444–446). Sap activity has been directly implicated to contribute to tissue damage in human oral candidiasis model *in vitro*, and some mutant strains of different SAP genes have shown reduced virulence in disseminated infection in murine models, highlighting the influence of multiple SAP genes on virulence (447–450). Lipases, another type of secreted hydrolase enzymes, also influence virulence in *C. albicans* strains. Like *SAPs,* there are 10 known gene members of the Lipase family (*LIP1-10*), and different lipase genes likely have different roles in virulence (451, 452). Less is known about the role of each lipase gene in virulence compared to *SAP* genes, but there is some evidence that some lipase genes are likely involved in virulence, as mutant strains of *LIP8* have shown reduced virulence in murine models compared to WT strains (453). Certain host cells (macrophages and hepatocytes) have been shown to display signs of toxicity when exposed to extracellular *C. albicans* lipase in vitro, indicating that the secretion of lipase by the organism may facilitate host cell damage, which is important for infection progression (454). Lipases are also well known to be involved with nutrient acquisitions in *C. albicans,* as lipases are able to catalyze the synthesis and the hydrolysis of triacylglycerols (455). Phospholipases, another secreted hydrolase enzyme by *C. albicans*, is responsible for catalyzing the hydrolysis of phospholipids, and has been implicated to be involved in both host cell penetration and cell lysis (456). Disseminated candidiasis infection was less severe in mice infected with a *C. albicans* strain with *PLB1* (a gene encoding phospholipase secretion) knocked out compared to wild-type *C. albicans* despite no detectable changes in growth or adherence, indicating the involvement of phospholipases in infection (456). *C. albicans* strain isolated from the blood of patients were found to have increased phospholipase production compared to commensal isolates, indicating that phospholipase production may be regulated by infection (457).

1.3.4.6 Secreted toxins

Beyond QSM and secreted hydrolases, *C. albicans* can also secrete other virulence factors like toxins, with candidalysin being a well characterized toxin in the pathogen. Candidalysin, a cytolytic peptide toxin encoded by the *ECE1* gene, is secreted by hyphae and has been shown to be able to both damage host epithelial cells and activate innate immune responses during infection (458). To cause damage, *C. albicans* secretes candidalysin on the surface of epithelial cells, which in turn reduces the ability of the cell to maintain its permeability barrier (458). The reduced permeability barrier is likely due to the ability of the toxin to form membrane pores on the surface of epithelial cells, allowing for an influx of calcium and other compounds that damage epithelial cells (458, 459). The toxin can also attack the membrane of macrophages and dendric cells *in vitro*, reducing the ability of these cells to undergo phagocytosis and damage *C. albicans* cells (460). Secretion of Candidalysin can directly influence virulence, as strains able to freely secrete the toxin have been shown to be more virulent than strains with reduced candidalysin secretion *in vivo* (461). Secreted factors by *C. albicans* play a major role in pathogenicity for the organism as they allow the organism to obtain nutrients from its host environment, reduce microbial competition, and damage host epithelial, endothelial, and immune cells.

1.3.4.7 Environmental pH stress tolerance and heat shock proteins

The ability of *C. albicans* to withstand environmental stresses is a major virulence factor for the pathogen, not only because it allows the organism to survive in many different environmental conditions, but it also reduces the ability of the host to challenge the organism using stress mechanisms that reduce viability in other microbes. For example, *C. albicans* is able to withstand extreme pH conditions as the organism has been noted to be able to survive a pH range of pH 2-10 (294). The ability of the organism to withstand rapid changes in pH is also critical for the organism, and this process is regulated by the *RIM101* pathway, which regulates the gene expression of pH response genes including *PHR1, PRA1, AND PHR2* (328, 462–464). A loss of *RIM101* function in *C. albicans* directly leads to reduced virulence in multiple infection types, highlighting the direct influence of pH sensing by the organism *in vivo* (465, 466). Environmental pH can directly induce the yeast-to-hyphae transition, which is a vital *C. albicans* virulence factor (302, 385). *C. albicans* is also able to modulate the extracellular pH of its environment, as the

organism can alkalinize is environment when beneficial to the survival of the organism, autoinducing hyphal formation (302). Another way *C. albicans* survives environmental stress is through the use of heat shock proteins (Hsps), which prevents protein unfolding in the organism when exposed to high temperature, starvation, or oxidative stress (311, 467). Hsps have also been implicated to be involved in the virulence of *C. albicans* beyond basic survival. For example, heat shock protein Hsp90 likely contributes to systemic candidiasis progression, as *C. albicans* infected mice treated with antibodies to Hsp90 had significantly less mortality compared to untreated mice (468). Functionality of Hsf1, a heat shock transcription factor, is also vital for virulence by *C. albicans*, as preventing Hsf1 activation attenuates virulence in a mouse model of systemic candidiasis (469). Environmental stress caused by reactive oxygen species (ROS) production by phagocytes can also be suppressed by *C. albicans in vitro* (470). The detoxification of ROS can be accomplished by *C. albicans* utilizing superoxide dismutase (SOD) and strains with SOD mutations show reduced virulence during infection *in vivo* (471, 472).

1.3.4.8 Biofilm formation by *C. albicans*

Biofilms, which are complex surface-associated cell population embedded in an extracellular matrix (ECM), are important for virulence in many microbes as the phenotypes and properties of these cells differ from their planktonic cell state (473–475) *C. albicans* can form biofilms on multiple adherent surface types, including both abiotic surfaces (especially catheters and dentures) and biotic surfaces (commonly mucosal cell surfaces) (475). The formation of biofilm typically occurs initially when yeast cells adhere to an abiotic or biotic surface, followed by the replication of yeast cells in this environment, then hyphal cells form on the upper surface of the biofilm while yeast cells become dispersed from the bottom of the biofilm (372, 476, 477). Biofilms have a direct role in virulence for *C. albicans* in multiple ways. For example, yeast cells dispersed from mature *C. albicans* biofilm have been shown to be more adherent *in vitro*, as well as more virulent in a disseminated infection mouse model compared to planktonic yeast cells (478). The growth stage of biofilm can also influence virulence, as mature *C. albicans* biofilm show resistance to neutrophilic killing, especially compared to developing biofilm, and these neutrophils are unable to trigger ROS production in mature biofilms, providing cells in mature *C. albicans* biofilms a survival advantage (479). Biofilms are also more resistant to antifungal drugs (including amphotericin B, fluconazole, flucytosine,

itraconazole, and ketoconazole) than planktonic cells (480). While the exact reasons for antifungal resistance by *C. albicans* biofilms is not fully understood, it is theorized that common resistance techniques found in many microbial biofilms, including reduced penetration by antifungal agents through the biofilm matrix, reduced growth rate, nutrient limitation, expression of resistance genes, or persister cells could all play a role in antifungal resistance by *C. albicans* biofilms (476, 481–483). Antifungal resistance by *C. albicans* biofilms causes major issues during infection in patients, as the ability of biofilm to survive on surfaces like implanted medical devices despite antifungal treatment creates a reservoir for *C. albicans* in patients that increases the risk of developing hard-to-treat systemic infections of tissues, organs, and the bloodstream by *C.albicans* (484).

1.3.5 Antifungal resistance by *C. albicans*

Antimicrobial resistance is a health concern globally, as rising rates of resistance to these drugs could lead to previously treatable infections becoming completely resistant to treatment in the future. Antifungal resistance by infectious fungal pathogens is complicated to combat, as developing new antifungals is difficult as fungi are eukaryotes, reducing the amount of cellular targets available in fungi that are not also present in human cells (485, 486). While antifungal resistance is more common in non-*albicans Candida* species like *C. glabrata, C. parapsilosis*, and *C. auris*, it is still possible to see resistance to certain antifungals by *C. albicans*, especially in patients who face prolonged exposure to antifungals due to chronic conditions that make them more susceptible to *C. albicans* infections (487–491). There are four major classes of antifungal drugs used to treat *Candida* infections (pyrimidine analogues, polyenes, azoles, echinocandins) and each class faces different mechanisms of resistance by *Candida species.* Resistance to antifungal drugs are considered primary (also called intrinsic), secondary (also known as acquired resistance), or clinical (491, 492). Primary antifungal resistance occurs when a species as a whole is known for all isolates having an innate resistance to a certain antifungal (485). Secondary/acquired resistance can occur in *C. albicans* and other pathogenic fungi when exposed to an antifungal agent, and this resistance can be reversible or persistent due to one or more genetic alterations following antifungal exposure (492). Clinical resistance is defined as isolates unable to be successfully eradicated by an antifungal agent in patients despite the antifungal still being effective against the same isolate *in vitro* (492). Clinical resistance

to an antifungal during a *C. albicans* infection is difficult to predict in a patient because of the success of the antifungal agent during *in vitro* testing, and is likely caused by a combination of host, antifungal, and pathogen factors (492). Overall, while antifungal resistance is difficult to combat for many infectious fungal pathogens, there has also been some research conducted investigating exactly how *C. albicans* can develop antifungal resistance against certain classes of antifungals.

1.3.5.1 Pyrimidine analogue antifungal resistance by *C. albicans*

Pyrimidine analogues, which is a class of antifungal drugs originally synthesized as an anti-tumor drug in the 1950s, only has one member: 5-Flucytosine (5-FC) (493, 494). 5-FC was first used to successfully treat systemic *Candida* infection in patients in 1968, and is occasionally used today in combination with azole antifungals for *Candida* infected patients (494, 495). The mechanism of action of 5-FC involves uptake of the compound by fungal cells by the enzyme cytosine permease, where 5-FC gets converted to 5-fluorouracil (5-FU) (496). The conversion to 5-FU is detrimental to the fungus, as 5-FU competes with uracil in fungal RNA, inhibiting RNA synthesis and subsequent protein synthesis (497, 498). 5-FU can also inhibit DNA synthesis, as the conversion of 5-FU to fluoro-deoxyuridylic acid inhibits thymidylate synthase, which ultimately causes DNA damage in fungal cells (497, 498). *C. albicans* can develop secondary resistance to flucytosine due to changes in the enzymes cytosine permease, cytosine deaminase, or uracil phosphoribosyl transferase (encoded by the *FCy2*, *FCy1*, and *FUR1* genes respectively), as changes to these enzymes can prevent the uptake of the drug, the conversion of 5-FC to 5-FU, or the conversion of 5-FU to 5-fluorouridine monophosphate, all of which are necessary actions for 5-Flucosytosine to be an effective antifungal (499). Secondary resistance to flucytosine commonly forms in *C. albicans* isolates when given to patients in monotherapy, so most administration of the antifungal is done in combination with amphotericin B, which exhibits synergy with flucytosine to prevent fungal growth (497, 500, 501).

1.3.5.2 Polyene antifungal resistance by *C. albicans*

Polyene family antifungals, which are natural antifungal compounds that are typically obtained from *Streptomyces* species bacteria, are utilized to combat infections caused by *C. albicans* (502). Polyene antibiotics are effective against numerous other fungal pathogens (including Aspergillus spp., Cryptococcus

spp., and Fusarium spp) and are considered to be a broad-spectrum antifungal (503, 504). Polyene antifungals cause damage to fungi by first binding to ergosterol, which is a necessary fungal sterol needed to maintain both membrane fluidity and integrity in fungal cells (505–507). Following the binding of polyene compounds to ergosterol, pores form in the plasma membrane of fungal cells, and ions and small organic molecules leak from the ion channels created by the pores, eventually leading to cell death (508–510). Amphotericin B and Nystatin are two antifungal drugs belonging to the antifungal drug class polyenes that are widely used clinically for patients facing infection by *C. albicans* (511). Amphotericin B (AmpB) is commonly used successfully to treat severe invasive infections of *C. albicans*, but harmful effects from the compound can occur, and nephrotoxicity can occur in patients treated with AmpB (512, 513). Nystatin is a topical antifungal available to patients infected with *Candida*, and is commonly used to treat oral candidiasis in both neonates and adults (514–516). While the development of resistance to polyene antifungals like AmpB and Nystatin are considered rare for *Candida* species, it is still possible and is important to monitor in patients (517). The cause of resistance to AmpB by *C. albicans* is not fully understood, but is theorized to be caused by a mutation in the *ERG3* gene, which is responsible for encoding the enzyme C-5 sterol desaturase, which is necessary for ergosterol biosynthesis (518, 519). When C-5 sterol desaturase is unable to be activated due to mutations in the ERG3 gene, it reduces the amount of ergosterol found in the fungal membrane, and therefore reduces the ability of AmpB to bind to ergosterol and form pores in the cell membrane of *C. albicans* (518). Resistance to nystatin is rare but has been seen in clinical samples, and *in vitro* studies have shown that inhibiting ergosterol production through gene mutation in *C. albicans* creates nystatin resistance in those strains (520–522). Cross resistance to polyene antifungals can occur with azole antibiotics (518, 523, 524).

1.3.5.3 Azole antifungal resistance by *C. albicans*

Azoles, which are heterocyclic synthetic antifungals commonly used clinically, inhibit fungal growth by inhibiting the fungal cytochrome P450-dependent enzyme lanosterol 14α-demethylase (also known as CYP51 or Erg11p), which is important for ergosterol biosynthesis (491, 525). This inhibition leads to an accumulation of toxic methylated intermediates, which ultimately disrupts fungal cell membrane function, inhibits growth, and occasionally causes cell death (507, 525, 526). While capable of causing cell death in

certain fungal species like *Aspergillus*, azole antifungals typically exhibit fungistatic activity against *Candida* spp (491). Antifungals belonging to the azole class include fluconazole, itraconazole, voriconazole, posaconazole, and isavuconazole (491). Fluconazole is one of the most commonly used azole drugs clinically used to treat *Candida* infections in both healthy and immunocompromised patients, likely due to their low cost, low toxicity to human cells, and their efficacy during treatment against *Candida* (489, 516, 527–529). Fluconazole is a very useful antifungal clinically as it can be used to treat both systemic and superficial infections by *C. albicans* (516). Fluconazole resistance is not very common in *C. albicans*, and resistance rates of clinical resistance in *C. albicans* strains has been estimated to be ~0.5-3% depending on the study (530–532). Despite not being very common clinically, resistance to fluconazole can still occur in *C. albicans* clinical isolates, and the pathogen possesses numerous mechanisms to develop resistance to this antifungal. First, *C. albicans* can overexpress *ERG11*, which is the gene encoding the drug target of fluconazole, and this overexpression reduces the activity of fluconazole due to the increased ergosterol production allowing for the organism to withstand fluconazole challenge and obtain resistance to the antifungal (533). Alterations in the drug target of fluconazole can also cause fluconazole resistance in *C. albicans*, as point mutations in the coding region of *ERG11* which results in amino acid substitutions that alters protein structure, ultimately decreases the affinity of fluconazole to bind to the organism and therefore cause toxic effects to the cell (534, 535). Fluconazole resistance can also occur in *C. albicans* if loss-of-function mutations occur in *ERG3*, as inactivation of the enzymes encoded by this gene does not allow for toxic methylated sterols to form in *C. albicans* when in the presence of fluconazole, reducing the ability of the drug to prevent fungal growth (518, 536). Increased drug efflux is also a major cause of fluconazole resistance by *C. albicans* isolates, as efflux of fluconazole utilizing transport proteins allows the fungal cell to reduce the amount of intracellular fluconazole, and therefore reduces the susceptibility of the cell to fluconazole (537).

1.3.5.4 Echinocandin antifungal resistance by *C. albicans*

Echinocandins, which are typically the first-line antifungal therapy for patients with invasive infection, are also typically very effective against *C. albicans* in patients and *in vitro* (516). This class of antifungals is the newest form of antifungals available for patients, and clinically available echinocandins

for patients infected with *C. albicans* include micafungin, anidulafungin, and caspofungin (538, 539). These antifungals are fungicidal against *Candida* species, and patients treated with echinocandins typically see less or milder side effects compared to polyenes and azoles, which may contribute to the popularity of this class of antifungals clinically (528, 540, 541). The mechanism of action for echinocandin antifungals is to inhibit β-(1,3)-glucan synthase, which is an enzyme that is required for the synthesis of 1,3-β-d glucan, a necessary component of the cell wall of fungi (542, 543). Resistance to echinocandins by *C. albicans* is very rare, with <3% of clinical isolates being estimated to be resistant to echinocandins (544, 545). Resistance can occur, however, when amino acid substitution mutations occur to the *FKS* genes, which encodes the catalytic subunit of β-(1,3)-glucan synthase (545, 546). Persister cells of *C. albicans* that are drug-tolerant can also occur following challenge by echinocandins, with these cells exhibiting elevated in vitro MIC values to the drug due to adaptations occurring to promote survival of *C. albicans* (545, 547, 548). While the development of resistance to echinocandin by *Candida* species is rare, it still can occur following prolonged or repeated exposure to the antifungal in patients, and subsequently should be monitored in patients receiving long-term echinocandin treatment (549, 550).

1.3.5.5 Antifungal resistance due to biofilm formation

While *C. albicans* isolates do not commonly develop resistance to most of the antifungals currently available to treat the pathogen, there are still challenges in treating the organism successfully in patients. The formation of biofilms by *C. albicans* can cause major issues in patients, and one of the major drivers of this difficulty is due to the ability of biofilms to withstand antifungal challenge. The structural environment of biofilms allows for increased drug tolerance as cells packed deeply within biofilms are protected from coming in contact with the antifungal drugs physically (551). Efflux pumps are often upregulated in *C. albican*s cells found in biofilms even without prior antifungal exposure, indicating that the upregulation of these pumps may be a feature of biofilms that allows the biofilm to be protected from antifungals and antimicrobial compounds secreted from competing microorganism both during early development and after maturity (484, 552, 553). Stress responses that are upregulated in *Candida* biofilms can also increase drug resistance for these biofilms, creating challenges in treating patients (554).

1.3.6 *C. albicans* infection in adults

As an opportunistic pathogen, *C. albicans* will likely never cause severe infection in most of the adult population it colonizes. However, unlike GBS, this organism can cause superficial infections in healthy people. Superficial infections by *C. albicans* should not be undervalued as a major issue for patients, as despite them being non-lethal, they can lead to morbidity in patients, and can happen frequently in certain patients. In patient groups such as those who are immunocompromised, elderly, or pregnant, this opportunistic pathogen can cause both superficial and severe infections (555–561). Infections caused by *C. albicans* are very prominent in patients suffering from certain medical conditions, such as HIV and diabetes, and these infections can often be complicated to treat in these patient groups (562, 563). Nosocomial infections are also commonly caused by *C. albicans*, likely due to its ability to adapt in difficult environments, as well as the immunocompromised health status of hospitalized patients (336, 555). *C. albicans* is a highly adaptive organism and can cause infection in different host tissues, including the skin, vaginal tract, respiratory tract, gastrointestinal tract, and the oropharynx. The ability of this organism to colonize such a variety of tissue environments allows it to cause a wide range of infection types, such as vulvovaginal candidiasis, oral thrush, respiratory infection, urinary tract infection, skin infections, and bloodstream infections. The gene expression of *C. albicans* can vary depending on infection type as well as patient type, leading to a wide variety of patient outcomes. Certain infections (like invasive Candidiasis) are difficult to treat, and emerging antifungal resistance by the pathogen highlights the need for an effective vaccine against *Candida* species. Investigating how this organism responds in different infection types as well as in different patient groups will give us a better understanding of the wide variety of virulence factors this organism possesses, and how we can best develop treatments to improve patient outcomes.

1.3.6.1 Vulvovaginal candidiasis

Vulvovaginal candidiasis (VVC) is diagnosed when symptoms of vaginal inflammation occur and a *Candida* species is present with no other infectious microbes (564) . While multiple *Candida* species can cause VVC, the most common *Candida* strain causing VVC is *C. albicans*, with roughly 90% of cases being attributed to the pathogen (565). VVC is a very common *C. albicans* infection type in women, as it is estimated that as many as 75% of women will experience at least one vulvovaginal infection caused by the

pathogen in their lifetime (566, 567). The ability to obtain effective over-the-counter treatments against VVC may also lead to these infections being underreported clinically as patients may not visit a medical facility for diagnosis and treatment (565). While the cause of VVC infection can't be identified in most patients, some known risk factors include antibiotic use, new sexual partners, oral contraceptive use, and hormone replacement therapy (568). Symptoms of VVC in women are not always the same on a case-to-case basis, but some common symptoms can include irritation or itching of the vaginal area, odorless white discharge, soreness, dysuria, and inflammation (569). When VVC infections are acute they can be treated easily with azole antifungals (565). While VVC is common in healthy women and is often treatable with either oral or topical antifungals, the burden these infections cause in certain patients should not be underestimated. For example, roughly 8% of women suffer from recurrent VVC (RVVC), where these patients have four or more episodes of VVC within a year (565, 568). RVVC in women is considered an incurable chronic condition and these patients likely have predisposing genetic factors that may make them more susceptible to *Candida* infections (565, 570). Patients with RVVC often suffer a decreased quality of life due to the frequency of the infections, negative mental health consequences (including feelings of stigmatization, embarrassment, and depression), as well as due to the cost of constant treatment (571–573). Treatment in RVVC patients with antifungal medications is often difficult, as symptoms of RVVC are reduced by antifungal treatment but often re-emerge once the treatment regimen is completed (571). VVC also occurs more frequently in patients with different medical conditions, such as those with diabetes, HIV, and those who are pregnant (347, 567).

1.3.6.2 Oral Candidiasis

Another well-known superficial infection that can be caused by *C. albicans* is oral candidiasis (OC), which is an opportunistic infection of the oral mucosa typically caused by an overgrowth of *C. albicans* in the mouth. While other Candida species can cause OC, *C. albicans* is the most common pathogen, with up to 80% of OC oral lesions being found to contain *C. albicans* (574, 575). Typically OC does not cause infection in healthy people, but instead is secondary to immune suppression caused by medical conditions including HIV, leukemia, and diabetes (294, 576). Other health factors that can also increase the risk of developing OC include certain medications (corticosteroids, chemotherapy drugs, antibiotics, etc), denture

use, smoking, dietary factors (low iron or high carbohydrate diet), exposure to radiation, and reduced salivary flow (294, 576). OC infections are categorized as either "white" or "erythematous" OC, with white OC symptoms including white lesions and plaques on the tongue, palate, buccal mucosa, and oral pharynx, and erythematous OC symptoms including erythematous red lesions on the palate, buccal mucosa, dorsal tongue, or in the oral mucosa in general depending on disease presentation (577–581). Acute pseudomembranous candidiasis, also commonly known as Oral Thrush, belongs to the white OC family and is the most common OC disease manifestation in not only immunocompromised patients but also in newborns (582). While not considered to be a lethal infection in most patients, OC can cause morbidity in patients due to symptoms that include discomfort and pain, altered sense of taste, and difficulty swallowing (583). These infections can be treated with a variety of antifungal treatments depending on the severity and antifungal resistance of the infection, with topical antifungals being very effective typically in mild OC cases, but oral and intravenous antibiotics are also available for patients who do not respond to topical agents (579, 584). While OC infections are considered to be superficial and treatable in most patients, in immunocompromised patients these infections can spread from the oropharynx and become a severe invasive infection (579, 585).

1.3.6.3 Invasive Candidiasis

Invasive infections caused by *Candida* species are very serious, and the increased prevalence of candidiasis in hospital settings has been theorized to be linked to medical advancements increasing the lifespan of immunocompromised patients(516). Invasive candidiasis (IC) has concerning mortality rates in infected patients, with the estimated mortality rates ranging between ~25%-60% depending on the patient population and region of the study (586–590). IC is diagnosed when an infected patient has *Candida* in their bloodstream (Candidemia) or in other typically sterile sites (also known as deep-seated tissue candidiasis) (591). How infection initiates in patients likely depends on multiple factors, including the initial infection site and if the infection later becomes disseminated to other tissue environments. For example, IC originating in the bloodstream can progress to deep-tissue candidiasis as the bloodstream can transport the pathogen to other tissue environments (592, 593). IC can also originate as deep-seated tissue candidiasis, remaining localized to the infected tissue environment or progressing to secondary candidemia

(594). *Candida* can also be introduced to sterile sites through the insertion of medical equipment or during surgery (594, 595). There are multiple risk factors for patients developing IC, some of which include extended hospital stays in the ICU, recent surgery (especially involving abdominal surgery), organ transplant, central vascular catheter use, chemotherapy, glucocorticoid use, and broad spectrum antibiotic treatment (557, 585, 596–598). Immunocompromised patients in general are also at an increased risk of IC (599–602). Candidemia can be caused by a variety of *Candida* species, with multiple studies finding that *C. albicans* was isolated from roughly half of the documented candidemia infections (596, 603, 604). This bloodstream infection is also very prevalent in hospital settings, as candidemia is the fourth most common type of nosocomial bloodstream infection in the United States (605). Candida species are also the most common cause of fungal septic shock in ICU patients (606). Treatment for IC often involves oral or intravenous antifungals, including caspofungin, fluconazole, and amphotericin B (516). While antifungal treatment is considered to be effective, this is usually when infections are treated early in disease progression (607, 608). IC can be challenging to diagnose in patients due to the common presence of yeast cells in many tissues, making positive blood cultures the standard way to positively diagnose IC, which often leads to a delay in treatment due to both the time it takes for microbial growth in lab testing cultures, as well as the volume of *Candida* cells necessary for detection often occurring later in infection progression (591–593, 609). Blood cultures testing for IC are also not always accurate, with an estimated ~50% sensitivity clinically, also leading to a delay in treatment for infected patients receiving false negatives (592, 593). Delay in antifungal treatment can be fatal for patients, with just a 12-24hr delay in the introduction of antifungal treatments being able double the risk of death in candidemia patients (610).

1.3.7 *C.albicans* infection in neonates and young children

While *C. albicans* is known to be able to cause both superficial and severe infections in adult immunocompromised patients, it is also possible for neonates and young children to become infected by the opportunistic pathogen. Especially concerning is when *Candida* infections occur in neonates, as the immune system is still developing at the time of birth, making them highly susceptible to infections caused by the fungus (611–613). The transition from a semi-sterile environment in the developing womb to a microbial-rich environment after birth can pose serious risks for these infants, who possess both a still-

developing immune system as well as a lack of developed immunological memory (613). In general, neonates are susceptible to a wide variety of microbial infections due to their underdeveloped immune system at the time of birth, with roughly 40% of neonatal deaths being attributed to infections (614). A variety of different infections caused by *C. albicans* can occur in neonates early in life, as well as in young children. While *Candida* infections in neonates and infants are sometimes superficial, these children are also at risk of invasive *Candida* infection, which can cause morbidity, long-term health complications (including neurodevelopment issues), or even death (600, 615, 616). A better understanding of the risk factors for these infections as well as how to recognize signs early in infection will likely lead to better treatment options and infection prognosis in these patient groups.

1.3.7.1 Superficial infections caused by C. albicans

Neonates and young children are at an increased risk of certain superficial infections caused by *C. albicans* compared to adults. For example, diaper dermatitis, which is categorized by rashes with confluent erythema with both satellite lesions and pustules, is often caused by *C. albicans* (617, 618). Children with diaper dermatitis are also often colonized with *C. albicans* in the GI tract, with positive cultures being isolated from the stool of these infants (618). Diaper dermatitis caused by *C. albicans* is often easily treated with topical nystatin with a high efficacy rate (619). *C. albicans* has also been implicated to increase the risk and severity of Early Childhood Caries (ECC), which is a condition where one or more decayed, missing, or filled tooth surfaces in the primary detention in children under 3 years old (620–624). Polymicrobial biofilms of *C. albicans* and *S. mutans* are common with ECC, as these organisms seem to synergize with one another to progress the severity of disease in patients (622, 625–627). Treatment for ECC is typically complicated and based on the severity of the disease, as some patients may be effectively treated with changes in diet and/or a topical fluoride, while others may require tooth extraction and extensive restoration (628). One of the most common infections caused by *C. albicans* that neonates are at an increased risk of developing is OC, with OC typically presenting in acute pseudomembranous candidiasis, theorized to be due to their underdeveloped immune system early in life (629). OC can occur in neonates soon after birth following the transmission of *C. albicans* from the mother to the child during vaginal delivery if the mother is suffering from vaginal candidiasis at the time of delivery (629). Other theorized transmission

sources include *C. albicans* contaminated bottle nipples or even the skin of mothers that happen to be colonized with the pathogen (629). Neonatal thrush (also referred to as OC in these patients) is often diagnosed when white patches are found in infants (under 1 year of age) on the tongue and/or oral mucosa (630, 631). OC in neonates can be asymptomatic, but can also lead to irritation and reduced feeding by infected neonates (632). While thrush can be treated in infected neonates with nystatin, spontaneous cure of OC can occur 3-8 weeks following initial infection for some infants (582, 633). OC is not common in healthy children, but can be seen in children with HIV, with the formation of oral lesions caused by *C. albicans* often being seen as a sign of severe disease progression in these children due to increased immunosuppression (634). Superficial mucocutaneous candidiasis infections are often treatable in otherwise healthy infants, in very-low-birthweight neonates superficial infections can sometimes progress to serious invasive infections (635).

1.3.7.2 Invasive Candidiasis

Invasive candidiasis can also occur in neonates, especially those of very-low-birthweight (VLBW), and often the infections lead to concerning rates of morbidity and mortality (600, 636–638). It is thought that ~10% of nosocomial sepsis cases in VLBW infants can be attributed to *Candida* spp, with *C. albicans* being the most prominent *Candida* pathogen found to infect neonates (639, 640). Like oral candidiasis, the development of invasive candidiasis in newborns often starts with vertical transmission of the pathogen from *C. albicans* colonized mothers during delivery leading to colonization by the pathogen (639). Colonization of *C. albicans* in newborns can also occur when the infant is exposed to contact with the skin of mothers or healthcare providers that are colonized with *C. albicans* (639). While colonization of *C. albicans* is a risk factor for infection in neonates in general, additional risk factors have been identified for colonization progressing to infection in these infants. Use of broad spectrum antibiotics, especially cephalosporins, have been implicated in also increasing the risk of neonates developing invasive candidiasis (641). Neonatal patients who have received multiple days of mechanical ventilation or abdominal surgery are also at an increased risk of developing invasive candidiasis (16, 636, 637, 641, 642). Invasive candidiasis can present in multiple infection types, with candidemia with end organ involvement being the most common, and other infections manifestations include urinary obstruction, endophthalmitis,

endocarditis, and meningitis (643, 644). The mortality rates of invasive candidiasis in neonates is concerning, with mortality rates as high as 20-30% being recorded (600, 615, 616). Morbidity is also common for neonates who survive invasive candidiasis, with neurological impairments being seen in as many as 73% of survivors (600, 615, 616). Like with adults, delayed antifungal treatment significantly increases mortality in neonates, and delayed treatment for these neonates may occur due to challenges in diagnosing invasive candidiasis in these patients (610, 645).

1.3.7.3 Treatment of invasive candidiasis in neonates

Treatment for neonates with invasive candidiasis involves utilizing antifungal compounds, including fluconazole, amphotericin B deoxycholate, liposomal amphotericin B, and micafungin (646). It has been theorized that preventative measures may also increase the survivability and reduce morbidity for neonates infected with *C. albicans.* Vaccine development has begun with some success in Phase 1 clinical trials, but skepticism exists for the long-term success of these vaccines due to the adaptability of *C. albicans* as an organism (647–649). Empirical antifungal treatment or fluconazole prophylaxis has been proposed as possible protocol for neonates who are at a high risk of developing invasive candidiasis, but while initial studies are promising, more clinical research needs to be done before this is considered a standard practice (650–652). Overall, invasive candidiasis is common in neonates, especially those who are premature or of extreme low birthweight, and prompt treatment of those infections are vital in preventing mortality and morbidity in those patients.

1.3.8 Polymicrobial interactions of *C. albicans* and oral streptococcal species

Polymicrobial interactions involving *C. albicans* and oral streptococcal species have been well researched, as mixed biofilms involving the pathogens are common in the oral cavity of both children and adults. Interactions between *C. albicans* and oral streptococcal species are thought to mostly occur through adherence to one another, with the *C. albicans* adhesin protein Als3 binding to streptococcal AgI/II familty peptides (653). The most studied *C. albicans-Streptococcal spp.* interactions involving Als3 involve the oral streptococcal species *S. gordonii, S. mutans, S. mitis, S. sanguinis,* and *S. oralis*. *C.albicans-Streptococcal spp*. interactions *in vitro* have been shown to promote biofilm growth, enhance the ability of these organisms to adhere to surfaces, as well as enhance the virulence of these pathogens (94, 654–661). *ALS3* gene

expression, which has been implicated as a virulence factor for *C. albicans*, can be modulated by members of its microbiota. *In vitro* mixed biofilms of *C. albicans, S. sanguinis,* and *S. mutans* led to an upregulation of *ALS3*, while mixed biofilms of *C. albicans* and the bacterium *P. gingivalis* did not alter the expression of the adhesion gene compared to solo *C. albicans* biofilms (409, 662). *In vivo* studies involving interactions between *C. albicans* and oral streptococcal species have also shown that these interactions directly influence pathogenesis during infection. For example, co-infection of *C. albicans* and *S. mutans* in the mouth of young rats led to a rapid onset of severe carious lesions on their teeth, while solo-infected and uninfected rats had less severe lesions in comparison to co-infected mice, indicating that synergy exists between the two pathogens to enhance their virulence during co-infection (97). The pathogenicity of *C. albicans* and *S. oralis* has also been shown to be enhanced in oral co-infections with one another compared to solo infections *in vivo*, as mice co-infected with both pathogens developed more severe tongue thrush lesions compared to mice infected in-solo with either pathogen, implicating a synergy between the two pathogens to enhance virulence (98). While interactions between *C. albicans* and oral streptococcal species have been well defined, until recently there was not much known about how *C. albicans* may interact with non-oral *Streptococcal spp in vitro* and *in vivo*.

1.3.9 Known polymicrobial interactions of *C. albicans* and GBS

C. albicans and GBS, despite their genetic differences as fungal and bacterial pathogens respectively, share some traits that indicate that these organisms may interact under certain environmental conditions (Figure 1). Both organisms typically reside as asymptomatic colonizers in most of the population, but can cause serious infection in certain patient groups, such as those who are newborn, pregnant, elderly, or immunocompromised (2, 555–561). *C. albicans* and GBS are both highly adaptable organisms that can effectively colonize difficult tissue environments, including the gastrointestinal tract and the vaginal tract (45, 47, 294, 338, 339). Patient studies identifying colonized microbes of the vaginal flora in pregnant women have also shown that *C. albicans* and GBS are often co-isolated from the vaginal tract, indicating that there may be a synergistic relationship between the two organisms to survive in that difficult tissue environment (288, 663). Co-infections between GBS and unspecified *Candida* species have also been recorded clinically in neonatal sepsis cases, although the survival outcome of these co-infections have not

been investigated (6). Recent research has shown that interactions between *C. albicans* and GBS likely depend on environmental conditions both *in vitro* and *in vivo*. Pidwill et. al recently reported that multiple GBS strains belonging to different serotypes can bind with *C. albicans in vitro* under planktonic conditions, indicating that the ability of these organisms to interact may be conserved amongst strains (53). The ability of these organisms to bind to one another are mediated by both Als3 in *C. albicans* and BspC in GBS, as mutant strains of the organisms lacking these proteins showed reduced binding to one another *in vitro* (53). GBS and *C. albicans* were shown to synergize to enhance the ability of these microbes to adhere to vaginal epithelial cells (VEC), and this effect no longer occurred when GBS was cultured with an *Δals3 C. albicans* mutant strain (53). The reduced ability of GBS and *C. albicans* to synergize to adhere to VECs when Als3 function was removed in mutant strains led this group to conclude that direct physical contact can influence interactions between these organisms (53). There has been evidence that secreted factors by the microbes may also influence the interactions between the two organisms, as it has been shown *in vitro* that GBS can inhibit hyphal formation by *C. albicans* by inhibiting *EFG1/*Hwp1 expression (664). As some groups have indicated that interactions between GBS and *C. albicans* are modulated by hyphal-cell wall adhesions and others have shown that hyphal formation can be inhibited by GBS, it is likely that the role of *C. albicans* hyphae in GBS-*C. albicans* is dependent on specific strain traits and environmental conditions (53, 664). Interactions between GBS and *C. albicans* have also been shown to directly influence the ability of these organisms to colonize certain tissue environments. Interactions between GBS and *C. albicans* directly enhanced the ability of GBS to adhere to bladder epithelium compared to solo GBS infection using HTB-9 (which is a human bladder epithelial cell line), but did not alter the ability of *C. albicans* to adhere to bladder epithelium.. Vaginal co-colonization by the microbes has been studied in mice *in vivo.* These studies showed that mice co-infected with both pathogens saw increased *C. albicans* colonization compared to solo infection, indicating that GBS can promote *C. albicans* colonization in the vaginal tract (664). While prior research has given us insights on how these pathogens may interact to influence colonization, there is still a gap of knowledge about how interactions between GBS and *C. albicans* can influence their growth and viability in different nutrient environments, their ability to tolerate antimicrobial treatment *in vitro* and *in vivo*, as well as influence infection pathogenesis during co-infection in comparison to solo infections with the pathogens, and the research contained in this thesis helps close this gap.

1.4 Zebrafish as an infectious disease model for GBS and *C. albicans* infections

Zebrafish (*Danio rerio*), while not a mammalian host model, has been well established as an effective and advantageous infectious disease model for both *Streptococcal* and *Candida* infections for multiple reasons (315, 665–670). Immune system similarities to humans are vital for determining a suitable live animal model for studying infectious disease, and zebrafish have a well-developed immune system very similar to that of mammalian immune systems (665, 671–673). The zebrafish genome is fully sequenced, and this sequencing has revealed a high degree of genetic homology to vertebrates, with at least 70% of human genes having at least one zebrafish orthologue (674, 675). Zebrafish can also be genetically modified with high success rates using multiple genome editing tools (676–679). Larval zebrafish also provide a great option for studying diseases that primarily affect immunocompromised individuals, as the adaptive immune system in zebrafish is not functionally mature until 4-6 weeks post fertilization, indicating that studies using larval zebrafish before this stage of development are using a immunocompromised model (680). This is a very suitable immune model for studying both GBS and *C. albicans* infections, as patients typically infected by these pathogens are immunocompromised by nature. Beyond immune system similarities between zebrafish and mammalian hosts, there are additional advantages to utilizing a zebrafish infection model. Zebrafish are small in size and have a rapid generation time, which allows for the use of many zebrafish for experiments in just a short time span (681). Zebrafish are translucent during early development and do not need to be sacrificed in order to be imaged using fluorescent microscopy, allowing for live-imaging of infection pathogenesis using fluorescent microbes (682). The existence of multiple transgenic zebrafish lines that have fluorescently labeled immune cells also allows for the imaging of the immune response to a live infection (683). Zebrafish are also able to be used to study the effectiveness of antimicrobial treatment during infection, as adding the chosen antimicrobial treatment to the water of infected fish allows for the fish to uptake the drug without injuring the animal through injection, and has been shown to effectively alter microbial populations in the zebrafish (684–686). Overall, the larval zebrafish has provided a suitable in vivo model for co-infections with GBS and *C. albicans,* which has allowed us to expand our knowledge of their interactions during infection.

CHAPTER 2

INTERACTIONS BETWEEN STREPTOCOCCUS AGALACTIAE AND CANDIDA ALBICANS AFFECT PERSISTENCE AND VIRULENCE

2.1 Introduction

Streptococcus agalactiae, also known as Group B streptococcus or GBS, is a Gram-positive, opportunistic pathogen that colonizes the gastrointestinal and/or vaginal tract of roughly 20-30% of healthy people worldwide (27). While this microbe is a commensal in most of the population, certain patients are at a higher risk of serious infection by GBS, including the elderly, immunocompromised, patients suffering from chronic conditions like diabetes and cancer, pregnant women, and newborns (2, 151, 687, 688). GBS colonization of the vaginal tract in pregnant women is common, with 10-30% of pregnant women estimated to be colonized (689). This colonization is a major risk factor for invasive GBS disease in neonates if exposed to the pathogen in utero through ascending infection or at the time of delivery. Chorioamnionitis caused by GBS can lead to severe complications for the fetus including miscarriage, preterm delivery, or stillbirth (43, 690). Newborns exposed to GBS at time of birth are at risk of contracting serious, and often fatal, early-onset (EOD) or late-onset (LOD) bacterial meningitis infections (173, 689). Children surviving GBS infection often face life-long complications such as seizures and neurological complications (18, 691). There is currently no commercially available vaccine for GBS, and there are very limited treatment options for mothers colonized with GBS prior to or during delivery (143, 692). To treat pregnant mothers found to be colonized with GBS, intrapartum antibiotic prophylaxis (IAP) is administered directly before and during delivery (689). IAP administration has been shown to prevent the incidence of GBS infection in newborns born to GBS colonized mothers by 86-89%, but universal screening for GBS colonization in pregnant women is not a worldwide standard (143, 689). IAP treatment is also not able to reduce the risk of GBS infection in utero and has not been effective in preventing the development of LOD GBS bacterial meningitis infections (693). While IAP has been shown to be effective in countries with universal GBS screening at 35 to 37 weeks of pregnancy, the consequences from this treatment, including the possibility of developing antibiotic resistant GBS strains and disruptions to the microbial flora of both the mother and their offspring, are not well understood (689, 694–697). A better understanding of the factors that contribute to colonization,

treatment effectiveness and infection caused by GBS will lead to the development of more effective drugs and vaccines.

Historically, research on an infectious pathogen is conducted by investigating the pathogen in solo. However, many tissue environments are microbially diverse, unique to each patient, and can be rapidly altered depending on environmental conditions. Multiple factors can influence the vaginal microbiome, including changes in hormones, alterations in pH, stress, diet, and antimicrobial treatments (698–701). Polymicrobial interactions have been shown to directly influence treatment effectiveness in vitro, as well as affect treatment and infection outcome in patients clinically (685, 702–706). Polymicrobial interactions between GBS and other microbes co-colonizing the vaginal tract and how these interactions affect the growth, cell viability, virulence, and treatment effectiveness against these microbes is poorly understood. While the most common microbes found to be colonizing the vaginal tract belong to the Lactobacillus genus, other microbes can colonize the vaginal tract and overgrow (sometimes leading to infection) following disruptions to the Lactobacilli population, including Candida albicans (707, 708).

C. albicans is an opportunistic pathogenic yeast that commonly colonizes the vaginal tract of ~30% of women (709). Like GBS, *C. albicans* functions as a commensal in most of the population, but can cause severe infections in immunocompromised patients, which include pregnant women and newborns (564, 710–712). Oropharyngeal Candidiasis (Thrush) caused by *C. albicans* can occur in newborns due to passage from a colonized mother to the newborn during delivery (713). *C. albicans* is also the leading cause for Vulvovaginal Candidiasis (VVC), with 75% of women having a VVC infection at least once in their lifetime, and ~8% of those cases being recurrent (568, 714). Pregnant women are considered to be at a higher risk of developing VVC caused by *C. albicans*, as estrogen levels and glycogen content are found to be increased in the vaginal secretions of pregnant women compared to a non-pregnant patient (715). Because *C. albicans* and GBS have the ability to colonize and infect the same types of patients and tissue environments, interactions between these microbes in vivo could influence factors like treatment effectiveness and infection outcome following challenges by these opportunistic pathogens.

The relationship between *C. albicans* and other streptococcal species has been well documented as C. albicans has been shown to exhibit synergy with multiple oral streptococcal species, including

Streptococcus mutans (97, 716), *Streptococcus oralis* (98, 717) and *Streptococcus gordonii* (655, 718) to enhance biofilm formation and virulence. While there has been less reported on the interactions between GBS and *C. albicans*, there is clinical evidence that *C. albicans* and GBS are often co-isolated together from colonized patients (288, 289). Previous research indicated that co-association between GBS and *C. albicans* is modulated through hyphal-specific surface adhesion protein *Als3* (53), while another study indicated that GBS can prevent *C. albicans* hyphal development (664), indicating that interactions between these two pathogens are complex and likely dependent on environmental conditions. *C. albicans* can also promote bladder colonization by GBS, further establishing the role that interactions between the two organisms have in the carriage of both microbes (287). While previous studies show that these microbes can co-associate to enhance the colonization of both the bladder and vaginal tract (287–289), the role this co-association has on treatment effectiveness and virulence during infection has not been explored. Beyond colonization, it is possible that interactions between *C. albicans* and GBS also influence the effectiveness of antimicrobials, as well as virulence, clinically.

This study demonstrated how co-association between GBS and *C. albicans* alters growth rate, treatment effectiveness and influences virulence during infection. Results demonstrated synergistic interactions during co-culturing of *C. albicans* and GBS. Interactions between *C. albicans* and GBS led to decreased susceptibility of GBS to the antibiotics erythromycin and clindamycin in vitro and reduced the effectiveness of clindamycin treatment against GBS infection *in vivo*. Results demonstrated a clear synergistic effect in survival outcomes of co-infections of GBS and *C. albicans* in comparison to solo infections by either pathogen utilizing a zebrafish infectious disease model. This research highlights how polymicrobial interactions can influence the treatment effectiveness and virulence of the pathogens GBS and *C. albicans* when co-colonized in comparison to solo colonization with either pathogen.

2.2 Results

2.2.1 GBS and *C. albicans* synergize to enhance growth *in vitro*

GBS and *C. albicans* are opportunistic pathogens that can adapt to colonize multiple sites in the body, including the vaginal tract. The vaginal tract can be a difficult environment for microbes to colonize due to low pH and limited nutritional availability, forcing microbes to adapt in order to survive (719, 720).

To investigate the role nutrient availability plays in the co-association between GBS and *C. albicans,* these organisms were co-cultured together or grown separately in nutrient rich (Todd Hewitt Broth with 0.2% supplemented yeast, THY B) or nutrient poor (serum free RMPI 1640) media environments and monitored for growth over time. In RPMI media, the growth of GBS was significantly increased in co-cultures with *C. albicans* at both 6 hours (4.8 fold, *p=0.04), and at 24 hours (11.6 fold, *p=0.01) following initial culture compared to solo cultures of GBS (Figure 1A). *C. albicans* viability was not significantly different in solo or co-cultures with GBS at either 6 or 24 hours post culture in nutrient poor media (Figure 1B). Interestingly, neither GBS or *C. albicans* cell growth was significantly different when in solo or co-cultures when grown in THY B (nutrient rich media) at 24hrs post culture (Figure A.1). The increased growth of GBS in the presence of *C. albicans* in RPMI media is not strain specific, as this effect was also observed in co-cultures of *C. albicans* and COH1 WT (a serotype III GBS strain), indicating that the increase of GBS growth in the presence of *C. albicans* in nutrient poor environments may be conserved amongst GBS strains (Figure B.1). The ability of *C. albicans* to significantly increase the growth of GBS in RPMI media is dependent on the viability of *C. albicans* cells, as GBS growth in RPMI media with heat-killed *C. albicans* cells was not significantly different compared to solo GBS cultures following 24 hours of incubation (Figure 2). To investigate early interactions between GBS and *C. albicans* in co-culture environments in RPMI meida, GBS and *C. albicans* were cultured both in solo and together in co-cultures, and cell growth was measured every 2 hours for a 6 hour time period. GBS growth was significantly increased by the presence of *C. albicans* compared to solo cultures early as 4 hours post co-culture (7.2 fold, **p=0.0010) (Figure 3A). Notably, *C. albicans* cell viability decreases initially when introduced into RPMI media, indicating that this media type is likely stressful to the organism. The presence of GBS in co-cultures with *C. albicans* reduced the loss of viability for the organism in comparison to solo cultures of *C. albicans* at both 2 hours (5.7 fold, **p=0.0051) and 4 hours (5.1 fold, *p=0.0144) co-culture despite competing for resources, indicating that the presence of GBS may be beneficial for the survival of *C. albicans* in stressful nutrient environments.

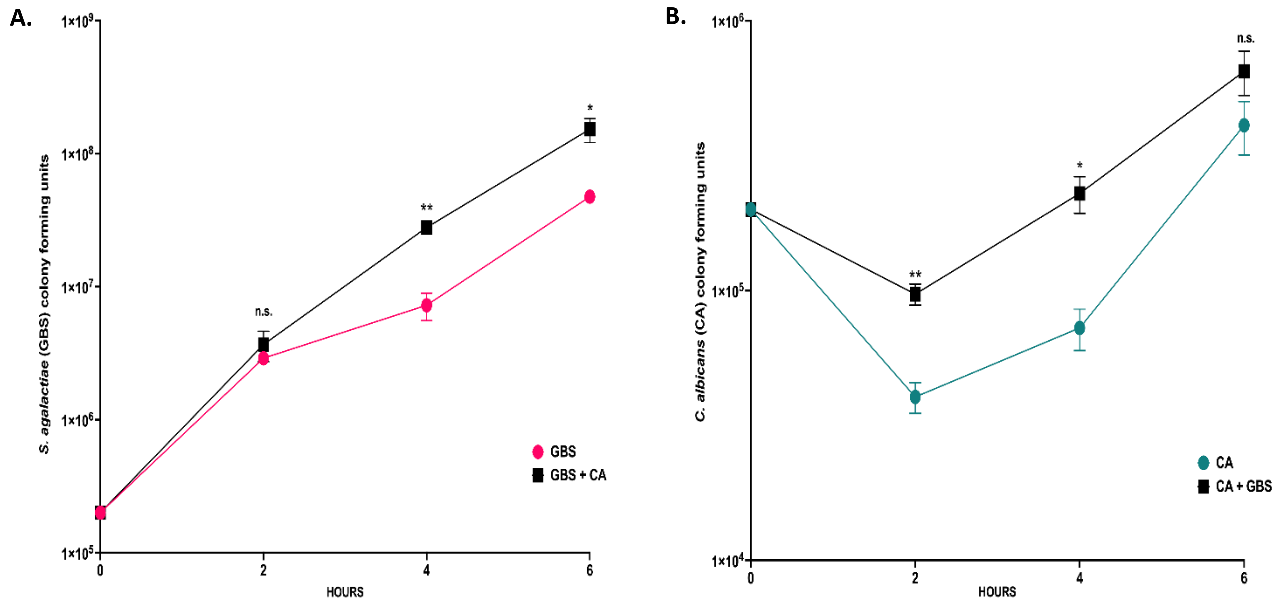


Figure 2.1: The growth rate of GBS and *C. albicans* in solo and co-cultures. **A.** Concentration of GBS cells (colony forming units) in solo (magenta) and co-cultures with *C. albicans* (black) in nutrient poor media. **B.** Concentration of *C. albicans* cells (colony forming units) in solo (blue) and co-cultures with GBS (black) in nutrient poor media. Experiments were performed in triplicate and statistical significance was calculated by unpaired two tailed student t-test for each time point, *p<0.05, **p<0.005, ****p<0.00005

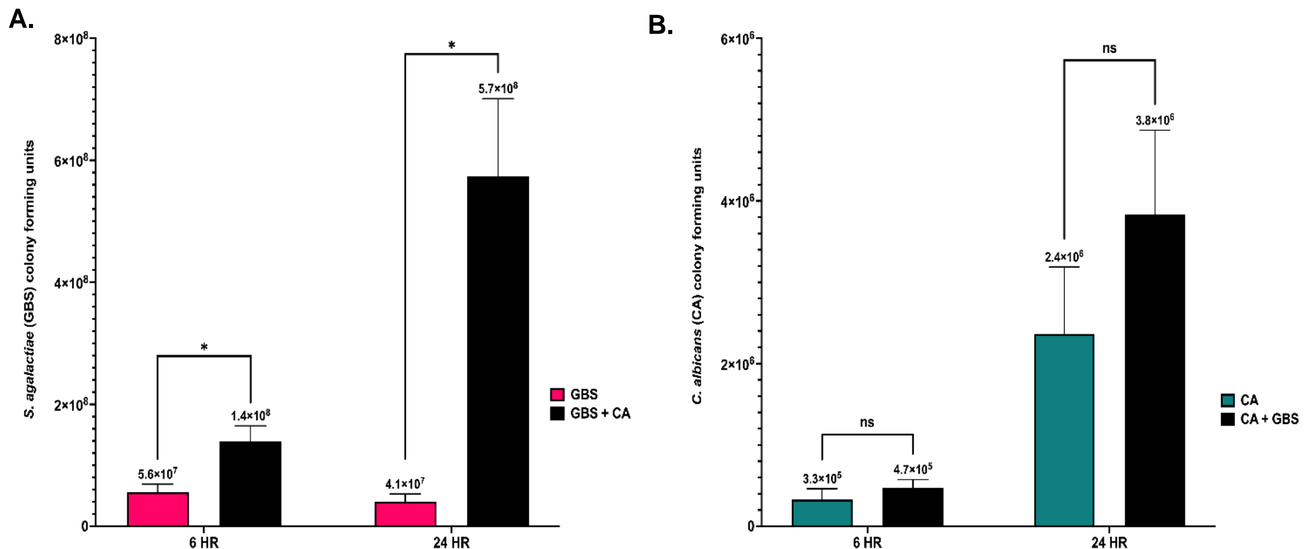


Figure 2.2: Interactions between GBS and *C. albicans* during co-culture in low nutrient media alters growth and viability A. Colony growth of GBS following solo or co-culture with *C. albicans* at 6 and 24hrs post culture in nutrient poor media **B.** Colony growth of *C. albicans* following solo or co-culture with GBS at 6 and 24 hours post culture in nutrient poor media. Experiments were performed in triplicate and statistical significance was calculated by unpaired two tailed student t-test, *p<0.05, **p<0.005, ****p<0.00005

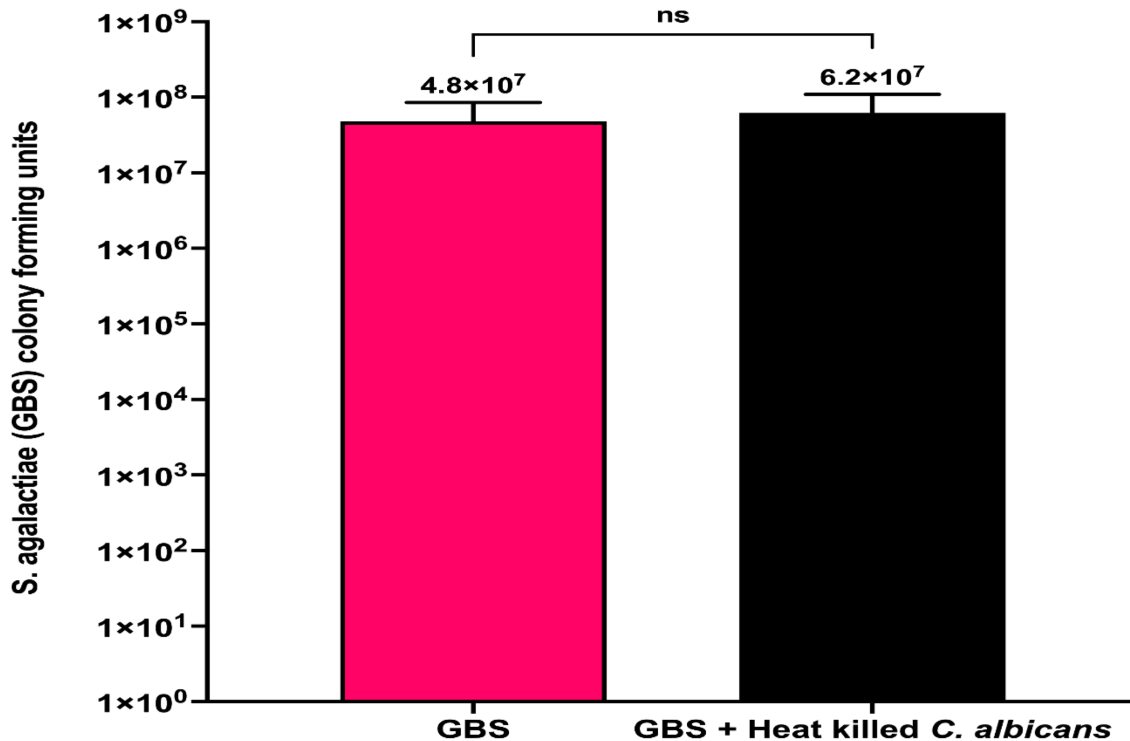


Figure 3: *C. albicans* must be viable to cause increase in GBS growth during co-culture. Growth of GBS alone or in co-culture with heat-killed *C. albicans* cells in nutrient poor media for 24 hours. Experiments were replicated 4 times (n=4) and statistical significance was calculated using an unpaired two- tailed student's t test.

2.2.2 *C. albicans* hyphal formation is not required for increased growth of GBS

C. albicans is a polymorphic microbe that can proliferate as yeast, pseudohyphal, or hyphal cells depending on environmental conditions. Research investigating the role of *C. albicans* hyphae in its interactions with GBS have shown conflicting results, as it has been shown that certain GBS strains may inhibit hyphal growth by *C. albicans*, while others have shown that GBS can actually benefit from attaching to hyphae *in vitro* (53, 664). To investigate the role of hyphae in the increased growth of GBS observed in the presence of *C. albicans* in RPMI media, GBS was co-cultured with either a strain of *C. albicans* that has overexpression of a repressor of hyphal formation and therefore remains in its yeast morphology *in vitro* (*NRG1^{OEX}-iRFP*) or the parent strain (*Caf2 FR*) (315, 316, 668, 721). Fluorescent images of co-cultures of GBS and the parent strain *Caf2 FR,* compared to growth of *Caf2 FR* alone, following a 24-hour incubation in RPMI media, demonstrated that *Caf2 FR* cells produced hyphae in the

nutrient poor media, while *NRG1^{OEX}-iRFP* cells were unable to do so and remained in a yeast morphology (Figure 4A & 4B). The presence of GBS did not alter the morphology of *NRG1^{OEX}-iRFP* or *Caf2 FR* (Figure 4C & 4D), indicating that the presence of GBS did not prevent *Caf2 FR* from forming hyphae (Figure 4C). The ability to form hyphae was not required for *C. albicans* to significantly increase the growth rate of GBS in RPMI media, as *C. albicans* strains *Caf2 FR* and yeast locked *NRG1^{OEX}-iRFP* caused significantly (p=0.001 & p=.0008, respectively) increased growth of GBS (Figure 4E) following a 24-hour co-incubation compared to the growth of GBS alone. The presence of GBSdid not significantly change the overall growth of *Caf2 FR* and *NRG1^{OEX}-iRFP* in nutrient poor media (Figure C.1).

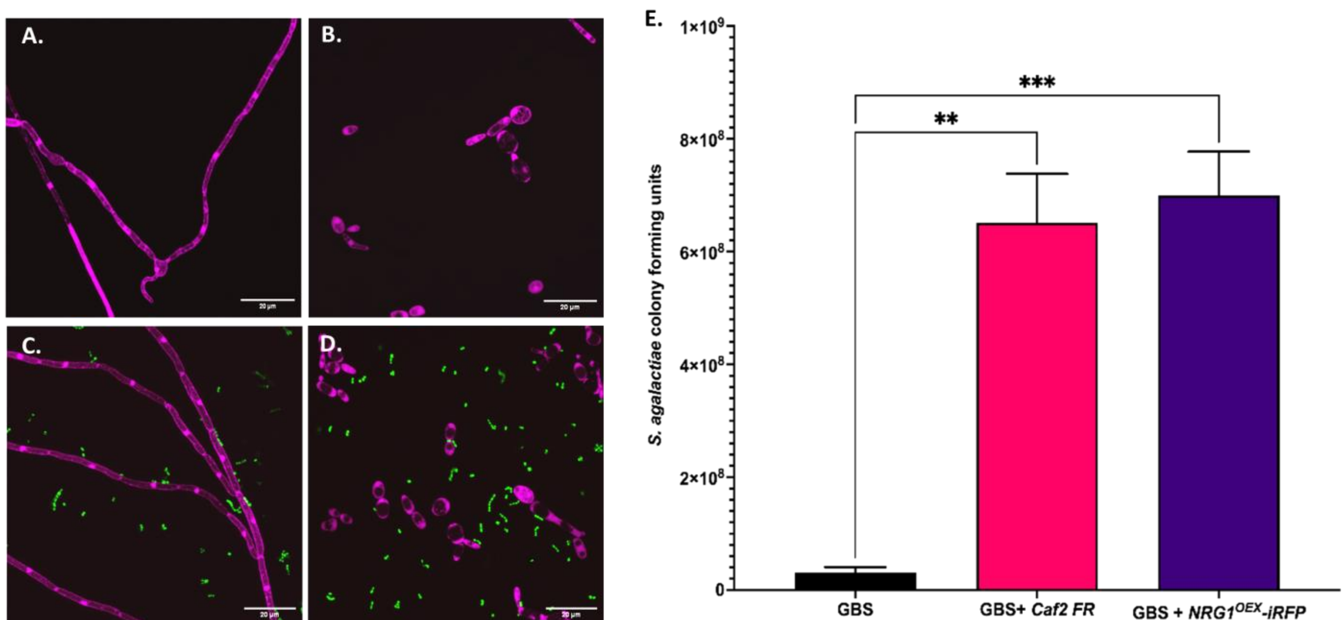


Figure 2.4: *C. albicans* hyphal formation is not required for GBS increased growth. GBS 515 was co-cultured with either a strain of *C. albicans NRG1^{OEX}-iRFP* (yeast locked) or the parent strain that is freely able to hyphae, *Caf2-FR* for 24hrs in nutrient poor media. Fluorescent images were taken at 1000X magnification using a point-scanning confocal microscope (RFP = *C. albicans*; GFP = GBS) **A.** *Caf2 FR* following 24 hours of incubation in nutrient poor media. **B.** Co-culture of GBS 515-GFP and *Caf2 FR* following 24 hour incubation in nutrient poor media**. C.** *NRG1^{OEX}-iRFP* following 24 hours of incubation in nutrient poor media. **D.** Co-culture of GBS 515-GFP and *NRG1^{OEX}-iRFP* following 24 hours of incubation in nutrient poor media. **E.** Growth of GBS 515 following solo or co-culture with *Caf2 FR* or *NRG1^{OEX}-iRFP*. Cultures were grown for 24 hours in nutrient poor media. Experiments were replicated 4 times (n=4) and statistical significance was calculated using one way ANOVA **p<0.005, ***p<0.0005.

2.2.3 GBS antibiotic susceptibility is decreased when co-cultured with *C. albicans in vitro*

Antibiotic resistant GBS is a major clinical concern for pregnant women, who have the risk of passing the bacteria to their offspring in utero or during delivery, potentially leading to life threatening bacterial infections in their newborns. GBS strains are showing increased rates of resistance to both erythromycin and clindamycin clinically, which is a concern for people allergic to beta lactam antibiotics who rely on these antibiotics as alternative therapies (7, 722, 723). Previous work demonstrated that *C. albicans* is able to enhance antibiotic tolerance of other Gram positive or negative bacteria including *Staphylococcus aureus,* and *S. gordonii, and Pseudomonas aeruginosa* (704, 724–726). To investigate if the presence of *C. albicans* in a culture can affect antibiotic susceptibility of GBS, both organisms were cultured together with erythromycin or clindamycin. The growth of *C. albican*s was not significantly altered in solo or co-cultures with GBS treated with either antibiotic, indicating that these antibiotics do not affect the growth of *C. albicans* (Figure 5B & 5D). Erythromycin and clindamycin were still effective at decreasing the growth of GBS in both solo and co-cultures with *C. albican*s compared to cultures grown without the antibiotic (Figure 2A). However, co-cultures of GBS and *C. albicans* have significantly ($p=0.002$ & $p=0.01$, respectively) more GBS growth following antibiotic treatment compared to solo cultures of GBS treated with erythromycin and clindamycin (Figure 5A & 5C). This indicates that *C. albicans* can alter the effectiveness of the antibiotics erythromycin and clindamycin against GBS *in vitro.*

The ability of *C. albicans* to synergize with GBS to enhance its antifungal tolerance was also investigated, as the effectiveness of certain antifungals against *C. albicans* has previously been shown to be altered in the presence of other bacterial strains including *Pseudomonas aeruginosa* (685). To investigate if antifungal effectiveness can be altered for *C. albicans* when cultured with GBS, growth was determined for both organisms when grown together and separately in RPMI media for 24 hours with the antifungals fluconazole and nystatin at the time of inoculation. Results indicated that the effectiveness of fluconazole and nystatin against *C. albicans* was not significantly different in solo vs co-cultures with GBS (Figure 6A & 6C), indicating that GBS does not affect the ability of *C. albicans* to tolerate these antifungals. GBS growth was also not significantly affected by any of the antifungals tested in solo or co-cultures with *C. albicans* (Figure 6B & 6D).

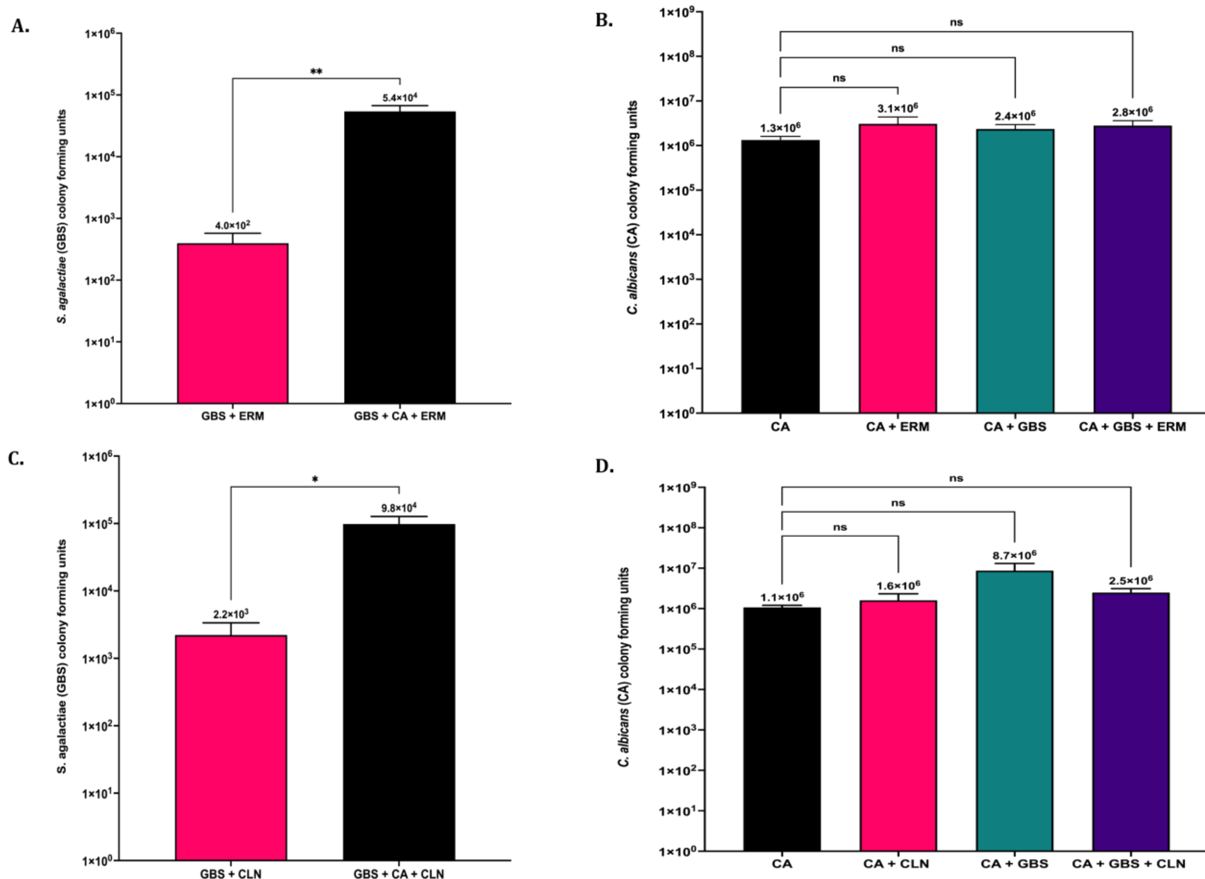


Figure 2.5: **Antibiotic effectiveness against GBS is reduced in the presence of *C. albicans***. **A.** GBS growth following solo or co-culture with *C. albicans* treated with 2µg/mL erythromycin. **B.** *C. albicans* growth following solo or co-culture with GBS treated with 2µg/mL erythromycin. **C.** GBS growth following solo or co-culture with *C. albicans* treated with 2µg/mL clindamycin. **D.** *C. albicans* growth following solo or co-culture with GBS treated with 2µg/mL clindamycin. Experiments investigating treatment of cultures with erythromycin were performed 6 times (n=6). Experiments investigating treatment of cultures with clindamycin were performed 5 times (n=5). Statistical significance was calculated by unpaired two-tailed student's t test for A & C, and One-Way ANOVA for B&D *$p<0.05$, **$p<0.005$.

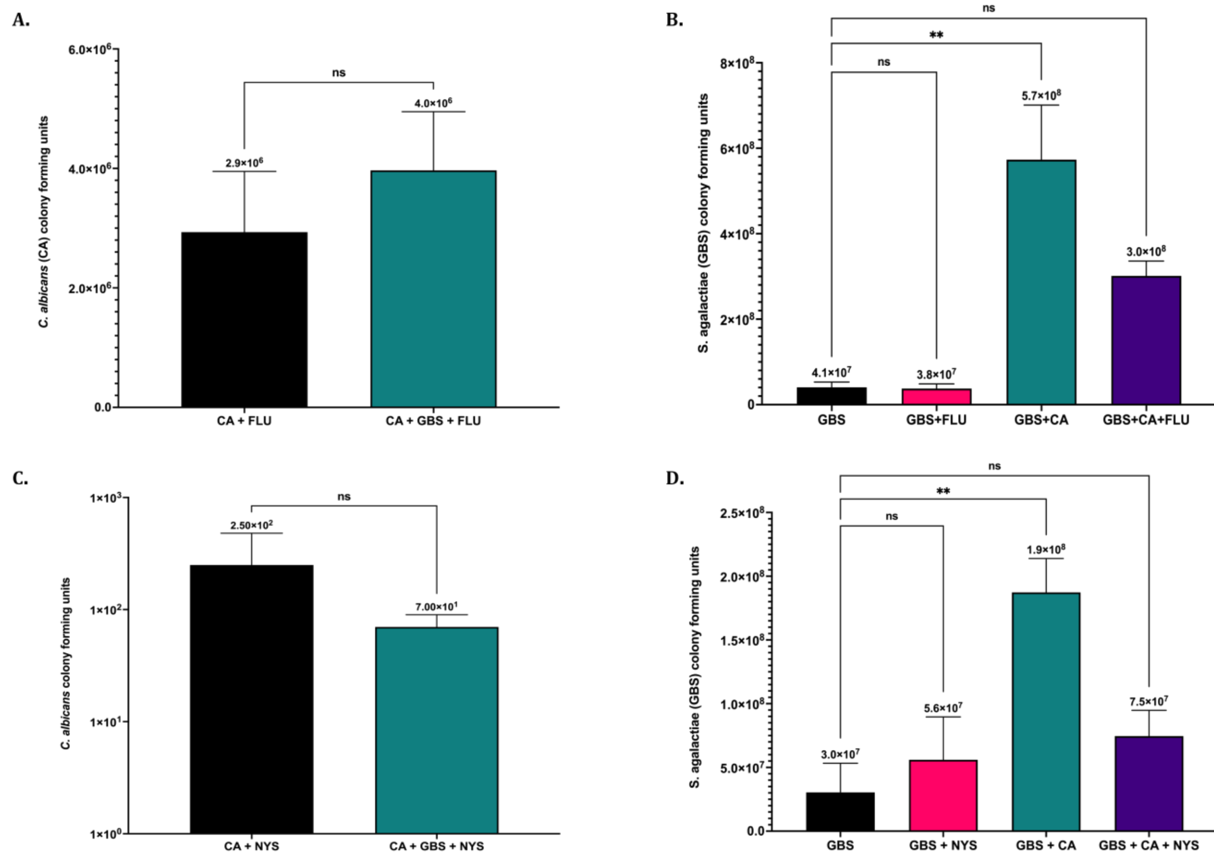


Figure 2.6: Antifungal effectiveness against *C. albicans* is not altered in the presence of GBS. A. *C. albicans* growth following solo or co-culture with GBS treated with 25µg/mL fluconazole. **B.** GBS growth following solo or co-culture with GBS treated with 25µg/mL fluconazole. **C.** *C. albicans* growth following solo or co-culture with GBS treated with 5µg/mL nystatin. **D.** GBS growth following solo or co-culture with GBS treated with 5µg/mL nystatin. Fluconazole experiments were performed in triplicate, and Nystatin experiments were performed 4 times. Statistical significance was calculated by unpaired two-tailed student's t test for A & C, and One-Way ANOVA for B&D. *p<0.05, **p<0.005, ****p<0.00005

2.2.4 Route of infection plays a role in virulence in *in vivo* co-infections with GBS and *C. albicans*

Data presented above revealed that interactions between GBS and *C. albicans* result in enhanced growth *in vitro.* Therefore, it was important to investigate if interactions between the two organisms can alter their virulence *in vivo*, as previous research had indicated that *C. albicans* virulence can be directly influenced by the presence of other microbes (727) The virulence of a co-infection compared to a solo infection of either GBS or *C. albicans* was compared using a zebrafish infectious disease model. Zebrafish are well established as infection models for both *Streptococcal spp.* and

Candida spp. infections (665, 667). Injection into the otic vesicle (OV) was used as a “localized” infection model as the organisms are inoculated into an enclosed cavity that is devoid of leukocytes prior to infection. For solo infections, 40 colony forming units (cfu) of GBS or 40 cfu of *C. albicans* was microinjected into the OV of 2 days post fertilization (dpf) zebrafish larvae. For co-infections, half the dose of both organisms (20 cfu of GBS and 20 cfu of *C. albicans* for a total of 40 cfu) was injected into the OV. Injections of sterile media (5% PVP in PBS) were used as a negative control. Following injection, fish were monitored for survival every 24 hours for 72 hours total. When *C. albicans* was injected alone into the OV almost no death was observed (Figure 7). Zebrafish larvae infected with GBS alone at 40 cfu had a survival rate of ~55% at 48 hours post infection (hpi). However, zebrafish co-infected with both GBS and *C. albicans* at half the dose of single infections showed significantly ($p=0.002$ & $p=<0.0001$, respectively) higher mortality rates by 48 hpi compared to solo infections with either GBS or *C. albicans* alone (Figure 7), with roughly 30% of the zebrafish surviving past 48 hours (Figure 7).

As shown above, localized co-infections with GBS and *C. albicans* are more virulent at half the dose than solo infections of either pathogen in zebrafish. The *in vitro* studies above show that the presence of *C. albicans* can increase the growth of GBS in nutrient poor environments. The increased virulence seen in co-infections may be caused by an increased bacterial and fungal burden in the larval zebrafish following co-infections. To investigate if the bacterial and fungal burden in larval zebrafish is increased in co-infections of GBS and *C. albicans*, 2dpf zebrafish were injected into the OV with either a streptomycin resistant GBS or *C. albicans* individually, or a co-infection of both pathogens. After 24hpi zebrafish were euthanized, homogenized, and bacterial and fungal burden was determined by plating serial dilutions of the homogenized zebrafish onto selective plates. Co-infections led to a significantly ($p=0.03$) higher GBS bacterial burden in larval zebrafish at 24hpi compared to solo infections of GBS, which may explain why co-infections lead to increased mortality compared to a solo infection of this bacterium (Figure 8A). Fungal burden of *C. albicans* at 24 hours was not significantly different between solo and co-infections (Figure 8B). The average fungal burden of the larval zebrafish at 24hpi was less than the original inoculum, suggesting the immune system was able to clear *C. albicans* from the OV at this dose.

While infections centralized in the vaginal tract are considered localized, both of these pathogens have the capability of disseminating into tissues, potentially leading to severe bloodstream infections with high mortality rates (4, 728). Yolk sac injection of the zebrafish larvae with either of these pathogens most often leads to a systemic infection. Therefore, to investigate if co-infections of these pathogens are more virulent than solo infections of either pathogen, an infection route that most often leads to a systemic infection was used. Systemic infections in zebrafish were performed by injecting either GBS, *C. albicans*, or half of the dose of both pathogens into the yolk sac (which results in bloodstream infection with no localized infection in the yolk) of 2 dpf larval zebrafish and monitoring survival for 72 hpi. At 72 hpi results showed that there were no significant differences in mortality rates between solo infections of GBS and *C. albicans* and co-infections with both pathogens (Figure 9A). However, it should be noted that co-infections where half the bacterial and fungal load of GBS and *C. albican*s were initially injected were just as virulent as the solo infections, indicating that interactions between these two pathogens may promote virulence for these pathogens (Figure 9A).

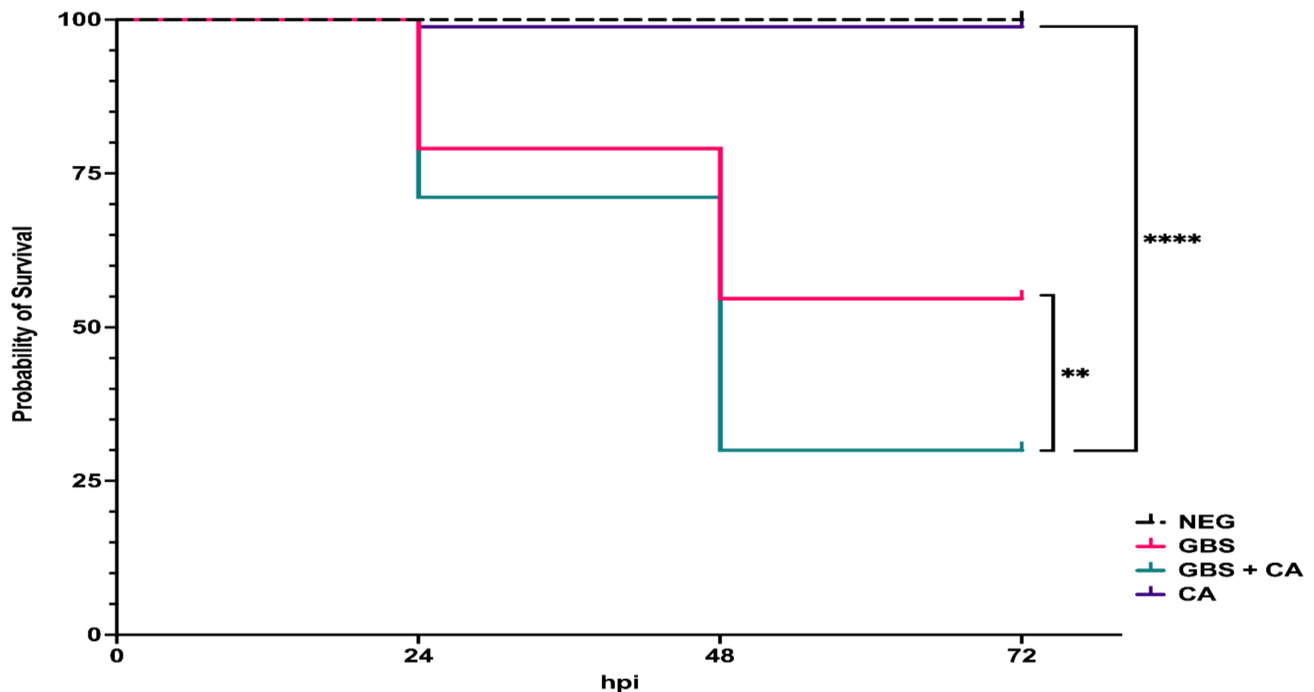


Figure 2.7: *C. albicans* and GBS synergize *in vivo* to increase virulence. Otic vesicle injection of solo or co-infections of GBS and *C. albicans* into 2dpf larval zebrafish. Solo injections had either 40 cfu of GBS 515 or 40 cfu of *C. albicans.* Co-infections had 20 cfu of GBS and 20 cfu of *C. albicans* (totaling 40 cfu) together. Negative control was 1 nL of 5% PVP in PBS. Infections were replicated 4 times with 20 fish per experimental condition (n=80). Statistical significance of Kaplan-Meier survival curves were calculated using a log rank (Mantel-Cox) test. **p<0.05, ****p<0.00005

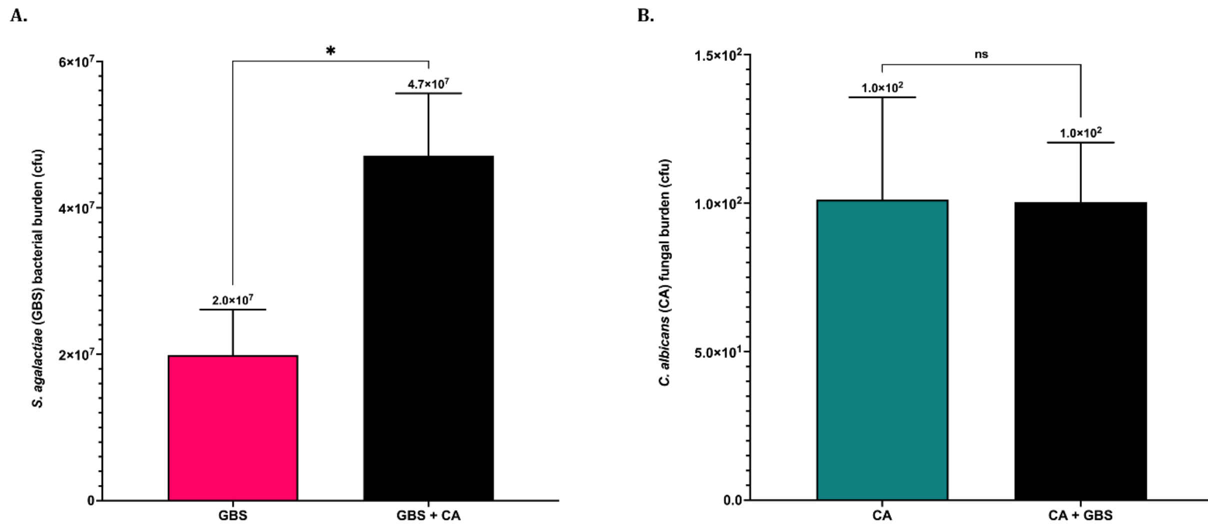
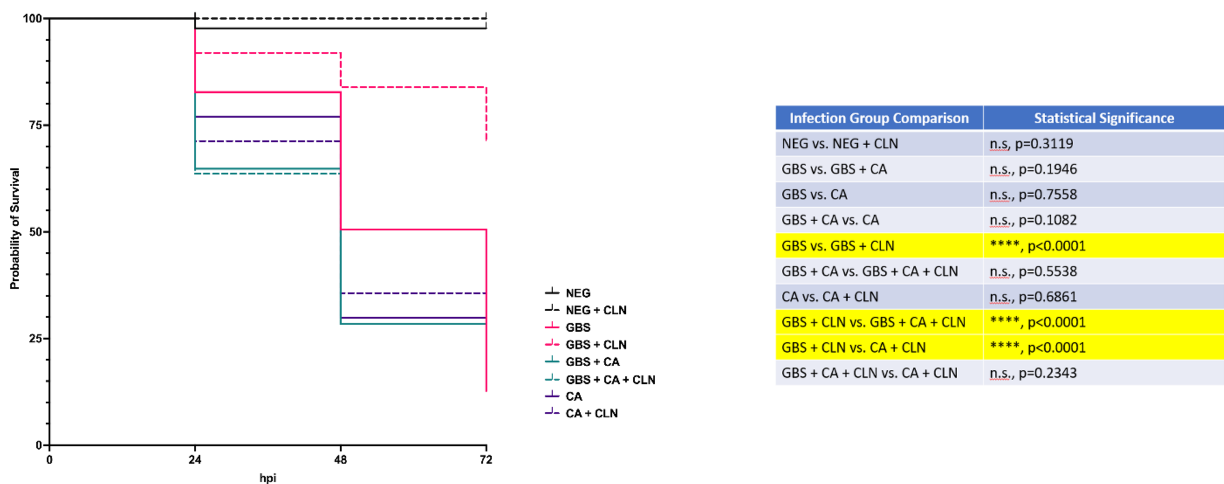


Figure 2.8: Localized co-infection with *C. albicans* increases GBS burden *in vivo.* A. The average GBS bacterial burden in zebrafish 24 hours following localized solo or co-infection with *C. albicans* **B.** The average *C. albicans* fungal burden of zebrafish 24 hours following localized solo or co-infection with GBS. Experiments were performed 5 times (n=5) and statistical significance was calculated using an unpaired two-tailed student's t test *p<0.05



Infection Group Comparison	Statistical Significance
NEG vs. NEG + CLN	n.s, p=0.3119
GBS vs. GBS + CA	n.s., p=0.1946
GBS vs. CA	n.s., p=0.7558
GBS + CA vs. CA	n.s., p=0.1082
GBS vs. GBS + CLN	****, p<0.0001
GBS + CA vs. GBS + CA + CLN	n.s., p=0.5538
CA vs. CA + CLN	n.s., p=0.6861
GBS + CLN vs. GBS + CA + CLN	****, p<0.0001
GBS + CLN vs. CA + CLN	****, p<0.0001
GBS + CA + CLN vs. CA + CLN	n.s., p=0.2343

Figure 2.9: The presence of *C. albicans* decreases effectiveness of clindamycin against GBS *in vivo.* Systemic solo or co-infections of GBS and *C. albicans* used a yolk sac injection of 2dpf zebrafish larvae. For solo infections zebrafish were injected with either 20 cfu of GBS 515 or 20 cfu of *C. albicans.* For co-infections zebrafish were injected with 10 cfu of GBS and 10 cfu of *C. albicans* (totaling 20 cfu) together. The negative control was 1 nL of 5% PVP in PBS. Experiments were replicated 3 times with 20 fish per experimental condition. **A.** Zebrafish survival of systemic solo and co-infection of GBS and *C. albicans* without antibiotics. **B.** Zebrafish survival of systemic solo and co-infections of GBS and *C. albicans* treated with 6µg/mL clindamycin in the tank water of the fish at the time of initial infection. Statistical significance of Kaplan-Meier survival curves were calculated using a log rank (Mantel-Cox) test. ****p<0.00005

2.2.5 GBS is less susceptible to antibiotic treatment during systemic co-infections *in vivo*

Antibiotic treatment effectiveness for *in vivo* co-infections between GBS and *C. albicans* have not been studied previously. *In vitro* results reported above indicated that when GBS is co-cultured with *C. albicans* in RPMI media that GBS is less susceptible to the antibiotics erythromycin and clindamycin (Figure 9B). To determine if systemic co-infections of GBS and *C. albicans* are less susceptible to the antibiotic clindamycin, a survival assay was performed and the same dose of clindamycin was administered to the water of the infected zebrafish for all infection groups directly following inoculation. Survival was monitored every 24 hours for 72 hours total. Results showed that the zebrafish mortality rate of solo infections of GBS treated with clindamycin were significantly ($p=<0.0001$) decreased by ~59% compared to untreated solo GBS infections. However, co-infections of GBS and *C. albicans* treated with clindamycin were just as virulent as untreated co-infections, with less than an 8% reduction in mortality following treatment, indicating that in co-infections with *C. albicans,* GBS are less susceptible to the antibiotic clindamycin.

2.3 Discussion

The role of polymicrobial interactions on treatment and infection outcomes in the vaginal tract has been understudied despite increased knowledge about the influence of the microflora on disease in other tissue environments. The vaginal tract is capable of rapidly changing the composition of its microbiome, while being a challenging tissue environment to colonize due to its acidic mucosa (698–701). While the vaginal tract is highly populated with the beneficial bacteria Lactobacillus in healthy individuals, the overgrowth of other microbes that can colonize this environment following disruptions to the *Lactobacilli* population can cause serious health issues in patients (698–701). Two of these microbes, *C. albicans* and GBS, are considered to be opportunistic pathogens as in most healthy individuals they act as commensals. However, in immunocompromised patients, such as newborns, patients with chronic diseases (diabetes, cancer, etc.), the elderly, and pregnant women they can cause either superficial or serious infections (2, 151, 687, 688). Previous reports demonstrated that GBS and *C. albicans* synergize to promote the ability of both organisms to colonize the bladder as well as the vaginal tract (53, 287–289),

but not much was known prior to this study about the effects of interactions between these two pathogens beyond their ability to co-associate in these specific tissue environments.

In this study, the effects of co-association between GBS and *C. albicans* beyond just colonization were further defined. The influence of interactions between the two microorganisms on their viability in specific media environments, the treatment effectiveness against each organism, and virulence in co-infection vs. solo infection was investigated. Understanding the outcomes of interactions between these two organisms is important, as enhanced colonization by these microbes can pose serious health risks. This is particularly true for pregnant women, who can pass these pathogens to their newborns in utero or during delivery, potentially leading to serious and sometimes fatal infections (43, 690). Throughout this study, *in vitro* co-culturing protocols used previously to study *C. albicans* and *P. aeruginosa* polymicrobial interactions were utilized to explore the effect of interactions between *C. albicans* and GBS with and without specific antimicrobials (685). In addition, *in vivo* methods using an infectious disease zebrafish model were used to define differences in virulence and treatment effectiveness following co-infection vs. solo infection of both pathogens in localized and systemic infections.

Results revealed that nutrient availability can mediate interactions between *C. albicans* and GBS *in vitro*. Nutrients available to microbes in the vaginal tract can be altered through vaginal secretions and the nutrient composition of those secretions can be influenced by factors like pregnancy, poor diet, hormones and stress (698–701). Nutrient availability has been shown to impact gene expression for both *C. albicans* and GBS, and the gene expression of both would likely influence the way these microbes interact with one another *in vitro* and *in vivo* (729–731). *C. albicans* is an adaptable microbe that can proliferate in both nutrient rich and nutrient poor conditions and can use a variety of different nutrient sources like glucose, lipids, proteins, amino acids and metals. The presence of these nutrients can also influence morphological changes for *C. albican*s. For example, glucose acts as a morphogen for *C. albicans*, regulating yeast-to-hyphae transition that is a well-known virulence factor for the microbe, while a lack of certain nutrients, such as iron, can also trigger a yeast-to-hyphae transition (732–734). Nutrient availability also heavily impacts the gene expression of GBS, as previous studies have shown that GBS can rapidly alter its transcriptome to adapt to a change of environment (and nutrients) when transferred

from a media culture to human amniotic fluid or human blood cultures (735, 736). Due to the fact that both these organisms have adaptations that allow for them to survive in nutrient rich and nutrient poor environments, and their gene expression likely is altered in each condition, it was important to investigate interactions between both microbes in nutrient rich and nutrient poor environments. Results showed that when grown in THY B (nutrient rich), no significant differences were observed for either *C. albicans* or GBS growth when co-cultured together compared to grown separately (Sup. Figure 1). However, when co-cultured in RPMI (nutrient poor) media, growth was significantly increased by 4 hrs. post co-culture for GBS compared to when grown in solo (Figure 3A). The increase of GBS growth in the presence of *C. albicans* compared to solo cultures of GBS also persisted to 24 hours (Figure 1A), indicating that the ability of *C. albicans* to promote the survival of GBS in stressful nutrient environment is present in both short and long term cultures. *C. albicans* initially lost cell viability when cultured in nutrient poor media, but that decrease of viability was significantly less when also cultured with GBS compared to solo cultures (Figure 3B). This is very notable, as enhanced survival of *C. albicans* in the presence of GBS despite both organisms likely competing for resources in the nutrient poor media indicates a synergy between the two microbes to promote their survival in stressful nutrient environments. Nutrient dependent interactions have been shown previously with interactions with *C. albicans* and other bacterial species. For example, *C. albicans* can deplete the oxygen in its environment, thereby promoting the growth of anaerobic bacteria in those cultures (737). *P. aeruginosa* can also inhibit the growth of *C. albicans* through iron sequestering as well as synergize with the fungistatic drug fluconazole to have fungicidal activity against *C. albicans* (685, 738). Sugar availability can affect interactions between *C. albicans* and *S. mutans*, as adherence of *S. mutans* to *C. albicans* in mixed biofilms with both microbes is only possible in the presence of this nutrient source (716, 739). The ability of GBS to co-associate with *C. albicans* may be nutrient and environmentally dependent, which may explain why certain patients are at a higher risk of infection and serious complications by these pathogens.

The role of *C. albicans* cell morphology on interactions with GBS *in vitro* was investigated in this study as the morphological state of *C. albicans* can alter the virulence of the microbe and likely also influence its interactions with GBS. *C. albicans* is polymorphic and can grow in three distinct cellular morphologies; yeast, pseudo hyphae, and hyphae and can alter their morphology based on

environmental factors such as nutrient availability, temperature, stress, and fluctuations in pH (307, 740, 741). Each morphology has a unique gene expression profile as well as distinct virulence traits and are responsible for different aspects of infection. Yeast cells of *C. albicans* are thought to aid in the spread of infection throughout the body, while hyphal *C. albicans* cells have been shown to be able to penetrate tissues, causing damage and destruction of the host cells in that environment (742). Less is known about the role of pseudo hyphae in infection, although it is considered to be a transition state between yeast and hyphal cells (742). Previous studies of interactions between GBS and *C. albicans* have had conflicting conclusions regarding the role of *C. albicans* cellular morphology in the co-association of these two microbes (53, 287, 664). Some studies have indicated that the co-association of GBS and *C. albicans* is dependent on hyphae, specifically through the expression of the hyphal-specific *ALS3* gene (53, 287). Als3p belongs to the agglutinin-like sequence (ALS) protein family in *C. albicans* and functions as both an adhesion and an invasin, leading to this gene being known as a well-established virulence factor of *C. albicans* (400). This gene is only expressed in *C. albicans* hyphae and pseudo hyphae, and has been indicated to be responsible for the synergy of *C. albicans* and GBS in their colonization of the bladder of mice (287, 400). Other studies have shown that GBS can actually inhibit *C. albicans* hyphal growth by decreasing the expression of *EFG*1-induced Hwp1, a cell-to-cell adhesion cell-surface protein of *C. albicans* whose presence is required for biofilm production (664). Results in this study demonstrate that *C. albicans* hyphal growth is not inhibited by the presence of GBS, indicating that the ability of GBS to inhibit hyphal growth may be environmentally influenced or strain specific. GBS growth was significantly increased in the presence of both yeast-locked and hyphal *C. albicans* in RPMI media, indicating that growth-enhancing interactions between GBS and *C. albicans* are likely not dependent on *C. albicans* cellular morphology, but instead rely on multiple factors such as environmental stressors, specific strain traits, and nutrient availability.

This study aimed to investigate how interactions between *C. albicans* and GBS can influence treatment effectiveness, as antimicrobial resistance by both pathogens can have serious health implications clinically. No significant changes in treatment effectiveness against *C. albicans* by multiple antifungal treatments were observed during co-culture with GBS. However, polymicrobial interactions between *C. albicans* and GBS significantly altered the effectiveness of erythromycin and clindamycin *in*

vitro against GBS compared to GBS cultured alone with these treatments. While vaccines against GBS have been in development for several years, there is no vaccine commercially available against GBS, and no alternative treatment options for these infections are available outside of antibiotic use. The main antibiotic used for both IAP administration and to treat GBS invasive infection is penicillin, a beta-lactam antibiotic considered to be highly effective against GBS (689). Erythromycin and clindamycin are two antibiotics prescribed to patients who cannot be treated with penicillin due to allergies to beta-lactam antibiotics. Clinically, GBS strains are showing rising resistance to both erythromycin and clindamycin, with clindamycin-resistant Group B *Streptococcus* strains specifically being implicated as a concerning threat by the CDC (743). Erythromycin and clindamycin belong to different families of antibiotics (macrolide and lincomycin, respectively) but have similar mechanisms of action that target the 50s ribosomal subunit, preventing protein synthesis (152). Commonly, GBS clinical strains show cross-resistance to erythromycin and clindamycin, leading to both being unavailable treatment options for many patients (152). Previous research has shown that vaginal candidiasis caused by *C. albicans* can occur following the use of antibiotics (707). *C. albicans* can also become less susceptible to amphotericin B when exposed to erythromycin, indicating that antibiotics can influence the behavior of *C. albican*s (744). The risk of *C. albicans* co-colonization with other multidrug resistant organisms has also been shown to be increased in hospital patients previously treated with macrolide antibiotics, highlighting the influence of antibiotics on *C. albicans* (745). Our results show that the antibiotics erythromycin and clindamycin were significantly less effective against GBS *in vitro* when co-cultured with *C. albicans* in nutrient poor media in comparison to solo GBS cultures. These results indicate that these antibiotics may not be the best option for patients colonized with both *C. albicans* and GBS, as they are not only less effective against GBS, but can potentially alter the susceptibility of *C. albicans* to certain antifungals following exposure to these antibiotics.

Little is known about differences in infection outcomes in co-infections of GBS and *C. albicans* compared to solo infections with both pathogens *in vivo* despite both pathogens being able to cause superficial or invasive infections in immunocompromised patients. Previous *in vivo* work investigating these two pathogens have not explored overall infection outcome in their infected murine models following inoculation of just GBS, *C. albicans*, or both pathogens together (287, 664). C*. albicans* can

cause co-infections with both Gram negative and positive bacterial species clinically. For example, co-infections of *C. albicans* and *P. aeruginosa* commonly occur in cystic fibrosis patients (746, 747). *C. albicans* has also been shown to be able to aid in the dissemination of *S. aureus* into tissues causing a systemic infection following oral co-infection in mice (748). Moreover, *C. albicans* can synergize with other *Streptococcal* species *in vivo* to enhance the virulence of both microbes (97, 98, 655, 716–718). However, how GBS and *C. albicans* co-infections alter infection outcomes in comparison to solo infections in either a localized or a systemic infection is not well understood. This study utilized larval zebrafish as an *in vivo* infection model for studying co-infection with GBS and *C. albicans.* Larval zebrafish have multiple routes of infection readily available at different stages of development. Investigating treatment effectiveness *in vivo* is easily performed with zebrafish due to treatments being able to be administered in the tank water without the need to inject, preventing unrelated secondary infections and complications. Zebrafish models of infection have also been well established for both *Streptococcal* bacteria and *C. albicans* (665, 667). Zebrafish are also effective models for specific infectious diseases in general at they are vertebrates with a strong similarity of mammals in both their innate and adaptive immune systems (671, 672). By utilizing both *in vitro* and *in vivo* experimental models we can better understand how interactions between these organisms can alter virulence and treatment effectiveness, and these findings can be used to further investigate how treatment effectiveness against these microbes when colonized together is altered in a mammalian host. Results reported above suggest that otic vesicle (OV) co-infections with GBS and *C. albicans* are significantly more virulent than solo infections of either pathogen in larval zebrafish, especially compared to solo infections of *C. albicans*, which rarely caused mortality in this infection route In OV co-infections of GBS and *C. albicans* the bacterial burden of GBS at 24 hpi was significantly increased compared to solo GBS infections, indicating that the increased mortality rates for co-infections compared to solo infections may be due to increased bacterial burden for those fish. The ability of *C. albicans* to worsen localized infection outcomes when co-infected with other streptococcal species has been reported previously in oral infections (97, 98, 655, 716–718). Co-infection with *C. albicans* and *Streptococcus oralis* in the oral cavity of mice significantly increases the severity of infection, increases biofilm formation, and exacerbates the severity of tongue lesions compared to solo infections with either microbe (98). A similar interaction has also been seen with

C. albicans and *S. mutans*, as the ability of both these microbes to infect and form biofilm in the teeth of rodents were enhanced in co-infection compared to solo infection, as co-infections led to severe teeth carious lesions (97). All these results indicate that *Streptococcal* species like GBS can synergize with *C. albicans* to enhance virulence and overall infection severity *in vivo*.

Investigation of changes in virulence of yolk sac infection (which most often results in systemic infection) of GBS and *C. albicans* in co-infections vs. solo infections was also important as both pathogens can cause severe bloodstream infections in patients. There were no significant differences in virulence for GBS and *C. albicans* yolk sac infection when infected with just one pathogen compared to co-infections, which is not unexpected considering the severity of systemic infections with these pathogens. Also to consider is that interactions between the two organisms would be less prevalent in a systemic infection where the organisms would not be in close contact compared to the OV localized infection, resulting in less synergy. Not surprisingly, when treating GBS systemic solo infections with the antibiotic clindamycin, the infections become significantly less virulent. However, clindamycin treated co-infections with *C. albicans* and GBS are just as virulent as untreated co-infections, indicating that the presence of *C. albicans* makes the antibiotic clindamycin less effective against GBS following co-infection in larval zebrafish *in vivo.* This correlates with the in *vitro* data reported above indicating that clindamycin is less effective against GBS when *C. albicans* is also present in the media. While the treatment effectiveness of antibiotics against GBS in the presence of *C. albicans* has not been researched prior to this study, it has been shown previously that mixed biofilms of *C. albicans* and other bacterial species are less susceptible to antimicrobial treatments (704, 726, 749). Results reported here suggest that the reduced effectiveness of clindamycin against GBS clinically may be influenced by the presence of *C. albicans*, as it is possible that the antibiotic is not as effective in patients that are also colonized with *C. albicans*. These results indicate that clindamycin may not be a suitable alternative antibiotic against GBS for patients that are also colonized with *C. albicans*, highlighting the importance of knowing the microbial composition of the vaginal tract for patients, especially expecting mothers.

The results from this study demonstrate that co-colonization between GBS and *C. albicans* can alter microbial growth, treatment effectiveness, and virulence of infections for both pathogens. Overall,

these results help further define the ability of GBS and *C. albicans* to co-associate in specific conditions and highlight important clinical issues for treatment effectiveness and infection outcomes for both pathogens.

2.4 Materials and Methods

2.4.1 Bacterial strains and growth conditions

The *Streptococcus agalactiae* strain GBS 515 was used in all experiments unless otherwise noted (Bacterial strains listed in Table 1). GBS 515 is a serotype 1a, ST-23 human clinical isolate from the blood of a patient diagnosed with neonatal septicemia and was generously provided by M.R. Wessels (90). For all experiments using GBS, the bacterium was inoculated in Todd-Hewitt medium (Acumedia) supplemented with 0.2% yeast extract (THY) in sealed conical tubes and grown statically at 37°C overnight. For selection of GBS after co-culture experiments, cultures are serially diluted and plated on Strep B ChromoSelect Selective Agar Base (Millipore Sigma) agar plates.

2.4.2 Fungal strains and growth conditions

The fungal strain of *Candida albicans* (*C. albicans*) used for co-culturing experiments with and without specific antifungals or specific antibiotics was SC5314-NEON unless otherwise noted (Fungal strains listed in Table 2). *C. albicans* was inoculated onto a THY agar plate for individual colonies and grown overnight at 37°C aerobically. For liquid overnight cultures, a single colony of *C. albicans* was selected from an agar plate, inoculated into THY media, and grown overnight with shaking aerobically in glass culture tubes at either 30°C or 37°C as noted in the experiment. For selection of *C. albicans* after co-culture experiments, cultures are serially diluted and plated onto THY plates supplemented with 2μg/mL ampicillin (Millipore Sigma).

2.4.3 Growth curve experiments

For growth curve experiments GBS 515 and SC5314 NEON were both grown overnight in THY media aerobically at 37°C. GBS 515 was grown statically overnight, while liquid cultures of SC5314 NEON were grown with shaking. Following 14 hr. overnight incubation, GBS 515 was sub-cultured into fresh THY media at a 1:100 dilution and subcultures were grown and normalized to an optical density (OD) 600 nm of

0.225 (mid log phase) prior to co-culturing. Prior to co-culturing, 1mL of the overnight culture of SC5314-NEON was added to a sterile Eppendorf tube, briefly vortexed, then centrifuged at 7000 RPM for 1 minute. Following centrifugation, the supernatant was removed, and the SC5314 NEON pellet was resuspended in 1mL of sterile 1X PBS. This step was repeated two times, then following the last resuspension the concentration of SC5314 NEON was determined by measuring the absorbance of a 1:100 dilution of the SC5314 NEON in 1mL of PBS at OD600 nm. Once concentrations of GBS 515 and SC5314 NEON were calculated, GBS 515 and SC5314 NEON were inoculated either together or in solo in 6 mL of nutrient rich (THY) or nutrient poor (RPMI 1640 with L-glutamine and 25mM HEPES) media at a concentration of 2 X 10^5 cfu/ml per organism. Following culturing, serial dilutions of solo and co-cultures of GBS 515 and SC5314 NEON were plated once an hour for 6 hours totals on selective media plates and grown for 24 hours at 37°C. Following growth on selective media, individual colonies were enumerated to determine overall growth concentration of solo and co-cultures of GBS 515 and SC5314-NEON for each time point.

2.4.4 Co-culture experiments with antimicrobials

For co-culture experiments with and without specific antimicrobials, GBS 515 and SC5314 NEON were both grown overnight in THY media aerobically at 37°C as described above for growth curve experiments. Once concentrations of GBS 515 and SC5314 NEON were calculated, GBS 515 and SC5314 NEON were inoculated either together or in solo in 4 mL of nutrient rich (THY) or nutrient poor (RPMI 1640 with L-glutamine and 25mM HEPES [LONZA]) media at a concentration of 2 X 10^5 cfu/mL per organism. Following culturing, serial dilutions of solo and co-cultures of GBS 515 and SC5314 NEON with or without supplemented antimicrobials were plated at either 6 hours or 24 hours post co-culture on selective media plates and grown for 24 hours aerobically at 37°C. When treated with fluconazole (Millipore Sigma) cultures were supplemented with a dose of 25μg/mL. When treated with erythromycin (Millipore Sigma) tubes were supplemented with a dose of 2μg/mL. When treated with clindamycin (Indofine Chemical Company, Inc, Hillsborough, NJ) tubes were supplemented with a dose of 2μg/mL. To enumerate GBS in experimental cultures serial dilutions from solo and co-cultures that were untreated were plated on Strep B ChromoSelect Selective Agar Base (Millipore Sigma), grown overnight at 37°C and individual colonies were enumerated. GBS cultures treated with antibiotics erythromycin or

clindamycin were plated on THY agar plates supplemented with 50 μg/mL Nystatin (Millipore Sigma), grown overnight at 37°C aerobically, and individual colonies were enumerated. To enumerate SC5314 NEON concentrations in experimental cultures serial dilutions were plated on THY agar plates supplemented with 2μg/mL ampicillin (Millipore Sigma), grown overnight at 37°C and individual colonies were counted.

To confirm that co-culture results with GBS were not strain specific, GBS clinical isolate, serotype III CC-17 strain COH1 was co-cultured without antimicrobial treatment as described above. Following 24 hours of culturing, the growth of solo cultures of COH1 and SC5314 NEON, as well as co-cultures of both organisms were calculated following the enumeration of individual colonies on selective plates following serial dilutions. COH1 and SC5314 NEON selective plates used for this experiment are the same as the selective plates described above.

2.4.5 Heat-killed experiments

For heat-killed *C. albicans* experiments, GBS 515 and SC5314 NEON solo cultures and co-cultures were prepared using the same co-culture procedure described above, but prior to adding SC5314 NEON to the cultures the *C. albicans* cells was boiled at 100°C for 15 minutes prior to introduction into nutrient poor media solo and co-cultures. Heat-killed *C. albicans* was also plated on THY agar supplemented with 2μg/mL ampicillin to confirm that cells were no longer viable. Solo and co-cultures of GBS 515 and heat killed SC5314 NEON were grown for 24 hours at 37°C aerobically with shaking, and GBS 515 growth was calculated by plating serial dilutions of cultures on the same selective media plates as stated above.

2.4.6 Hyphal *C. albicans* strain vs. Yeast-locked strain assay

For experiments investigating the role of hyphal growth on GBS growth rates, GBS 515 was grown statically 14 hours in THY media at 37˚C and then sub cultured at a 1:100 dilution into 5mL of fresh THY media. Subcultures were grown to an optical density (OD) 600 nm of 0.225 (mid log phase) prior to co-culturing with *C. albicans*. *C. albicans* strains *Caf2 FR* (reference strain capable of hyphal formation) and *NRG1^{OEX}-iRFP* (yeast locked) were grown at 37°C overnight aerobically with shaking. Following incubation at 37°C for 16 hours, 1mL of overnight stock was briefly vortexed, spun down at 7000 RPM for

1 minute and washed 2 times with 1X PBS before measuring the absorbance at OD 600 nm to determine concentration of each strain. After the concentration of cultures of GBS, *Caf2 FR*, and *NRG1^{OEX}-iRFP* were determined, culture tubes with 4mL of RPMI 1640 with L-glutamine and HEPES (Lonza) were inoculated with a $2X10^5$ cfu/ml concentration of 515 GBS, a $2X10^5$ concentration of *Caf2 FR*, a $2X10^5$ concentration of *NRG1^{OEX}-iRFP*, or $2X10^5$ concentration of GBS and a $2X10^5$ concentration of *C. albicans*. Cultures were grown for 24 hours and enumerated by counting individual colonies following serial dilutions plated on selective plates.

To image GBS 515, *Caf2 FR,* or *NRG1^{OEX}-iRFP* in solo or co-cultures, cultures were inoculated as described above and grown at 37°C with shaking for 24 hours. Following this growth period, 7 µL of each culture were added to a glass slide and covered with a 22 x 22 mm cover slip. After the cultures were mounted on the glass slides confocal images were taken using an Olympus IX-81 inverted microscope containing a FV-1000 laser scanning confocal system (Olympus, Waltham, MA). The fluorescent proteins EGFP (488nm/505 to 525 nm excitation/emission) and Far-Red (635nm/ 655 to 755 nm excitation/emission) were detected using laser/optical filters with a 100X oil objective (NA,1.40). Images were taken as Z stacks and were processed using FluoView (Olympus, Waltham, MA). Image brightness was enhanced for visualization using ImageJ (750).

2.4.7 Zebrafish care and maintenance

Adult zebrafish used for breeding were maintained at the University of Maine Zebrafish Facility at 29°C on recirculating systems. All zebrafish studies were approved by the University of Maine Institutional Animal Care and Use Committee (IACUC protocol #A2020-02-01). After collection, embryos were stored in petri dishes with sterilized water obtained from the recirculating system supplemented with 0.1% methylene blue. Embryos found to not be developing were removed after 24hpf to maintain cleanliness in petri dishes. Embryos were maintained at a temp of 29°C through development at a density of 50 embryos per dish. Following use in experiments, zebrafish were transferred to a 31°C incubator and were monitored for survival every 24 hours for 72 hours. Surviving zebrafish from experiments as well as zebrafish not used for experiments were humanely euthanized after 72 hours post experiment by an overdose of 0.64 µg/mL tricaine (Ethyl 3-aminobenzoate methanesulfonate

($H_2NC_6H_4CO_2C_2H_5 \cdot CH_3SO_3H$); Sigma-Aldrich). Experiments were conducted using the wild-type ZF1 strain.

2.4.8 Zebrafish microinjections for infection for different infection routes

Larvae at ~48 hours post fertilization (hpf) were manually dechorionated and anesthetized in 0.32mg/mL of tricaine prior to injection. Survival assays were performed by injecting either 1nL of GBS 515 at 4 X 10^7 cfu/ml dose, 1nL of SC5314 NEON at 4 X 10^7 cfu/ml dose, or 1nl of GBS at 2 X 10^7 cfu/ml and SC5314 NEON at 2 X 10^7 cfu/ml combined to equal an approximate dose to solo infections into the otic vesicle of larval zebrafish to simulate a localized infection (751). Mortality of the fish was determined by observation of a loss of heartbeat and no movement when gently probed on the tail. Survival of the zebrafish were monitored daily, and experiment was terminated at 72 hours. Surviving fish were euthanized with an overdose of tricaine (0.64 mg/mL) at the end of the experiment. Injection doses were confirmed by serial dilution and plating of the inoculum on THY agar plates and enumerating bacteria or fungi colonies.

Larvae at ~48 hpf were manually dechorionated and anesthetized in 0.32 mg/mL of tricaine prior to injection. Survival assays were performed by injecting either 1nL of GBS 515 at 2 X 10^7 cfu/ml, 1nL of SC5314 NEON at 2 X 10^7 cfu/ml, or 1nl of GBS at 1 X 10^7 cfu/ml and SC5314 NEON at 1 X 10^7 cfu/ml combined to equal an approximate dose equal to solo infections into the yolk sac of the larval zebrafish to simulate an infection as described previously (752). Mortality of the fish was determined by observation of a loss of heartbeat and no movement when gently probed on the tail. Survival of the zebrafish was monitored daily, and experiment was terminated at 72 hours. Surviving fish were euthanized with an overdose of tricaine (0.64 mg/mL) at the end of the experiment. Injection doses were confirmed by serial dilution and plating of the inoculum on THY agar plates and enumerating bacteria or fungi colonies.

To investigate if clindamycin effectiveness against GBS in solo vs co-infection is altered yolk sac infection, clindamycin was added to the tank water of the zebrafish immediately after injection at a dose 6μg/mL. Prior to these experiments a cytotoxicity screen was performed to determine if adding clindamycin to the tank water of zebrafish could cause harm to the fish by monitoring development in different doses of clindamycin over a 3-day period. Cytotoxicity was considered to be signs of deformity during early development of embryos including deformed or missing heads, hearts, or tails. No

cytotoxicity was found for zebrafish treated with clindamycin at doses as high as 10μg/mL (data not shown).

2.4.9 Creation of GBS 515 Streptomycin resistant strain

To select for GBS 515 after injection into a zebrafish, a streptomycin resistant strain of GBS 515 was created (515 SR) following a previously described protocol (753). To spontaneously induce antibiotic resistance to streptomycin, overnight cultures of GBS 515 were grown in THY media with different concentrations of streptomycin, and then plated on THY agar plates supplemented with streptomycin. Spontaneous antibiotic resistance by GBS 515 to streptomycin was achieved after exposure to 250 μg/mL of streptomycin in liquid cultures and 250μg/mL of streptomycin supplemented on agar plates. GBS 515 strain with streptomycin resistance was confirmed by streaking a liquid culture of the bacterium on an agar plate supplemented with 250μg/mL of streptomycin, then performing a 16S ribosomal RNA (rRNA) gene polymerase chain reaction (PCR) specific for GBS. Multiple virulence assays were performed in zebrafish to confirm that the GBS 515 SR strain had the same virulence as the wild-type strain (Figure D.1).

2.4.10 Bacterial and Fungal Burden Assay

Larvae at ~48 hpi were manually dechorionated and anesthetized using 0.32 mg/mL of tricaine prior to injection. Bacterial and Fungal Burden assay was performed by injecting either 1nL of GBS (515 SR) at 4 X 10^7, 1 nL of *C. albicans* (SC5314 NEON) at 4 X 10^7 or 1 nL of GBS at 4 X 10^7 and *C. albicans* at 4 X 10^7 into the otic vesicle. Doses were confirmed by plating serial dilutions of inoculum on THY agar. At 24hpi, 8 fish per infection group were euthanized by overdose of tricaine and homogenized using 200 uL of PBS and a pellet pestle (Thermo Scientific). Fish who were dead prior to euthanasia were not selected for homogenization. Following homogenization, the sample was plated on selective plates using serial dilution and approximate burden was calculated by enumeration of single colonies on selective plates. To calculate GBS bacterial burden, serial dilutions of the homogenized fish samples were plated on CNA agar with 250μg/mL Streptomycin. To calculate *C. albicans* fungal burden samples were plated on Candida BCG agar (Difco) supplemented with Neomycin (500μg/mL) and Ampicillin (10 μg/mL).

2.4.11 Statistical analysis

All experiments were analyzed for significance using a two-tailed student's t-test in Prism GraphPad unless otherwise noted in figure legends.

Tables

Table 2.1: *S. agalactiae* strains in this study

***Streptococcus agalactiae* strain**	**Origin**
GBS 515	Serotype Ia Clinical Isolate (754)
COH1	Serotype III Clinical Isolate (755)
GBS 515-GFP	Created for this study using previous GBS GFP strain (86)
GBS 515-SR	Created for this study

Table 2.2: *C. albicans* strains in this study

Candida albicans strain	**Description and genotype**	**Reference**
SC5314-Neon	Wildtype clinical isolate; pENO1-NEON-NAT	(756)
Caf2 FR	SC5314 background; *Δura3::imm434/URA3 pENO1-iRFP-NAT*	(721)
NRG1^{OEX}-iRFP	THE21 background: *ade2::hisG/ade2::hisG ura3::imm434/ura3::imm434::URA3- tetO ENO1/eno1::ENO1* *tetR-ScHAP4AD-3XHA-ADE2 pENO1-iRFP-NATR*	(315, 316, 668)

CHAPTER 3

ENVIRONMENTAL PH DIRECTLY INFLUENCES INTERACTIONS BETWEEN STREPTOCOCCUS AGALATIAE AND CANDIDA ALBICANS

3.1 Introduction

Human tissue environments can be challenging for microbes to colonize due to stress factors they encounter in those tissues. The ability of microorganisms to adapt to and withstand environmental stressors is crucial for the health of the microbe, as it not only promotes the ability of the microorganism to survive and colonize a host, but also often directly impacts the gene expression of the organism. There are many environmental stressors to which microbes are exposed in human tissue environments, some of which include nutrient stress, temperature changes, extreme pH conditions and fluctuations, and microbial competition, all of which can differ depending on the specific tissue environment (757–762). Microorganisms combat environmental stressors using different cellular processes depending on the species, and the cellular processes involved with surviving environmental stressors for one microbe may directly impact other microbes in the same colonizing environment in a symbiotic, commensal, or antagonistic fashion (763). For example, polymicrobial biofilms are often more resistant to antimicrobial treatment and may also be larger in biomass compared to biofilms consisting of just one microbe depending on the microbial species involved in these interactions (705, 764–767). Human tissue environments generally contain a diverse microflora, and interactions between microbes likely influence the ability of microbes to colonize and/or influences virulence in a host.

Streptococcus agalactiae (Group B Streptococcus or GBS) and *Candida albicans* are two opportunistic pathogens that often experience environmental stressors in the tissue environments they colonize, which include the skin, gastrointestinal tract, and the vaginal tract (27–32, 294, 338, 339). The vaginal tract is an especially harsh niche for microorganisms to survive due to its acidic environment caused by its lactobacilli population, microbial competition, and a lack of nutrients for microbes (43, 719, 720). Both GBS and *C. albicans* are considered to be highly adaptable organisms due to their ability to overcome host and environmental stressors, and this adaptability likely aids their ability to establish themselves in the vaginal microbiota. One of the stressors these microbes need to adapt to when colonizing the vaginal tract

is an acidic pH environment (708, 768–770). The pH of a heathy vaginal tract is considered to be in the 3.8-5.0 range, and an increase of pH in the vaginal tract has been linked to vaginal infections such as Bacterial Vaginosis (BV) (771–774). Both GBS and *C. albicans* can colonize the vaginal tract, which indicates that these organisms possess tools to combat the stress of that acidic pH environment (775, 776). Beyond the vaginal tract, both pathogens also face pH stress when challenged by certain phagocytic cells, as mature phagocytes like macrophages have acidic intracellular components that aid in the phagocytosis of microbes (777–780). The gene expression of both GBS and *C. albicans* is directly influenced by its environmental pH, with many genes upregulated or downregulated in an acidic pH environment compared to a neutral pH environment (781, 782). There is evidence that the regulation of gene expression for GBS in response to environmental pH is regulated by two-component system (TCS) CovR/CovS, (also known as CsrRS), which is also a TCS well known in GBS to be responsible for regulating major virulence genes in response to environmental conditions (116, 117, 781, 783). The influence of acidic pH is complicated, as some genomic studies have shown certain virulence genes in GBS to be downregulated or upregulated in acidic pH compared to neutral pH (84). There is also conflict about the role of pH in adherence to epithelial cells, as some studies state that GBS adheres more readily to epithelial cells at neutral pH, while others have concluded that acidic pH enhances adherence by GBS to epithelial cells (781, 784, 785). The relationship between *C. albicans* and pH fluctuations is also complicated, but like GBS the organism has genomic mechanisms available allowing it to adapt to and alter an acidic pH environment. *C. albicans* is known as a highly adaptable organism in part due to its ability to survive a wide range of pH environments, from extremely acidic at pH 2 to as high as pH 10 (786). The morphological state of *C. albicans* is directly related to pH, as fluctuations in pH levels leading to increased pH actually triggers the organism to undergo a yeast-to-hyphal transition, which is known as a major virulence factor for the organism (314, 385, 787). While *C. albicans* is able to survive in an acidic pH environment, it is not ideal for the organisms, as exposure to acidic pH can cause damage to the cell wall, which in turn makes the organism more susceptible to immune recognition (788). To reduce pH stress from either acidic or alkaline environments, *C. albicans* actively increases or decreases its extracellular pH to become neutral (302, 789).

While it is known that both GBS and *C. albicans* possess genomic mechanisms that help combat acidic stress in both the vaginal tract and phagocytotic cells like macrophages, it is unknown how

interactions between GBS and *C. albicans* are altered when exposed to an acidic pH media environment in comparison to a neutral pH environment. Previous work done in the chapter prior in this thesis discovered that *C. albicans* is able to promote the growth, survival, and resistance by GBS to antimicrobial treatment in certain media environments. However, these previous interactions have been studied in buffered media, where these microbes remained in a relatively neutral pH media during the experiments. It is also unknown how GBS may influence the ability of *C. albicans* to withstand acid stress or transform an acidic pH environment to a neutral pH environment. Overall, by investigating the influence of pH on GBS and *C. albicans* interactions we can get a better understanding of how these organisms may be interacting in certain host environments where acidic stress is common.

3.2 Results

3.2.1 *C. albicans* alters the pH of RPMI media when grown with or without GBS

Previous research has shown that *C. albicans* has the ability to alter the pH of some growth media from either an acidic or alkaline pH to a neutral pH in both solid and liquid media in as little as 12hrs post culture in some specific media types (302). However, it is unknown how efficiently *C. albicans* can alter the pH of media when also cultured with other microorganisms that also undergo pH stress response, especially in a stressful nutrient-poor environment. To investigate this, we co-cultured *C. albicans* with GBS in unbuffered (without HEPES) RPMI 1640 media (GIBCO) with either a starting pH value of 7 (neutral RPMI media) or 5.5 (acidic RPMI media) aerobically for 24hrs at 37°C. Following a 24hr co-culture or solo culture of both organisms in acidic or neutral pH media, culture supernatants were removed, filter sterilized, then the pH of the supernatants were measured. GBS, when grown by itself in neutral RPMI media (pH 7), slightly decreased the pH of the growth medium by 0.06 to 6.94 (Figure 1A & 1B). GBS when grown in acidic RPMI media (pH 5.5) makes the pH of that media even more acidic, decreasing by 1.13 and leading to a final media pH of 4.13 (Figure 1A & 1C). When GBS and *C. albicans* were grown in neutral RPMI media together the media became more alkaline, with a raise in pH by 0.79 from pH7 to pH 7.79 (Figure 1A & 1B). However, when grown in acidic RPMI media, the media did not become more alkaline and instead became slightly more acidic, decreasing in pH by 0.19 from pH 5.5 to pH 5.31 (Figure 1A & 1C). While the culture containing GBS and *C. albicans* did still become more acidic when initially grown in acidic RPMI media, the

culture did not become as acidic as when GBS is grown alone in that media (pH 5.31 compared to pH 4.31, respectively), indicating that the presence of *C. albicans* in the culture with GBS helps reduce the acidification of the media caused by GBS. *C. albicans*, when grown alone in either neutral RMPI media or acidic RMPI media was able to alkalize its growth media, with the pH raising by 0.92 from pH 7 to pH 7.92 in the neutral RMPI media, and raising by 1.12 in RPMI acidic media, changing the media from pH 5.5 to almost neutral at pH 6.92 (Figure 1A-1C). Overall, it is obvious that the presence of GBS alters the ability of *C. albicans* to completely alkalize its media environment, but *C. albicans* can still help reduce the acidification of the growth medium, potentially reducing acid stress to GBS in those cultures.

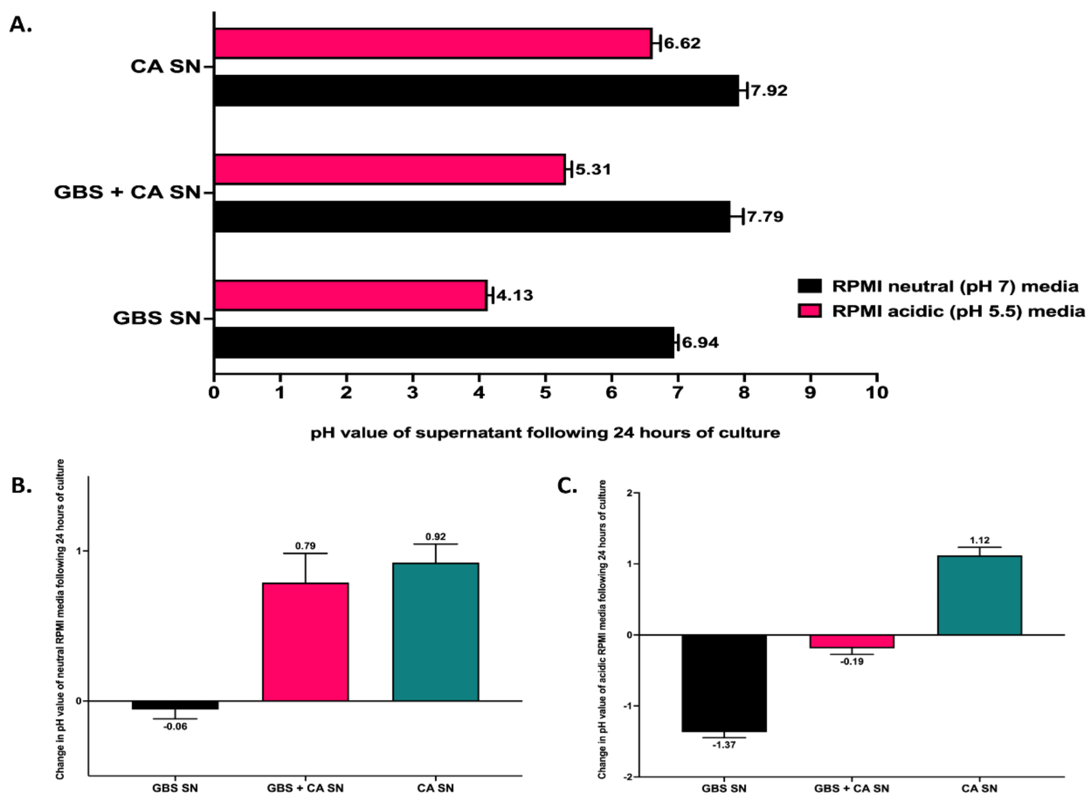


Figure 3.1: The ability of *C. albicans* to modulate media pH is altered in the presence of GBS GBS and *C. albicans* were grown together or separately in RPMI media with different starting pH for 24hrs at 37°C, then the pH of culture supernatants was measured to determine how pH value may change in solo and co-cultures of GBS with different initial pH values. **A.** Final pH value of culture supernatants of GBS and *C. albicans* solo and co-cultures following 24hrs of growth in RPMI neutral (pH 7) media and RPMI acidic (pH 5.5) media **B.** average change in pH value of solo and co-cultures following 24hrs of growth in RPMI neutral media **C.** average change in pH value of solo and co-cultures following 24hrs of growth in RPMI acidic media

3.2.2 *C. albicans* reduces the ability of GBS to acidify growth media in nutrient rich THY media with or without additional glucose supplementation

Previous data in this study has shown that GBS can acidify its growth media, especially in media that is already acidic initially, and that *C. albicans* can help reduce that acidification and potentially protects GBS from acid stress (Figures 1A-1C). However, the previous media studied (RPMI media) is nutrient poor and therefore an already stressful media environment for these microbes. To investigate how pH may be altered in a nutrient rich media type, GBS and *C. albicans* were grown separately and together for 24hrs at 37°C in Todd-Hewett supplemented with 0.2% yeast extract (THY) media, which is a media rich in nutrients due to its contents including beef heart infusion, peptones, and dextrose (790). THY media prior to microbial growth typically has a pH value of 7.4-7.6, which is slightly alkaline. Following 24hrs of growth, culture supernatants were removed, filter sterilized, then the pH levels of the culture supernatants were measured. GBS, when grown alone in nutrient rich THY media, produced acid in its culture media, as media cultures averaged a reduction of pH by a value of 1.80 to a final average pH value of 5.78 (Figures 2A & 2B). However, when GBS was grown with *C. albicans* in THY media cultures did not become as acidic, with the average acidification of the media reduced to 0.77, for a final average pH of 6.82 (Figures 2A & 2B). *C. albicans* barely modified the media when grown alone, with an average increase of pH by a value of 0.18, with a final average pH value of 7.63 (Figures 2A & 2B). Overall, like with RPMI media, GBS can acidify the media during growth, but that acidification is reduced in the presence of *C. albicans*.

To further investigate a nutrient rich environment, GBS and *C. albicans* were also grown together and separately in THY media with an additional supplementation of glucose (THY + GLU), bringing the final glucose concentration in the media to 0.04% and monitored for changes in pH after 24 hours of growth. Investigating the role of additional glucose availability on the ability of GBS and *C. albicans* to modify the pH of their growth media was performed for multiple reasons. First, there is an increased glucose availability in diabetic patients (especially in the blood), and diabetic patients are at an increased risk of infection by both GBS and *C. albicans* (3, 258, 344, 791–794). GBS also utilizes glucose in both aerobic and anaerobic growth as its primary carbon source and produces lactic-acid as an end-product of glucose fermentation,

therefore acidifying its environment (795, 796). To investigate if an increase in glucose availability can further influence the ability of GBS and *C. albicans* to modify their media pH, GBS and *C. albicans* were grown separately or together for 24hrs at 37°C in THY + GLU media. Following 24hrs of growth, culture supernatants were removed, filter sterilized, then media pH was measured. When GBS was grown in THY + GLU media separately the media became more acidic than when grown in THY media without glucose supplementation, with an average decrease of pH value of 2.87, with a final average pH of 4.73 (Figures 2A & 2C). Like with THY media without glucose, GBS cultures grown with *C. albicans* saw reduced acidification in comparison to cultures with GBS alone, with an average decrease of pH by a value of 1.87, with an average final pH value of 5.73 (Figures 2A & 2C). *C. albicans*, when grown alone in THY + GLU barely modified its media pH, with an average increase of pH value by 0.04, leading to an average final pH value of 7.55 (Figures 2A & 2C). The final pH values of *C. albicans* cultures grown in THY media with or without additional glucose supplementation are very similar (7.63 and 7.55, respectively), indicating that additional glucose supplementation did not affect the ability of *C. albicans* to modify the pH of its environment. Overall, *C. albicans* was able to maintain a relatively neutral pH media environment in both THY and THY + GLU media when grown alone and helped reduce the ability of GBS to acidify its pH environment when grown together in comparison to GBS that was grown alone (Figure 2). Additionally, GBS cultures became more acidic when grown in THY + GLU media compared to THY media, indicating that additional glucose availability possibly allows for an increase in lactic acid production (Figure 2A).

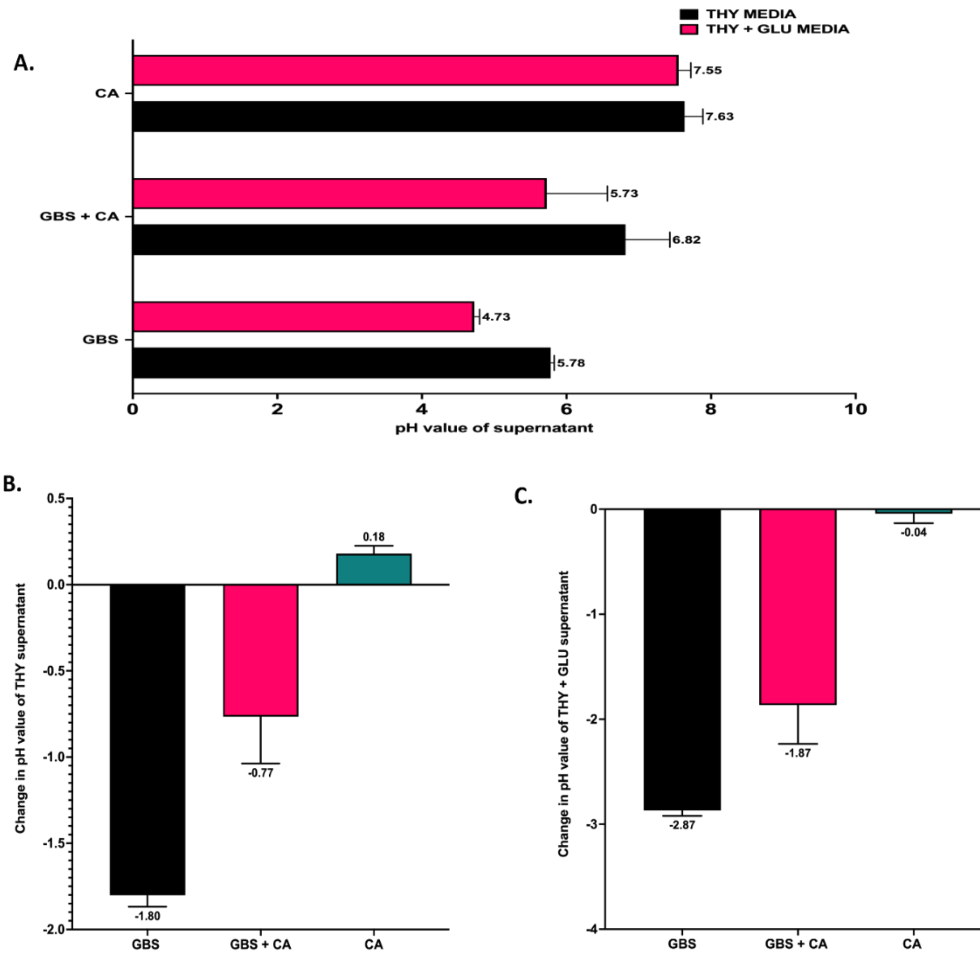


Figure 3.2: *C. albicans* can reduce acid stress to GBS caused by glucose supplementation GBS and *C. albicans* were grown together or separately in THY media with or without additional glucose supplementation for 24hrs at 37°C, then pH of culture supernatants was measured to determine how pH value may change in solo and co-cultures of GBS with different initial pH values. **A.** Final pH value of culture supernatants of GBS and *C. albicans* solo and co-cultures following 24hrs of growth in THY media and THY + GLU media **B.** average change in pH value of solo and co-cultures following 24hrs of growth in THY media **C.** average change in pH value of solo and co-cultures following 24hrs of growth in THY + GLU media

3.2.3 *C. albicans* promotes the viability of GBS following increased acid stress caused by glucose supplementation in culture media

Previous data in this study has shown that GBS can acidify its culture media during growth, and that this acidification is more prominent with increased glucose supplementation in THY media. Acidification of THY media with or without supplemented glucose by GBS is also reduced when GBS is grown in the presence of *C. albicans*. However, it is unknown how the reduction of acid by *C. albicans* can aid in the viability of GBS in those cultures. An acidic pH has a microbicidal effect on some microbes in the vaginal

tract, and while GBS is known to have acid stress response tools available, it is unknown how extreme pH levels may affect the viability of GBS, or if slightly increased pH caused by *C. albicans* can lead to increased GBS viability (775, 797). To determine if the reduction of acid by *C. albicans* in GBS and *C. albicans* co-cultures allows for increased viability of GBS in comparison to solo GBS cultures, GBS and *C. albicans* were grown separately and together in THY and THY + GLU media as previously described for 24hrs at 37°C. Following 24hrs of culture, serial dilutions were performed, and dilutions were plated on selective agar plates for GBS and *C. albicans* isolated growth for 24hrs at 37°C. Individual colonies on selective agar plates were enumerated to determine a concentration of viable cells from each original culture. Results indicated that when GBS is grown by itself in THY + GLU Media viability is significantly decreased compared to when it is grown in THY media (***p=0.001), as GBS THY+GLU cultures had more than 3-log fold less viable GBS cells compared to THY media (Figure 3A). When GBS was grown with *C. albicans* in THY + GLU media the viability of GBS cells is still significantly less than when grown in THY media (**p=0.011), but this decrease in viability is much less severe in these cultures, with only a roughly ½ log-fold difference in viable cells (Figure 3A). *C. albicans* viability was not significantly altered in THY or THY + GLU media compared to co-cultures of GBS and *C. albicans* in both media types (Figure 3B).

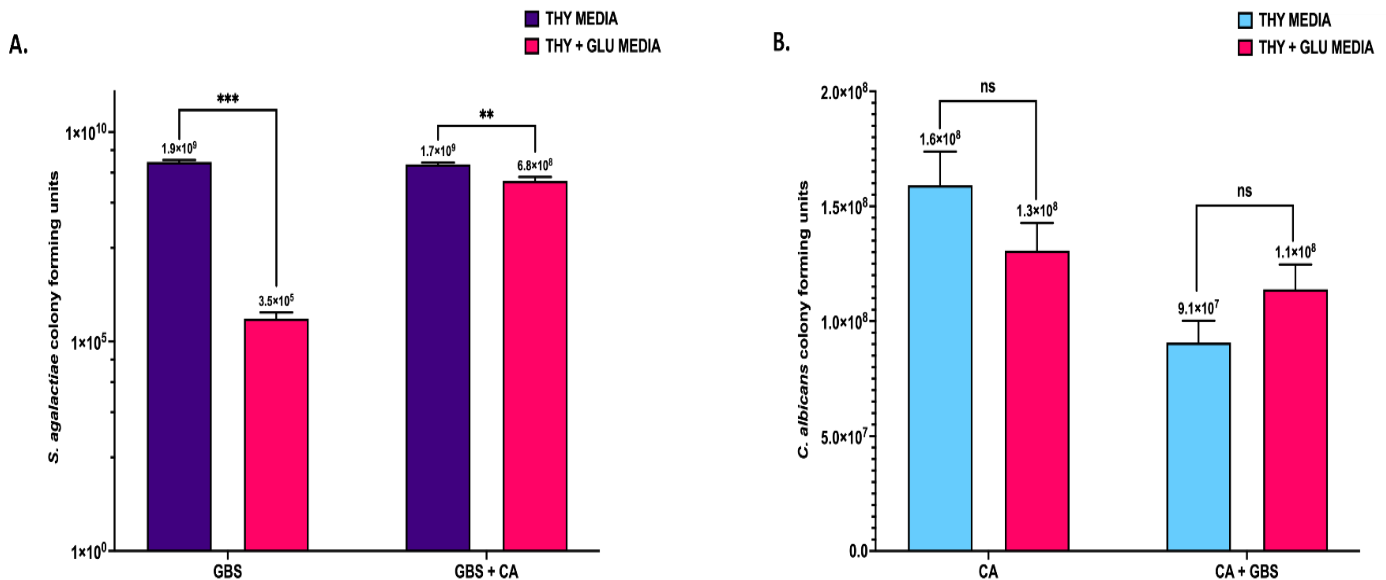


Figure 3.3: *C. albicans* promotes the viability of GBS in glucose supplemented media GBS and *C. albicans* were grown together or separately in THY media with or without additional glucose supplementation for 24hrs at 37°C, then cultures were serial diluted and plated on selective plates to enumerate concentration of viable GBS and *C. albicans* in culture tubes **A.** Viable GBS cells when grown with or without C. albicans in THY and THY + GLU media **B.** Viable *C. albicans* cells when cultured with or without GBS in THY and THY + GLU media **p<0.005, ***p<=0.0005

3.2.4 The morphology of GBS and *C. albicans* is altered in THY media supplemented with glucose compared to media without glucose supplementation

Following the discovery that GBS viability is significantly reduced in THY + GLU media compared to THY media without glucose supplementation, and that this reduction is not as severe when also grown with *C. albicans*, morphology of GBS and *C. albicans* in solo and co-cultures was investigated to determine if any differences in morphology for the pathogens occurs in the different media types due to differences in pH in these cultures. The effects of acidic pH on the morphology of GBS has not been explored previously, and data about biofilm formation by GBS in acidic pH environments have been conflicting, as some groups have indicated that biofilm formation is more prominent in pH neutral media environments, while others have reported the opposite (112, 798). The effect of pH on the morphology of *C. albicans* is better defined, as *C. albicans* can actively alkalize its environment in response to acid stress, and this transition to a more alkaline pH can auto-induce a yeast-to-hyphal transition for the pathogen, which has also been indicated to be a virulence factor for the organism (302, 314, 385, 787). To determine possible morphological differences in THY and THY + GLU cultures, solo and co-cultures of both fluorescent GBS (GBS 515 GFP) and *C. albicans* (Caf2 FR) in both media types were inoculated and allowed to grow for 24hrs at 37°C. Following 24hrs of culture, samples were imaged using a fluorescent confocal microscope to investigate possible differences in morphology in each media type for solo and co-cultures. The ability of *C. albicans* to transition from yeast-to-hyphal cells was not altered in solo or co-cultures with GBS in either media type (Figure 4). Interestingly, the ability of GBS 515 GFP to be fluorescent was severely inhibited when GBS was grown by itself in THY + GLU media but was not affected when grown with *C. albicans* in the same media type (Figure 4). While GBS seemed to adhere to *C. albicans* in both THY and THY + GLU media, the adherence of GBS to *C. albicans* in THY + GLU seems to be enhanced, with GBS readily adhering to both hyphae and yeast cells in that culture media environment.

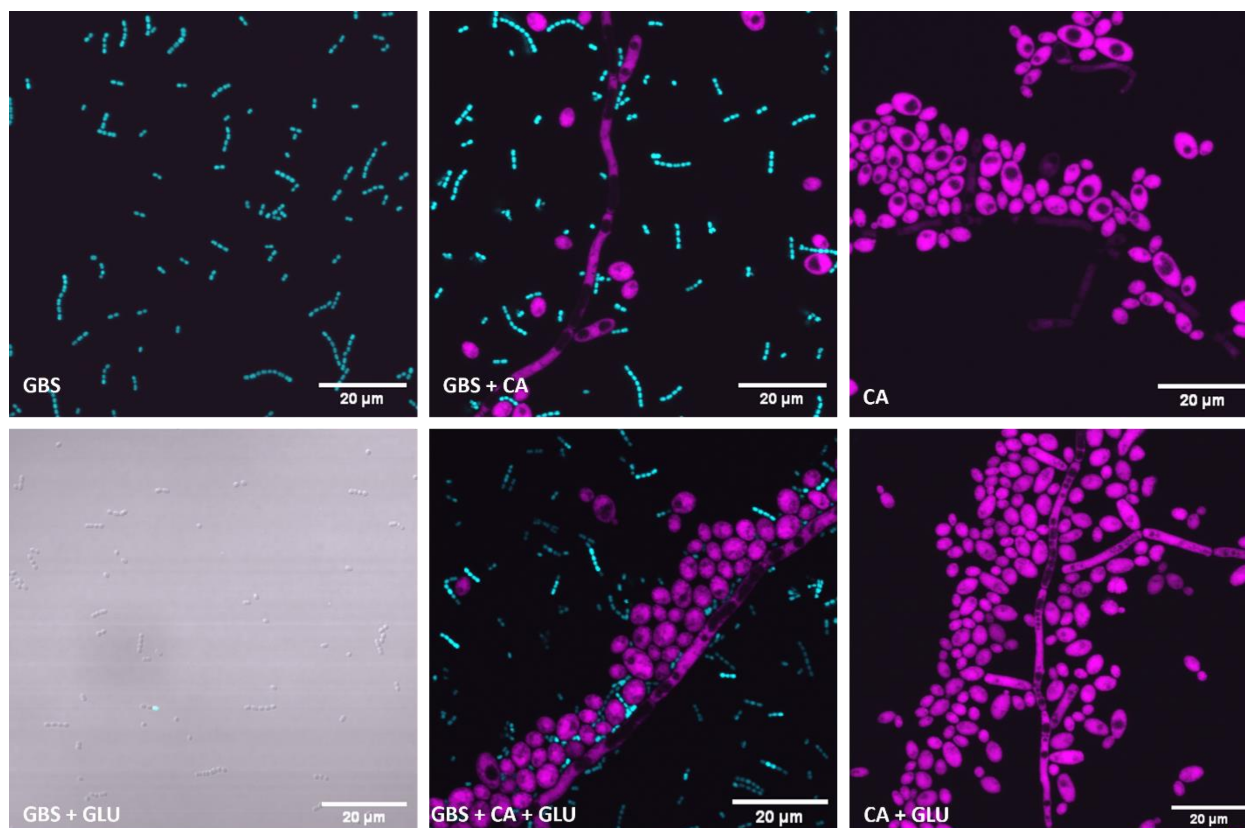


Figure 3.4: Changes in media pH following growth can alter the morphology of GBS and *C. albicans* in solo or co-cultures GBS 515 GFP was grown in solo or co-cultures with fluorescent *C. albicans* strain *Caf2-FR* for 24hrs in THY or THY + GLU media for 24hrs at 37°C and fluorescent images were taken at 1000X magnification using a point-scanning confocal microscope (Cyan = GBS; Magenta = *C. albicans*) and investigated for morphological differences between cultures.

3.3 Discussion

Stressors encountered by a microbe when colonizing or infecting different host niche environments not only alter the fitness of the organism, but also directly influences its gene expression. One of the most prominent stressors encountered by microbes in the human host is pH, as microbes must adapt to the pH of a host tissue environment to colonize and/or infect. While the pH of host niche environments can vary widely (pH 2-pH 10), most tissue environments in humans range from pH 7.35-7.4 and are considered neutral-to-slightly alkaline (302, 799). The vaginal tract is typically more acidic than most human tissues and can be a difficult host environment to colonize due to its acidic mucosa. Organisms colonizing this tissue environment must not only adapt to the initial pH of the vaginal tract, but also pH fluctuations in this tissue environment due to changes in the resident microbiota influencing vaginal pH (698–701, 800).

Both GBS and *C. albicans* are highly adaptable organisms in part due to their ability to colonize the vaginal tract. For many patients, colonization of the vaginal tract by GBS and *C. albicans* is asymptomatic, with no symptoms ever appearing in these patients (48, 567, 572). However, asymptomatic colonization of these pathogens in the vaginal tract can increase the risk of infection for certain groups of patients. For example, colonization of GBS in the vaginal tract of pregnant women is not only a cause stillbirth or preterm delivery in some patients, but can also lead to mothers passing GBS to newborns during delivery, which can cause life threatening conditions like bacteremia, pneumonia, and meningitis (217, 801, 802). Colonization of *C. albicans* in the vaginal tract can also cause serious complications for certain patient groups, especially when overgrowth of *C. albicans* leads to the development of vulvovaginal candidiasis (VVC) (572). Some patients suffer from recurrent vulvovaginal candidiasis (RVVC), a condition where patients have three or four episodes of VVC in one year, which can impact quality of life (803, 804). *C. albicans* can also cause neonatal thrush infection in newborns, some of whom acquire the pathogen during delivery through the birth canal of colonized mothers (805). Previous research has demonstrated that GBS and *C. albicans* are often co-isolated from the vaginal tract, which indicates that there may be a synergistic effect between the organism that promotes their ability to colonize this difficult tissue environment (288, 289, 663). What is unknown is how environmental pH alters the ability of these organisms to interact and/or synergize, as the pH of the vaginal tract can fluctuate due to changes in hormones, pregnancy, diet, and changes in the vaginal microbiota (806). The experiments in this study have expanded upon how GBS and *C. albicans* alter the pH of their growth media, as well as how that process is altered when they are co-cultured together.

This study first aimed to determine how GBS and *C. albicans* were able to modulate the pH of their growth media when cultured together or separately, and how that process differs when you start at an acidic pH or neutral pH in their nutrient-limited growth media. While *C. albicans* is known to be able to modulate its media pH to become more neutral in some media types, less is known about how GBS is able to modulate culture pH, or how these organisms interact in co-cultures to change culture pH following 24hrs of growth (302). The experiments in this study have shown that when *C. albicans* is cultured in nutrient limited media at either a starting pH of 5.5 (RPMI acidic media) or 7 (RPMI neutral media), culture media pH was altered following 24hrs of growth. In the RPMI acidic media, *C. albicans* was not able to fully neutralize the media pH but did raise the final pH of the media cultures close to neutral at pH 6.28. Culture

supernatants from *C. albicans* grown originally in RPMI neutral media, rather than maintaining a neutral pH, became more alkaline, changing from pH 7 to pH 7.79. These results were not surprising, as previous research indicates that *C. albicans* can neutralize extracellular growth media from an acidic pH by utilizing amino acids and subsequently releasing ammonia as a byproduct, which in turn increases the extracellular pH (302). The release of alkaline peptides in response to extracellular pH has been seen in other fungal species, and has been implicated to impact virulence for some of them (807–809). *C. albicans* can not only modulate the extracellular pH of its growth media, but can also neutralize the pH of the acidic macrophage phagosome, inducing the transition from yeast-to-hyphal cells and increasing the ability of *C. albicans* to escape macrophages (777). Overall, it appears that starting at an acidic media pH reduced the ability of *C. albicans* to alkalize its media environment compared to pH media culture, but in both cases *C. albicans* was able to modulate the extracellular pH to reduce acid. GBS, when grown alone in RPMI neutral media, minimally altered the pH of the culture media to pH 6.94. Interestingly, when grown in RPMI acidic media, the pH of the media became even more acidic, dropping from pH 5.5 to pH 4.13. The inability of GBS to acidify RPMI neutral media but further acidify RPMI acidic media was unexpected, as GBS is a lactic acid producing bacterium who releases this acid during either anaerobic or aerobic metabolism, which is a process that was likely not disrupted in pH neutral media (796, 810). However, this increase of acid production by GBS in pH acidic media compared to pH neutral media may be linked to GBS gene expression, as certain GBS metabolic genes involved in respiration are upregulated in acidic pH environments, possibly leading to increased lactic acid production by GBS as a byproduct of respiration (84). Further testing will need to be conducted to truly determine why differences in GBS response to acidic and neutral nutrient-limited media occur. Prior to this study, it was unknown how interactions between GBS and *C. albicans* may influence the extracellular pH of their growth environment following co-culture in comparison to solo cultures. The ability of *C. albicans* to alkalize its media environment was not altered when co-cultured with GBS in pH neutral media with a final pH of 7.79 seen in those cultures. However, when co-cultured together in acidic pH media, the ability of *C. albicans* to neutralize the pH acidic media was hindered, with the initial pH dropping from pH 5.5 to pH 5.31. It is possible that lactic acid production by GBS harms *C. albicans* and therefore its ability to modify culture pH, as previous work describing interactions between *C. albicans* and some lactobacilli species indicates that the production of lactic acid

by these bacteria can inhibit fungal growth and harm cell viability of *C. albicans in vitro* (811). Additionally, as this media type is nutrient-limited, it is possible that the nutrients needed by *C. albicans* to fully combat acid production by GBS in the media through alkalization were not abundant enough (302). While *C. albicans* was not able to fully neutralize RPMI acidic media when grown with GBS, it is still possible that the increase of pH in these cultures compared to GBS grown by itself in the same media type is beneficial to GBS. For example, an increase of pH in the vaginal tract is a risk factor for BV, and patients with BV have been shown to be at an increased risk of being colonized by GBS (812). Increased environmental pH also reduces acid stress to GBS in general, which is likely beneficial to the fitness of the bacterium in stressful environments like a nutrient-limited media environment.

Previous data in this study has indicated that the ability of GBS and *C. albicans* to modulate their extracellular media pH in nutrient-limited media is different in co-cultures in comparison to solo cultures. The previous media environment is stressful to microbes due to the lack of nutrients, and it is possible that both a lack of nutrients and additional stress inhibited the ability of *C. albicans* to fully neutralize its media pH when grown in acidic media and GBS. To investigate the ability of GBS and *C. albicans* to alter its extracellular media pH in solo or co-cultures these pathogens were inoculated together or in solo in either THY media alone, or THY media additionally supplemented in glucose. THY media is very nutrient rich, with components like dextrose, beef heart infusion, and peptide digest of animal tissue (790). The influence of additional supplementation of glucose in THY media (THY + GLU media) on the ability of these pathogens to modulate their extracellular media pH was also explored, as both pathogens can utilize glucose as a carbon source during growth (795, 813). The initial pH of both THY and THY + GLU media was slightly alkaline and averaged between pH 7.4-7.6 prior to microbial growth. When grown in THY or THY + GLU media, *C. albicans* extracellular pH or cell viability was not significantly altered in either media type. When grown in THY media, GBS was able to acidify its extracellular media to pH 5.78. This acidification was even more prominent in THY + GLU media, with the final pH of the GBS growth media averaging pH 4.73. The viability of GBS in THY + GLU media following 24hrs of growth was significantly decreased compared to GBS grown in THY media alone, with an almost 4 log-fold decrease in viability. It is likely that the increased acidification of the THY + GLU growth medium by GBS reduced its viability, as lactic acid is the end product of sugar fermentation in GBS and lactic acid production can actually be harmful

to GBS (814). Lactobacilli supplementation has been proposed as a probiotic treatment against GBS for this reason, as GBS growth has been shown to be inhibited when exposed to lactobacilli strains known to be strong producers of lactic acid *in vitro* (168, 815). The reduction of GBS viability in THY + GLU media cultures is still seen in GBS + *C. albicans* co-cultures, but this viability reduction is much less severe, with GBS viability only being reduced by ~1/2 log fold when also cultured with *C. albicans*. This increase in viability of GBS in *C. albicans* co-cultures compared to solo GBS cultures in THY + GLU media may be due to reduced acid in those supernatants, as the pH of THY + GLU media when GBS was cultured alone dropped on average to pH 4.73, while the pH of the GBS and *C. albicans* co-culture in THY + GLU media was much closer to a neutral pH at pH 5.73. Previous research has indicated that the ability of *C. albicans* to produce ammonia and thereby alkalize its environment can be repressed in the presence of glucose, but this was not evident in this study, as the pH of the GBS and *C. albicans* cultures when grown in THY + GLU media was still elevated compared to GBS solo cultures, indicating that *C. albicans* likely modulated the pH in those cultures raise the pH closer to neutral (302).

Finally, this study aimed to determine if any morphological differences can occur in solo or co-cultures of GBS and *C. albicans* when grown in THY and THY + GLU media as pH has been shown to directly influence the ability of *C. albicans* to transition from yeast-to-hyphal cells (302). The results of fluorescent imaging of GBS and *C. albicans* solo and co-cultures in THY and THY + GLU media, while not quantitative, can provide a qualitative look at possible interactions between the pathogens in these different media (and pH) environments. *C. albicans* hyphal formation was not inhibited in any culture despite lower environmental pH in co-cultures with GBS. The ability to transition from a yeast-to-hyphal cell is a virulence trait for *C. albicans*, so the ability to maintain this transition state is crucial for the fitness of the pathogen (385). Interestingly, in co-cultures of GBS and *C. albicans* grown in THY + GLU media, the adherence of these pathogens to one another seemed to be enhanced compared to GBS and *C. albicans* co-cultures in THY media. Adherence of GBS to *C. albicans* has been shown to promote the ability of both pathogens to adhere to vaginal epithelial cells *in vitro*, so it is possible that increased access to glucose may be beneficial to these pathogens in adherence to vaginal epithelial cells (53). Finally, GBS cells grown alone in THY + GLU media were not able to effectively fluoresce, while GBS cells growth with *C. albicans* in that same

media type were able to fluoresce with no visible defects. This was likely due to acid production in the cultures with GBS alone, as GFP is pH sensitive and fluoresces less under acidic conditions (816).

Overall, this study gives us a better understanding of not only how GBS and *C. albicans* influence the pH of their growth environment, but also how interactions between the two pathogens may influence their ability to modulate the pH of their environment. However, multiple gaps of knowledge still remain. For example, little is known about the ability of GBS to modulate its environmental pH beyond genomic studies revealing pH regulating genes similar to those found in other streptococcal species exist in the GBS genome (84, 775). Also, while the data in this study has indicated that interactions between GBS and *C. albicans* alter their ability to modulate their media pH *in vitro*, there is no data about how these pathogens may interact with one another to influence pH *in vivo*. Further research investigating how interactions between GBS and *C. albicans* are influenced by pH will give us a better idea of how these pathogens adapt to colonize the vaginal tract, and therefore allow us to hopefully develop methods to prevent colonization of the vaginal tract by these opportunistic pathogens.

3.4 Materials and Methods

3.4.1 Bacterial strains and growth conditions

Streptococcus agalactiae strain GBS 515 used for all experiments with the exemption of the microscopy experiment, which utilized a fluorescent GBS strain created for this study that originates from GBS 515. GBS 515 is a serotype 1a, ST-23 human clinical isolate from the blood of a patient diagnosed with neonatal septicemia and was generously provided by M.R. Wessels (90). For all experiments using GBS, the bacterium was inoculated in Todd-Hewitt medium (Acumedia) supplemented with 0.2% yeast extract (THY) in sealed conical tubes and grown statically at 37°C overnight. Following overnight growth, GBS was sub-cultured into fresh THY media and allowed to grow to mid-log phase prior to co-culture experiments.

3.4.2 Fungal strains and growth conditions

The fungal strains of *Candida albicans* (*C. albicans*) used in this study are listed in Table 2: With the exception of the microscopy experiment, all experiments were performed with the *C. albicans* strain

SC5314-Neon. For all experiments using *C. albicans,* the fungus was first inoculated onto a THY agar plate for individual colonies and grown overnight at 37°C aerobically. For liquid overnight cultures, a single colony of *C. albicans* was selected from an agar plate, inoculated into THY media, and grown overnight with shaking aerobically in glass culture tubes at 37°C.

3.4.3 Creating different pH media types for Co-culturing experiments

To investigate the role of pH on the interactions between GBS and *C. albicans*, different media types with different pH levels and nutrient availability were utilized. For experiments investigating the role of pH during co-culture in nutrient poor media, RMPI 1640 with no HEPES (manufacturer or other buffering agents at two different pH levels (pH 5.5 and pH 7-7.2) were utilized. RPMI 1640 (no HEPES) media from the manufacturer originates at pH 7-7.2 and was used for the pH neutral media. To create RPMI pH 5.5 media the neutral media was supplemented with 3M HCL until measured at ~pH 5.5 using a pH meter (manufacturer), filter sterilized, and stored at 4°C until used for experiments. To investigate the role of pH during co-culture in nutrient rich media, THY agar was supplemented with sterile glucose to reach a final glucose concentration of 0.04% in the medium, filter sterilized, then stored at 4°C until used for co-culturing experiments.

3.4.4 Measuring pH following 24 hour Co-culture of GBS and *C. albicans*

To investigate the final pH levels of the growth media of GBS 515 and SC5314-Neon following 24hrs of solo-or co-culture in different media types GBS 515 and SC5314-Neon were grown separately or co-cultured together as described in the “Materials and Methods: Growth Curve Experiments” section found in Chapter 2. Following 24hrr incubation at 37°C with shaking, cultures were centrifuged at 10,000 RPM for 10 minutes to form a pellet. Following centrifugation, supernatant from culture tubes were removed and filter sterilized using a 0.22μm filter (manufacturer) into fresh 15mL plastic tubes. Following filter sterilization, the pH of the supernatant was measured using a pH meter (Mettler Toledo).

For experiments using THY supplemented with glucose media, cultures were vortexed briefly for 10 second and 100 μL from each culture tube was removed and used for serial dilutions prior to centrifugation and pH measurements. Following serial dilution, cultures were plated on selective plates. To

enumerate GBS in experimental cultures serial dilutions from solo and co-cultures were plated on Strep B ChromoSelect Selective Agar Base (Millipore Sigma), grown overnight at 37°C and individual colonies were enumerated. To enumerate SC5314-Neon concentrations in experimental cultures serial dilutions were plated on THY agar plates supplemented with 2µg/mL ampicillin (Millipore Sigma), grown overnight at 37°C and individual colonies were counted.

3.4.5 Images protocol

To image GBS 515 and Caf2 FR in solo or co-cultures, cultures were inoculated as described above in THY and THY + GLU media and grown at 37°C with shaking for 24 hours. Following this growth period, 7 µL of each culture were added to a glass slide and covered with a 22 x 22 mm cover slip. After the cultures were mounted on the glass slides confocal images were taken using an Olympus IX-81 inverted microscope containing a FV-1000 laser scanning confocal system (Olympus, Waltham, MA). The fluorescent proteins EGFP (488nm/505 to 525 nm excitation/emission) and Far-Red (635nm/ 655 to 755 nm excitation/emission) were detected using laser/optical filters with a 100X oil objective (NA,1.40). Images were taken as Z stacks and were processed using FluoView (Olympus, Waltham, MA). Image brightness was enhanced for visualization using ImageJ (750).

3.4.6 Statistical analysis

All experiments were analyzed for significance using a two-tailed student's t-test with Welch's correction in Prism GraphPad unless otherwise noted in figure legend

Tables

Table 3.1: *S.agalactiae* strains used in this study

***Streptococcus agalactiae* strain**	**Origin**
GBS 515	Serotype Ia Clinical Isolate (754)
GBS 515-GFP	Created for this study using previous GBS GFP strain (86)

Table 3.2: *C.albicans* strains used in this study

Candida albicans strain	**Description and genotype**	**Reference**
SC5314-Neon	Wildtype clinical isolate; pENO1-NEON-NAT	(756)
Caf2 FR	SC5314 background; *Δura3::imm434/URA3 pENO1-iRFP-NAT*	(721)

CHAPTER 4

FUTURE DIRECTIONS AND CONCLUSIONS

The research presented in my dissertation has investigated the role of interactions between *Streptococcus agalactiae*, a gram-positive bacterium also known as Group B Streptococcus or GBS, and *Candida albicans*, an opportunistic fungal pathogen. This research has shown that interactions between these pathogens influence their growth rate, survival in stressful media and pH environments, ability to withstand antimicrobial treatment, and their virulence during infection using a larval zebrafish model. Historically, microbial research involving investigating the traits of infectious microbes is conducted using only that microbe. However, human tissue environments are polymicrobial in nature, and interactions between different microbes may influence their ability to colonize or infect a human host, as well as influence treatment outcomes in infected patients.

Interactions between microbes can be in an antagonistic, commensal, or synergistic fashion depending on the microbes involved, so investigating how specific microbes interact in stressful and competitive environments can help us determine how a microbe may influence the ability of another microbe to infect a human host. Both GBS and *C. albicans* are typically harmless commensals for many of the population they colonize. However, both pathogens can cause severe and sometimes fatal infections in certain patient groups, including newborn (especially those who are premature), immunocompromised, diabetic, and elderly patients. GBS and *C. albicans* often colonize the same tissue environments, which may lead to polymicrobial interactions between these two infectious pathogens.

Polymicrobial interactions between *C. albicans* and other oral streptococcal species have indicated that these microbes can synergize to enhance the virulence of these pathogens *in vivo*. GBS and *C. albicans* interactions has also been briefly researched in the context of adherence and colonization. Previous research has indicated that GBS and *C. albicans* can enhance the ability of these organisms to adhere to vaginal epithelial cells (VEC) using cell culture experiments (53). Previous *in vivo* murine infection experiments also discovered that co-colonization of GBS and *C. albicans* enhances the bacterial burden of GBS in the bladders of colonized mice, indicating that interactions between the two pathogens in beneficial to GBS in certain colonizing environments (287). Previous colonization studies in human patients

have discovered that GBS and *C. albicans* are often co-isolated from pregnant women, indicating that these organisms may interact in these patients due to their ability to colonize the same environments (288, 663). While the research cited above has helped define some interactions between GBS and *C. albicans* during colonization, little was known prior to the research described in this dissertation about how interactions between these two pathogens influences their ability to withstand environmental stress, their ability to withstand antimicrobial challenge, or their ability to infect a host when both pathogens are present compared to by themselves.

Both GBS and *C. albicans* can colonize the vaginal tract, which is a difficult environment for microbes to colonize and survive in due to multiple factors, including a low environmental pH, secreted antimicrobial peptides by other microbes, as well as competition for nutrients and epithelial cell adherence sites with other colonizing microbes. Our research investigated how factors like pH and nutrient availability can influence interactions between GBS and *C. albicans* utilizing *in vitro* co-culturing experiments using different nutrient and pH media. The results of our experiments indicated that interactions between GBS and *C. albicans* are directly influenced by both environmental pH and nutrient availability, as the growth rate and viability of GBS is enhanced by the presence of *C. albicans* in both low pH and nutrient limited media environments. This research identified that polymicrobial interactions between GBS and *C. albicans* can directly benefit GBS despite being in stressful environments like low pH and limited nutrient media, which can help us further understand why GBS and *C. albicans* are commonly co-isolated from colonized pregnant women vaginal isolates despite the difficulties involved with vaginal colonization for these pathogens.

While we have shown that the presence of *C. albicans* is beneficial for the survival and growth of GBS in stressful environments *in vitro,* we do not know exactly what genes may be responsible for these interactions. There are multiple possible causes for how *C. albicans* protects GBS from environmental stressors, including secreted factors by the fungal pathogen, direct adherence to one another, changes to the environmental pH caused by *C. albicans*, or other unknown factors. To better determine how exactly *C. albicans* protects GBS in certain environments, we plan to utilize high-throughput experimental methods like mass spectrometry to help determine which proteins are upregulated and downregulated in the culture

media of GBS and *C. albicans* when cultured separately or in co-cultures. Following these experiments, we plan to create or obtain GBS and *C. albicans* genetically mutated strains knocking out the genes responsible for the production of these identified proteins of interest. Once we have these strains, we can repeat co-culture experiments to determine if the loss of these genes influences the ability of *C. albicans* to promote GBS growth and viability in stressful environments like those that are acidic or nutrient limited.

Our research has also shown that interactions between GBS and *C. albicans* can influence the susceptibility of GBS to the antibiotics erythromycin and clindamycin. These two antibiotics have become less effective against GBS strains clinically, with a sharp increase of antibiotic resistance rates by GBS strains to these antibiotics being seen in the last 20 years. There is evidence that some polymicrobial biofilms are less susceptible to antimicrobials compared to single species biofilms, which led us to hypothesize that GBS may be less susceptible to certain antibiotics in the presence of *C. albicans*. We tested four different antibiotics: erythromycin, clindamycin, penicillin, and vancomycin. The experiments exploring the influence of *C. albicans* on the effectiveness of the antibiotics penicillin and vancomycin against GBS were inconclusive and were not reported upon in this dissertation. However, our results have indicated that the presence of *C. albicans* in erythromycin or clindamycin treated GBS cultures reduced the susceptibility of GBS to these antibiotics *in vitro*. This reduced susceptibility of GBS to erythromycin and clindamycin in the presence of *C. albicans* did not cause permanent resistance to these antibiotics by GBS (data not shown). While this finding could have clinical relevance, more research needs to be done to determine exactly how *C. albicans* reduces GBS susceptibility to these antibiotics. It is possible that *C. albicans* may inactivate these antibiotics which would benefit GBS in the culture media. It is also possible that polymicrobial biofilms form between GBS and *C. albicans* in the treated media culture, which would protect the GBS cells within the biofilm structure. Further experiments exploring these possibilities could help us better understand exactly how *C. albicans* reduces the susceptibility of GBS to erythromycin and clindamycin.

Another major component to this research was investigating how interactions between GBS and *C. albicans* influenced the ability of these pathogens to infect and resist treatment *in vivo.* We have discovered that co-infections between GBS and *C. albicans* are significantly more virulent in larval zebrafish

compared to solo infections of either pathogen depending on the infection site in the zebrafish. While it has been shown before that *C. albicans* can enhance the ability of GBS to colonize certain tissue environments *in vivo*, this is the first time that co-infections of GBS and *C. albicans* has been shown to be more virulent than solo infections in an *in vivo* model. To determine the cause of increased virulence by GBS and *C. albicans* in co-infected zebrafish, we plan to utilize multiple experimental techniques. For example, we hope to perform RNA-sequencing on solo and co-infected zebrafish to determine differences in gene expression for each of these infection groups. By determining which genes may be differentially expressed in solo or co-infected zebrafish we can better determine why GBS and *C. albicans* co-infected fish have decreased survival compared to solo infections with either pathogen. We can also utilize live imaging experiments using fluorescent microscopy to better determine interactions between these two infectious pathogens at different stages of infection in a live infection model.

Our data also indicated that larval zebrafish infected with GBS could be effectively treated with the antibiotic clindamycin during infection, as this treatment increased the survival of infected zebrafish by almost 50%. However, when the zebrafish were co-infected with both GBS and *C. albicans*, there was no statistical difference in survival in clindamycin treated fish compared to fish infected with only GBS. This research could have clinical implications, as if this is also happening in human hosts it could indicate that patients colonized with both pathogens should not be treated with the antibiotic clindamycin due to reduced efficacy. While these findings are notable, there is still a gap in knowledge of how exactly *C. albicans* reduces the ability of these co-infected fish to be treated with clindamycin. While we have determined that co-infections with GBS and *C. albicans* may be harder to treat with certain antibiotics, we have not determined how antifungal effectiveness may be affected by co-infections compared to solo infection. Having a better understanding of how antimicrobial effectiveness is altered in co-infections compared to solo infections could help us treat patients with antimicrobials more effectively clinically.

Overall, these findings highlight how interactions between the infectious pathogens GBS and *C. albicans* influence the ability of these pathogens to grow, survive in harsh environments, withstand antimicrobial challenges, as well as infect hosts in co-infection scenarios compared to solo infections. While much work is still to be done to determine exactly how these organisms interact in different environments,

this research establishes that interactions between these pathogens can directly impact infection pathogenesis and treatment outcome. Identifying the factors influencing interactions between GBS and *C. albicans* will hopefully lead to increased knowledge about virulence traits of both of these pathogens and help us better develop strategies to treat infections caused by these pathogens when both are present in a host.

APPENDICIES

APPENDIX A

GBS and C. ALBICANS VIABILITY IS NOT ALTERED IN NUTRIENT-RICH THY MEDIA

Previous work in this dissertation has identified that C. albicans is capable of increasing the viability of GBS cells in nutrient-limited RPMI culture media. RPMI culture media is very nutrient limited which likely induces a stress response in both GBS and *C. albicans*, as well as potentially leads to competition between the two pathogens for nutrients. The data suggests that *C. albicans* and GBS likely interact to promote their survival in the stressful nutrient limited environment. To determine if this same effect could be seen in a nutrient rich media environment, GBS and C. albicans were cultured separately and together in nutrient rich media Todd-Hewitt Broth supplemented with 0.2% yeast extract. This media type is specifically designed to cultivate the growth of streptococcal bacterial species due to its rich nutrient complex composition including peptic digests, vitamins, minerals, and amino acids. When cultured separately and together in this nutrient rich media, we did not see any significant differences in cell viability from GBS or *C. albicans*, indicating that interactions between GBS and *C. albicans* may be nutrient and stress dependent.

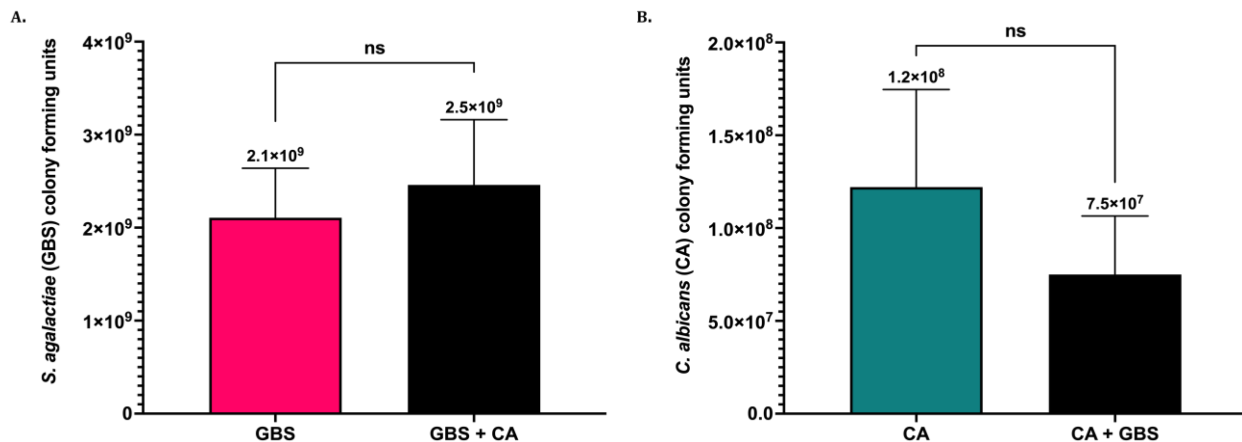


Figure A.1: GBS and *C. albicans* growth was not altered in co-cultures compared to solo cultures of the pathogens following 24hr culture in nutrient rich (THY) media GBS and *C. albicans* were incubated in nutrient rich media in solo and in co-cultures and viable colony counts were calculated following 24 hours of incubation **A.** GBS growth in solo and co-cultures with *C. albicans* following 24 hours of incubation **B.** *C. albicans* growth in solo and co-cultures with GBS following 24 hours of incubation. Experiments were performed in triplicate and statistical significance was calculated using an unpaired two-tailed student's t-test *$p<0.05$.

APPENDIX B

THE ABILITY OF *C. ALBICANS* TO PROMOTE GBS VIABILITY IN NUTRIENT LIMITED MEDIA IS NOT GBS STRAIN SPECIFIC

Previous work investigating interactions between GBS and *C. albicans* has indicated that *C. albicans* is able to enhance the cell viability of GBS in nutrient-limited media compared to GBS grown alone in that media type. What is not known about this interaction is if the ability of C. albicans to aid in the survival of GBS is strain specific. To determine if the ability of C. albicans to increase the viability of GBS in nutrient limited media following 24hrs or growth, GBS strain COHI WT, a Serotype III clinical GBS isolate originally isolated from an GBS infected newborn suffering from sepsis, was grown separately or co-cultured together with *C. albicans* in RPMI media at 37°C, with cell viability being measured after 24 hrs by enumerating GBS individual colonies on selective agar plates following serial dilution. Results showed that COH1 WT GBS cell viability was increased in the presence of *C. albicans*, just like what was seen with GBS strain 515, indicating that the increase of GBS cell viability in the presence of *C. albicans* in nutrient limited media is not GBS strain specific.

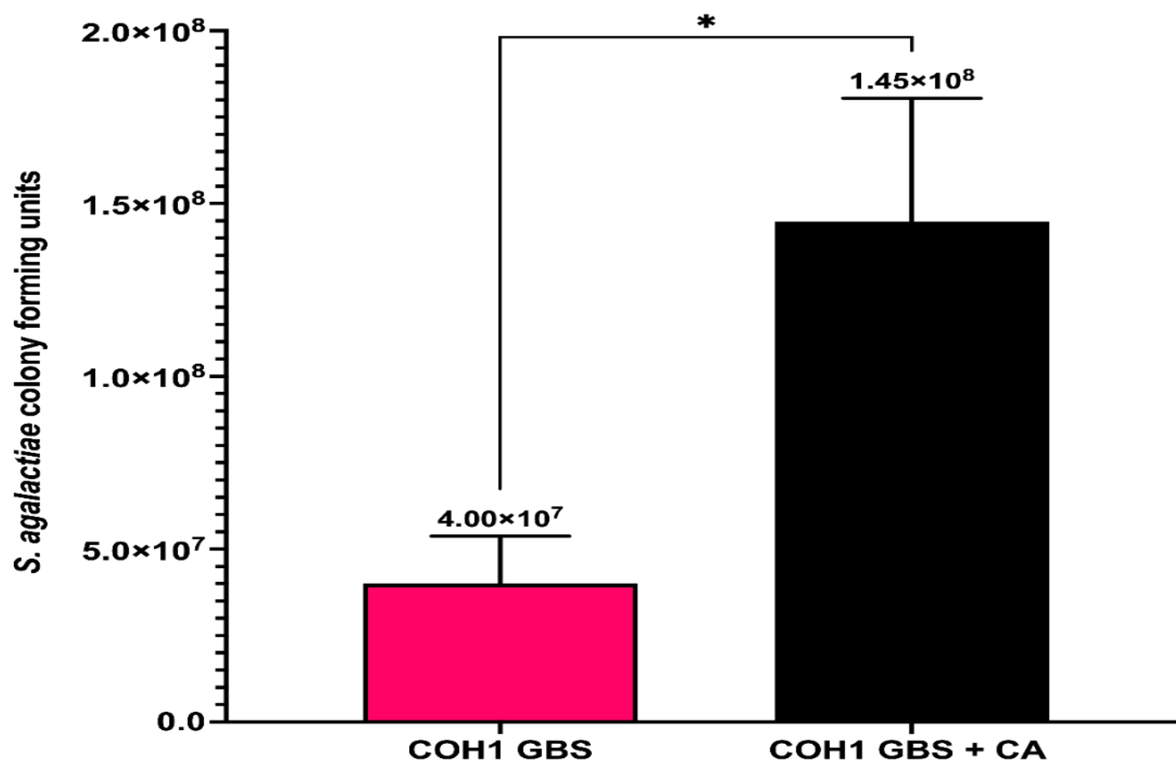


Figure B.1: GBS increase in growth when co-cultured with C. albicans is not serotype dependent. Colony growth of GBS COH1 (serotype III) following solo or co-culture with *C. albicans* at 24 hours post culture in nutrient poor media. Experiment was repeated 4 times and statistical significance was calculated using an unpaired two-tailed student's t-test *p<0.05

APPENDIX C

THE VIABLITY OF *C. ALBICANS* IN NUTRIENT LIMITED MEDIA IS NOT ALTERED BY THE PRESENCE OF GBS REGARDLESS OF *C. ALBICANS* CELL MORPHOLOGY

Previous work in this dissertation has indicated that the ability of *C. albicans* to enhance the cell viability of GBS following growth in nutrient limited media is not dependent on the morphology of C. albicans. This is a significant finding as previous research has shown some interactions between GBS and C. albicans are dependent on the ability of *C. albicans* to undergo the yeast-to-hyphal transition and adherence between GBS and hyphal filaments of *C. albicans*. What was unknown about the interaction of GBS and C. albicans in nutrient limited media was if the viability of C. albicans is altered when cultured with GBS in nutrient limited media. GBS was co-cultured with either yeast-locked *C. albicans* strain *NRG1^{OEX}-iRFP* or reference strain *Caf2 FR* and viable C. albicans cells were enumerated by counting individual *C. albicans* colonies on selective agar plates following the plating of serial dilutions from these growth cultures. Results showed that viable C. albicans cells from either strains were not significantly different when grown separately or with GBS, indicating that GBS does not alter the viability of *C. albicans* in nutrient-limited media regardless of the ability of the *C. albicans* strain to alter its morphology.

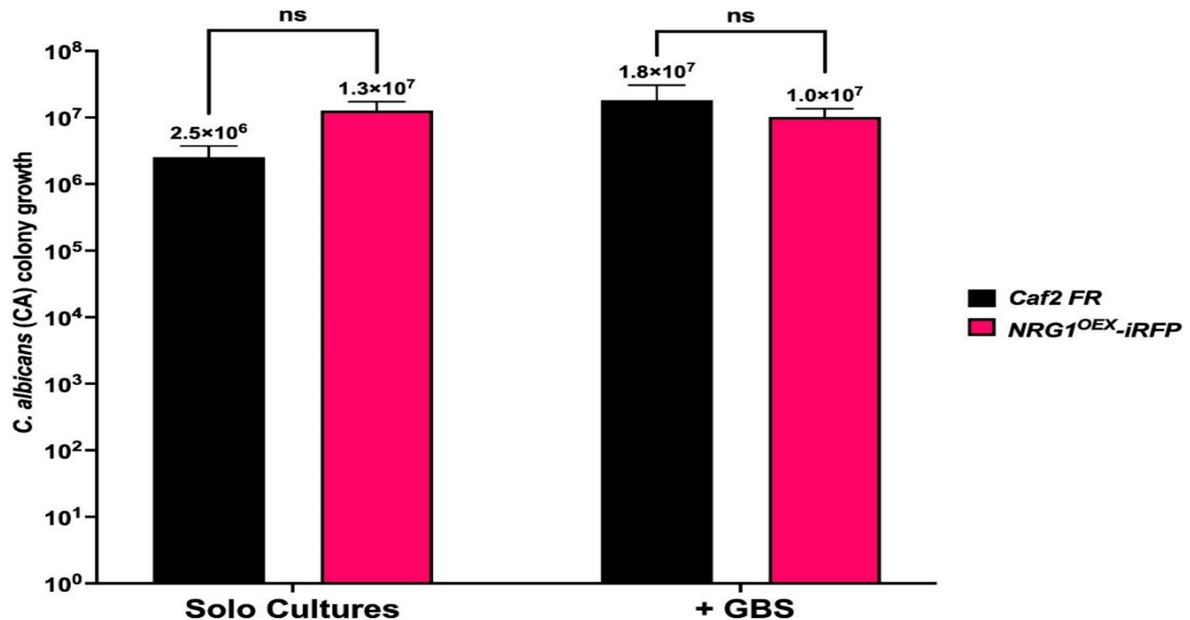


Figure C.1: Growth of *C. albicans* strains *Caf2 FR* and *NRG1^{OEX}*-iRFP 24 hours post culture in nutrient poor media in solo *C. albicans* cultures or co-cultures with GBS 515. Experiments were performed in triplicate and statistical significance was calculated using unpaired two-tailed student's t-test $^{*}p<0.05$.

APPENDIX D

THE VILUENCE OF GBS 515 IS NOT ALTERED WHEN STREPTOMYCIN SPONTANEOUS ANTIBIOTIC RESISTANCE IS INTRODUCED

To be able to perform bacterial burden assays following larval zebrafish infection as seen in section 2.2.4 of this dissertation, an antibiotic resistant GBS strain was created to be able to enumerate the bacterial burden of GBS in larval zebrafish following solo or co-infection of GBS with *C. albicans*. To cause spontaneous antibiotic resistance to the antibiotic streptomycin, GBS 515 was exposed to a large dose of streptomycin (250µg/mL) in its THY growth media, then plated on agar plates containing 250µg/mL streptomycin. An individual colony of GBS was selected from the streptomycin agar plate and grown again in THY media containing 250µg/mL to confirm permanent streptomycin resistance. The strain of GBS with the newly acquired streptomycin resistance was then confirmed to be GBS through 16S rRNA gene sequencing PCR for identification of GBS. Following the confirmation of the creation of the GBS strain resistant to streptomycin (GBS SR), survival assays involving infecting 2dpf zebrafish in the yolk sac with 100cfu of either GBS 515 WT or GBS 515 SR were performed, with survival of the fish monitored for every 24 hrs for a 72 hour timespan. The results from the survival assay determined that there were no significant virulence differences in the infections caused by GBS 515 WT or GBS 515 SR, which indicated that the GBS strain GBS 515 SR was suitable to use for bacterial burden assays.

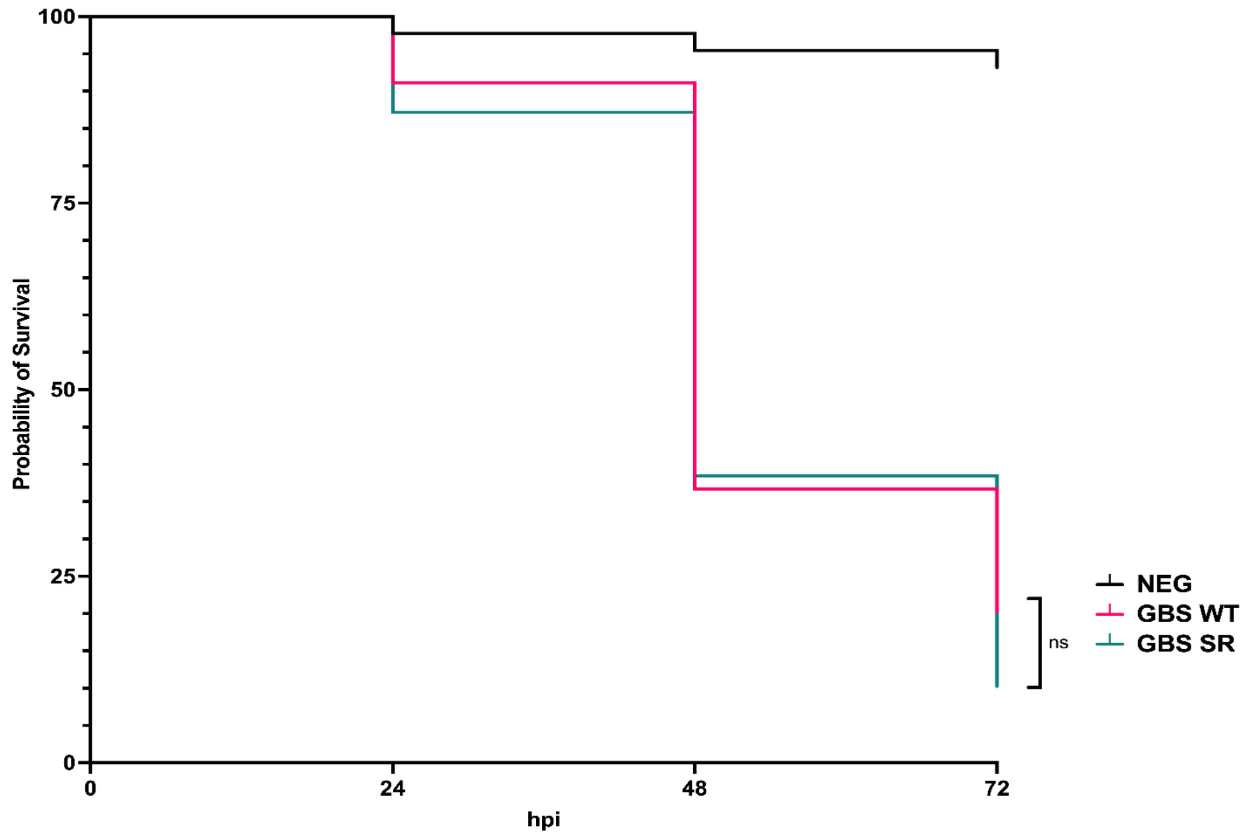


Figure D.1: Spontaneous antibiotic resistance to streptomycin does not affect virulence of GBS 515 Solo systemic infection was induced by injecting 2dpf larval zebrafish with either 40cfu of wild-type GBS 515 (GBS WT) or streptomycin resistant GBS 515 (GBS SR). Survival of zebrafish was monitored ever 24 hrs for 72hrs total. Experiments were performed in triplicate and statistical significance of Kaplan-Meier survival curves were calculated using a log rank (Mantel-Cox) test. $*p<0.05$

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