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The Impact of Lemongrass, Oregano, and Thyme Essential Oils on Candida albicans' Virulence Factors

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Abstract

The Impact of Lemongrass, Oregano, and Thyme Essential Oils on *Candida albicans'* Virulence Factors

by

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BS, Colorado State University, 1989

Dissertation Submitted in Partial Fulfillment

of the Requirements for the Degree of

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Abstract

Increased systemic infections and growing resistance of *Candida* species in immunosuppressed people have prompted research for additional treatment options. The purpose of this quantitative study was to investigate the potential of lemongrass, oregano, and thyme essential oils tested individually, combined, and combined with the antifungal agents fluconazole and caspofungin to kill *Candida albicans* isolates in a controlled laboratory setting. This study was grounded on the theoretical concepts of the epidemiologic triangle model. The experimental data collected were used to investigate risk factors related to age, gender, race, and comorbidities. Kill rates of lemongrass, oregano, and thyme essential oils individually and combined, kill rates of fluconazole, caspofungin, and the kill rates when the antifungals were each combined with the 3 essential oils were compared using 117 isolates recovered from bloodstream infections between January 2009 through August 1, 2017. The data collected were analyzed using 2-way repeated ANOVAS. According to study results, there were statistically significant increases in kill rates when the isolates were exposed to any of the combinations of essential oils tested. Using binomial and multinomial regression to analyze age, gender, race, and comorbidities resulted in the age group 25-34, kidney failure, and solid organ tumor cancer all being statistically significantly associated with an increased risk for *Candida albicans* bloodstream infections, and multiple organ failure negatively associated with the risk. Health care practitioners can use the results of this study to reduce the number of patients becoming infected with life-threatening yeast infections, which could reduce the costs associated with infections.

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Chapter 1: Introduction to the Study

Introduction

Candida infections are considered the second most numerous cause of fungal infections worldwide (Brown et al., 2012). Although most infections are superficial, invasive fungal infections, including those caused by *Candida*, are responsible for over an estimated 1.5 million infections annually worldwide (Brown et al., 2012). An accurate measurement of the burden of fungal disease is likely underestimated due to misdiagnosis and the lack of required reporting in the United States for all invasive fungal disease except for Coccidiodes, which causes Valley Fever (Brown et al., 2012). The Centers for Disease Control and Prevention (CDC, n.d.[2]) collects data on invasive candidiasis through the Emerging Infections Programs (EIP) from 10 state health departments; these states' local health departments; academic institutions; as well as other federal agencies, public health and clinical laboratories, and health care facilities. Increased numbers of successful organ transplants, as well as improvements in medical treatments for HIV/AIDS and cancers, have created an increasing number of patients who are immunosuppressed for extended lengths of time (Pellegrino, Onder, & Schmidt, n.d.). This rise in at-risk populations has caused an increase in organisms that are considered pathogenic, as well as the development of *Candida* isolates that are resistant to the limited available treatment options (Cleveland et al., 2012). Additional difficulties occur with being able to detect and treat these infections quickly as initial symptoms may be nonspecific for invasive *Candida* infections (Brown et al., 2012).

Background

Through the CDC's EIP program, data are also collected through active, population-based surveillance for candidemia in seven of the current EIP sites: Georgia, Maryland, Minnesota, New Mexico, New York, Oregon, and Tennessee (CDC, 2017). However, epidemiology studies on overall incidence of invasive *Candida* have been difficult to determine due to a lack of required reporting in other areas and the potential for misdiagnosis (Brown et al., 2012). Some scholars have evaluated the status of antifungal resistance epidemiology in haematological malignant patients (Alcazar-Fuoli & Mellado, 2014). Researchers have reported on the mechanisms of the virulence factors of *Candida* species and the ability to cause severe infections (Bliss et al., 2012). This knowledge added to the need to analyze the potential for *Candida* species to possess adaptive resistance mechanisms, the prevalence of these resistant organisms in certain patient populations, and the discrepancy that exists between in-vitro and in-vivo results due to how the host responds differently between colonizing *Candida* and invasive *Candida* (Lewis, Viale, & Kontoyiannis, 2012).

Studies on various essential oils and the effect on survival of yeast and bacteria have brought additional areas for further investigation. Groundwork for treating *Candida* with essential oils in-vivo and the chemical compounds that cause disruption or death to yeast cells have also been studied using essential oils extracted from oregano plants (Cleff et al., 2010). The inhibition of *Candida albicans* and *Aspergillus fumagatus* with thyme essential oil has also been studied. Thyme sterilized culture growth of both organisms (Bellete et al., 2012). Sajjad Ahmad Khan, Malik, and Ahmad (2012) used 21

essential oils against two quality control strains of *Candida albicans* alone and in conjunction with fluconazole and amphotericin B. All of these essential oils had various degrees of efficacy against strains, including the resistance strain. Sajjad Ahmad Khan et al. (2012), however, did not test any essential oils against patient recovered isolates. Rath and Mohapatra (2015) studied the effect of black cumin, curry leaf, Ajwain, and Betel leaf against *Candida albicans*, *Candida tropicalis*, *Candida glabrata*, and *Candida parapsilosis*. Rath and Mohapatra found that essential oils showed efficacy against the various strains of *Candida*, even after the oils were autoclaved. Although Rath and Mohapatra had multiple species of *Candida*, the essential oils that were tested would not be readily available in the United States. Rath and Mohapatra, however, tested the efficacy of essential oils after exposure to intense heat (from an autoclave). Different mechanisms of delivery of essential oils to *Candida* isolates were evaluated by testing the minimum inhibitory concentration in vapor and gas phase for various essential oils, and it was determined that Cymbopogon citratus (lemongrass) was highly effective in the vapor phase against *Candida* due to the ability for the essential oils to change surface structures on the yeast cell walls in a short time (Tyagi & Malik, 2010). The potential to diffuse essential oils into the air may provide a treatment option not only for a patient but also for the environment and, thus, disrupt the ability of the yeast to maintain contact with the patient through the host environment.

Age, gender, and an immunocompromised state have been shown to be associated with the risk of blood stream infections (Kaye et al., 2011; Yang et al., 2014). Scholars have not examined race as a risk factor or examined age and gender specifically to

Candida albicans blood stream infections. The opportunity to examine data and investigate *Candida albicans* isolated from blood stream infections from a large teaching hospital with patients who are immunocompromised for various reasons provided an area of study that has not been previously published and could add significantly to the body of knowledge. This study could provide a foundation for further research involving patients to improve outcomes and empower individuals in treatment options.

Problem Statement

Improvements in medical treatments for cancer, HIV/AIDS, as well as a rise in successful organ transplants have created an increased number of long-term immunosuppressed people (Pellegrino et al., n.d.). This immunosuppression has caused an increase in life-threatening fungal and yeast infections (CDC, 2011). There is a growing problem with ineffective treatment methods for life-threatening yeast infections in immunocompromised patients. Treatment options for *Candida* infections have become limited due to resistance of the organism to multiple antifungal medications (Brown et al., 2012). This problem has caused not only an increased cost for treatment, but also an elevated risk of mortality for patients infected with *Candida* (Zaoutis et al., 2005). Fewer traditional treatment options, along with increased mortality rates in both children and adults with *Candida* infections, necessitates different methods and new tactics to treat these life-threatening infections (Zaoutis et al., 2005).

Traditional treatments for *Candida* infections include antifungal medications, such as fluconazole and amphotericin B (CDC MASTER, n.d.). These drugs, however, are known to be toxic to the body, and resistance to these medications has continued to

rise (CDC, 2011). There are limited treatment options against *Candida* infections when the organism has become resistant to multiple medications (Brown et al., 2012). This problem has increased the risk of mortality in infected patients (Zaoutis et al., 2005). Essential oils may provide a synergistic treatment to improve antifungal medication activity against *Candida albicans* (Sajjad Ahmad Khan et al., 2012). In investigations of mechanisms that create antifungal activity in the chemical compounds found in essential oils naturally derived from plants, Khan et al. (2011) suggested that these compounds can cause lesions in the membrane of the yeast cell wall, alter the permeability of the plasma membrane, or cause oxidative stress to the organism. Despite results from scholars who indicated promising in-vitro results from the efficacy of essential oil treatment against *Candida* and its virulence properties, there are limited treatment options used for multidrug resistant *Candida* infections (Tyagi & Malik, 2010).

Purpose of the Study

The purpose of this study was to develop a system to examine the potential of three essential oils (lemongrass, oregano, and thyme) tested individually, combined, and then combined in conjunction with antifungal medications to kill *Candida albicans* isolates in a controlled laboratory setting. An experimental, quantitative method was used to evaluate the impact that the use of these oils, in combination with antifungal medications, had on the ability to minimize or prevent virulence factors, as well as the potential to kill *Candida albicans* isolates in laboratory in-vitro testing. *Candida albicans* isolates were collected from previously saved organisms that were recovered from blood cultures of patients hospitalized in a large teaching hospital. Information on

age, gender, race, and comorbidities were also collected from the isolates to investigate any significance related to *Candida albicans* infections and these covariates. In this study, I wished to develop a framework to examine the in-vitro effectiveness of three separate essential oils used separately and together in the presence of any antifungal medications an immunocompromised patient may be prescribed.

Research Questions and Hypotheses

1. RQ1: How does the use of lemongrass essential oil compare to standard antifungal medications for *Candida albicans* kill rates in laboratory testing?

   H_0^1: There is no significant difference in kill rates for the use of lemongrass essential oil compared to standard antifungal medications for *Candida albicans* in laboratory testing.

   H_a^1: There is a significant difference in kill rates for the use of lemongrass essential oil compared to standard antifungal medications for *Candida albicans* in laboratory testing.

2. RQ2: How does the use of oregano essential oil compare to standard antifungal medications for *Candida albicans* kill rates in laboratory testing?

   H_0^2: There is no significant difference in kill rates for the use of oregano essential oil compared to standard antifungal medications for *Candida albicans* in laboratory testing.

   H_a^2: There is a significant difference in kill rates for the use of oregano essential oil compared to standard antifungal medications for *Candida albicans* in laboratory testing.

3. RQ3: How does the use of thyme essential oil compare to standard antifungal medications for *Candida albicans* kill rates in laboratory testing?

   H_0^3: There is no significant difference in kill rates for the use of thyme essential oil compared to standard antifungal medications for *Candida albicans* in laboratory testing.

   H_a^3: There is a significant difference in kill rates for the use of thyme essential oil compared to standard antifungal medications for *Candida albicans* in laboratory testing.

4. RQ4: How does the combined use of lemongrass, oregano, and thyme essential oils compare to standard antifungal medications for *Candida albicans* kill rates in laboratory testing?

   H_0^4: There is no significant difference in kill rates for the combined use of lemongrass, oregano, and thyme essential oils compared to standard antifungal medications for *Candida albicans* in laboratory testing.

   H_a^4: There is a significant difference in kill rates for the combined use of lemongrass, oregano, and thyme essential oils compared to standard antifungal medications for *Candida albicans* in laboratory testing.

5. RQ5: How does the combined use of lemongrass, oregano, and thyme essential oils when used with standard antifungal treatments compare to standard antifungal medications for *Candida albicans* kill rates in laboratory testing?

   H_0^5: There is no significant difference in kill rates for the combined use of lemongrass, oregano, and thyme essential oils when used with standard antifungal

medications compared to standard antifungal medications for *Candida albicans* in laboratory testing.

H_a^5: There is a significant difference in kill rates for the combined use of lemongrass, oregano, and thyme essential oils when used with standard antifungal medications compared to standard antifungal medications for *Candida albicans* in laboratory testing.

6. RQ6: How does the combined use of lemongrass, oregano, and thyme essential oils compare to standard antifungal medications for disruption of the virulence factor of germ tube production of *Candida albicans* in laboratory testing?

H_0^6: There is no significant difference in the combined use of lemongrass, oregano, and thyme essential oils compared to standard antifungal medications for disruption of the virulence factor of germ tube production of *Candida albicans* in laboratory testing.

H_a^6: There is a significant difference in the combined use of lemongrass, oregano, and thyme essential oils compared to standard antifungal medications for disruption of the virulence factor of germ tube production of *Candida albicans* in laboratory testing.

7. RQ7: How do the covariates age, gender, race, or comorbidities contribute to the risk of *Candida albicans* bloodstream infections?

H_0^7: Age, gender, race, or comorbidities do not significantly contribute to an increased risk of *Candida albicans* bloodstream infections.

H_a^7: Age, gender, race, or comorbidities do significantly contribute to an increased risk of *Candida albicans* blood stream infections.

Theoretical Framework

The epidemiological triangle was used as the framework for the research. The epidemiological triangle provides a model that is based on the premise that disease does not occur randomly throughout a population; instead, certain members of this population may have a higher risk for a disease that does not randomly occur throughout the rest of the population (Soumpasis, Knapp, & Pitt, 2015). The triangle can also be used to help to identify increased risk factors for development of disease in these subpopulations (CDC, 2016). This model of causation is composed of a susceptible host, an external agent, and the environment in which the host and the agent are in contact with each other (Orr, 2005). Contact brings about the disease as the environment provides the means of transmission of the infectious agent into the susceptible host (CDC, 2016). In order to develop public health control measures of infectious diseases, all three components and how they interact need to be assessed (CDC, n.d.). Using the epidemiologic triangle as a framework for the research provided a foundation of an immunocompromised host, which can be placed into the susceptible host vertex, *Candida albicans*, which is placed at the agent vertex, and the environment which is at the final vertex of the triangle. In this study, I evaluated potential means to disrupt this interaction from causing disease by breaking one of the sides of the triangle. In addition, the relationship of these three components that enables disease to occur was also used for interpretation of the results from the study. For example, the potential to prevent virulence factors from forming

would disrupt the interaction of the agent to enter and cause disease in the host. Other areas of disruption could involve prevention of the agent from replicating and surviving in either the host or the environment. Although I focused on prevention of virulence factor development, as well as yeast cell inhibition and death, this framework also has future implications for disruption of the yeast in the environment (see Figures 1 and 2).

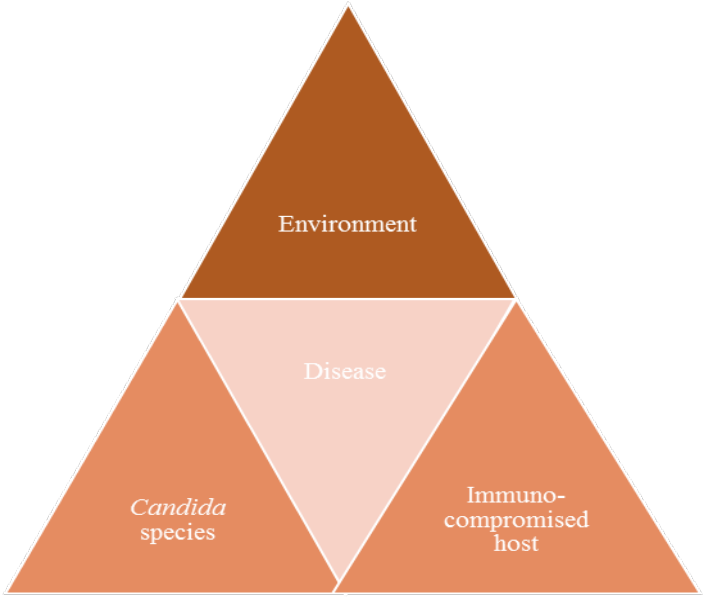


Figure 1. Epidemiological triangle.

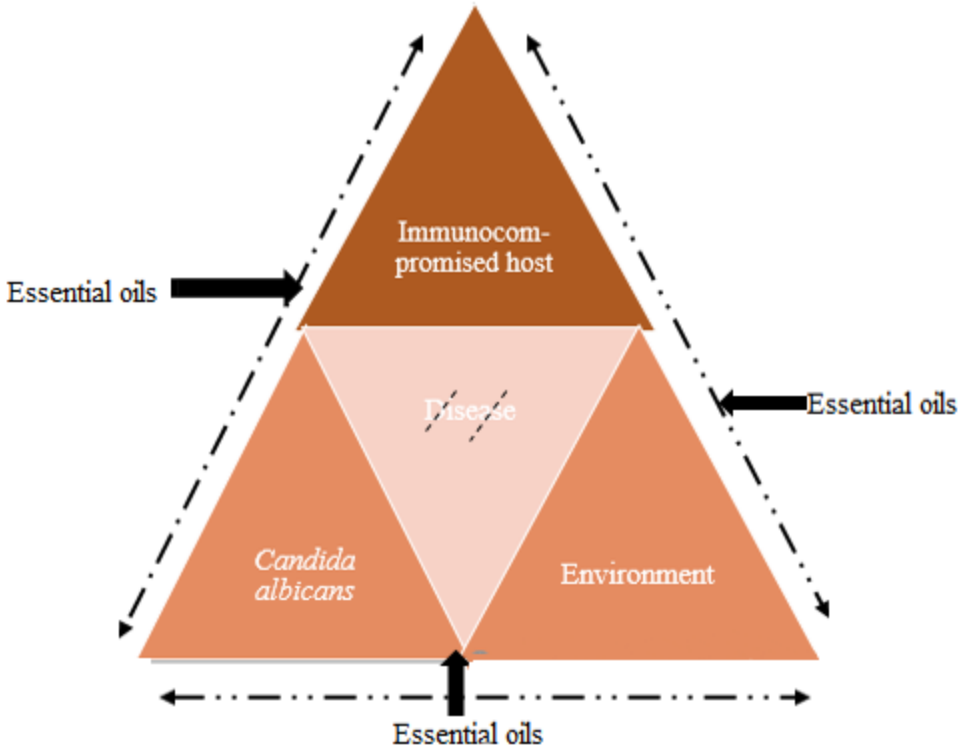


Figure 2. Disruption of epidemiological triangle.

Nature of the Study

The nature of this study was quantitative. An experimental study design was used to evaluate any impact that the use of lemongrass, oregano, and thyme essential oils individually had on *Candida albicans* kill rates in the laboratory, followed by the combined use of these essential oils compared to standard antifungal medications kill rates of *Candida albicans* in laboratory testing. Next, the combined use of lemongrass, oregano, and thyme essential oils, along with standard antifungal treatments, were compared to standard antifungal medications for *Candida albicans* kill rates in laboratory testing. Finally, the combined use of lemongrass, oregano, and thyme essential oils were

compared to standard antifungal medications for the ability to cause disruption of germ tube production of *Candida albicans* in laboratory testing. This design method provided a way to assure a high degree of validity with the ability to culture each *Candida* isolate before and after testing, as well as enabling the research to be duplicated (Frankfort-Nachmias & Nachmias, 2007).

Definitions

Chirality: Pertains to a molecule's characteristic mirror image, which was used to assess that the essential oil tested does not contain synthetic constituents (doTERRA CPTG Certified Pure Therapeutic Grade Quality Testing, 2016).

Cytotoxic effects: Causes harm to cells primarily through cell wall disruption, which can decrease the cell's functions or cause cell death (Ramage et al., 2012).

Comorbidities: Preexisting conditions or chronic diseases that are listed upon admission to the hospital.

Fournier transfer infrared spectroscopy: Used to identify structural components of compounds found in essential oils. This was used to determine the quality of the sample based upon previously recognized high-quality standards (doTERRA CPTG Certified Pure Therapeutic Grade Quality Testing, 2016).

Fungicidal: A compound that kills fungus including yeast (Bachmann et al., 2002).

Fungistatic: A compound that inhibits fungus but does not kill it. When the compound is removed, the fungus can resume growth activities (Bachmann et al., 2002).

Germ tube: A pseudo-hyphal extension that is used by *Candida albicans* to invade the host mucosal membrane (Bliss et al., 2012).

Haematological malignant patients: Patients with cancer involving the blood and lymphatic system (Alcazar-Fuoli & Mellado, 2014).

Invasive fungal disease: A fungal infection that has spread to one or more systems or organs in the host (Enoch, Ludlam, & Brown, 2006).

Minimum inhibitory concentration: The lowest concentration of an antimicrobial that will inhibit visible growth (Clinical and Laboratory Standards Institute [CLSI], 2015).

Nitric oxide: A free radical found in the body that is used for multiple cellular processes (Leyva-Lopez, Nair, Bank, Cisneros-Zevallos, & Heredia, 2016).

Prophylaxis: An antifungal medication taken to prevent an infection (Sanguinetti, Posteraro, & Lass-Florl, 2015).

Reactive oxygen species: A byproduct from cellular oxygen metabolism that can cause severe cellular damage in high amounts (Leyva-Lopez et al., 2016).

Tissue conditioner: A temporary lining that is placed between dentures and the gum to promote healing of the gum tissue (Amornvit, Choonharuangdej, & Srithavaj, 2014).

Assumptions

Essential oil components are effective against *Candida albicans* isolates (Bellete et al., 2012; Khan et al., 2011; Sajjad Ahmad Khan et al., 2012). Essential oil components effective against yeast isolates consistently produce similar results on

Candida albicans strains isolated from patients as well as established *Candida albicans* quality control strains (Bellete et al., 2012; Kalemba & Kunicka, 2003; Khan et al., 2011; Korenblum et al., 2013; Rath & Mohapatra, 2015; Shah et al., 2011). In this study I assumed that the samples taken from a large teaching hospital also provided a variety of patient demographics due to the diversity of adult patient population seen at the hospital. The methods used to evaluate sensitivity of *Candida albicans* isolates to the essential oils and antifungals were assumed to be accurate based upon established scientific methods used to determine antimicrobial susceptibility ranges for various organisms including *Candida albicans* (CLSI, 2015). Time to kill rates were also assumed to be accurate based upon previously established laboratory standards and would be reproducible upon repeat testing (Woolfrey, Lally, & Tait, 1986).

Scope and Delimitations

I used patient demographics and isolates recovered from the patient population of a large teaching hospital. The purpose of this research was to not only evaluate the effect of lemongrass, oregano, and thyme essential oils against *Candida albicans* but to also investigate any potential risk factors of people who acquire *Candida albicans* bloodstream infections during their patient stay at the hospital. Other variables and conditions that could further impact risk for *Candida albicans* bloodstream infections, such as potential ubiquitous nature of *Candida albicans* in this hospital as opposed to other hospital locations and risk factors based upon population ethnicities that are not generally seen in this geographic location, were outside the scope of this research. The impact of long-term prophylaxis of antifungal medications on risk factors was not

assessed. Isolates and patient information spanned 7 years in order to obtain an adequate sample, and there was potential that the risk factors as well as the characteristics of the *Candida albicans* isolates may vary over this time period and may, therefore, not provide accurate information of current risk factors.

Limitations

Limitations for this study included potential changes in treatment protocols for the time-period examined, the ability to grow the organisms that have been previously frozen, and using a local patient population and not a nationwide population. Collecting data from a large teaching hospital's patient population may also limit the validity of overall effectiveness of essential oil treatment in other patient populations. Other limitations included not being able to use actual patients' interactions with essential oils in conjunction with the antifungal treatments. Only using *Candida albicans* strains isolated from blood stream infections instead of isolates from less life-threatening *Candida albicans* infections and not investigating other emerging yeast pathogens were also known limitations for this study.

Significance of the Study

To address these gaps in knowledge, I evaluated a large number of clinically isolated *Candida albicans* with various susceptibility patterns. The impact that the essential oils have on *Candida* isolates was assessed to determine whether essential oil treatments could be used to improve outcomes of patients infected with *Candida*. This research is important for people who are immunocompromised and living with chronic infections of *Candida* that can turn life-threatening or for those who have been on long-

term prophylactic treatment with antifungal medications who are at risk of developing infections with multidrug resistant *Candida* species (Wanke, Dos Santos Lazera, & Nucci, 2000). The findings from this research could provide positive social change by reducing the number of patients becoming infected with life-threatening yeast infections, which not only would reduce mortality but would also reduce the costs associated with serious infections. There is the possibility for the results of this research to provide alternative options for treatment, to provide methods for improving treatment, and for future research to enable patients to feel empowered in treatment options. This study also has potential to become a foundation for other studies including in-vivo treatment with immunocompromised adults as well as within other populations including pediatrics, where treatment options due to patient age are even more limited (Bliss et al., 2012).

Summary and Transition

This research dissertation has five sections. In Chapter 1, I presented an overview of the problem and why the research was performed. Current limited treatment options have provided opportunities to seek additional methods to improve treatment capabilities. Few studies have been done using widely available essential oils and commonly used antifungal medications with a large number of clinically significant isolates of *Candida albicans*. This study provided an opportunity to examine experimental attributes as well as investigating covariates as risk factors for *Candida albicans* infections.

Chapter 2 provides an in-depth literature review on attributes of *Candida albicans*, alternative treatments, and covariates associated with risk of infection. This chapter includes an overview; theoretical framework; risk factors associated with blood

stream infections; immunocompromised patients and prophylaxis; virulence and resistance mechanisms of *Candida albicans*; alternative treatment options; host defense mechanisms; essential oil treatment testing; chemical mechanisms of actions for lemongrass, oregano, and thyme; combining use of essential oils and antimicrobials; host cell toxicity to essential oils; and methodology. In Chapter 3, I describe the design of the study, methodologies used, the study population, testing procedures, and variables tested. In Chapter 4, I discuss the results of the research that was performed including data collection, treatment methods, analyses of statistical test, and a summary of these results. In Chapter 5, I finish with the conclusion of these results, limitations of the study, recommendations, and implications for social change.

Chapter 2: Literature Review

Introduction

The CDC (2013) reported that *Candida* species is the fourth leading cause of health-care-associated bloodstream infections. Hospital life-threatening yeast infections in immunocompromised patients have become more difficult to treat due to resistance to multiple antifungal medications (Brown et al., 2012). This growing problem of finding effective treatment options has not only caused the cost for treatment to rise but has also contributed to rising mortality rates in both adults and children. Traditional treatment for *Candida* infections include fluconazole and amphotericin B (CDC MASTER,n.d.); however, although these drugs are known to be toxic to the body, limited available antifungal medications have left few other options (CDC, 2011).

Researchers have proposed that chemical compounds found in essential oils naturally derived from plants can inhibit yeast and fungus and may also provide a synergistic treatment to improve antifungal medication activity against *Candida albicans* in-vitro (Sajjad Ahmad Khan et al., 2012). Although there has been promising in-vitro results indicating efficacy of essential oil treatment options for multidrug resistant *Candida* infections, there are limited options used for treatment of these dangerous infections (Tyagi & Malik, 2010).

In this chapter, I review the literature related to *Candida* virulence factors, the disruption of virulence properties using antifungal medications, drug resistance mechanisms of *Candida albicans*, and outcomes for *Candida* species when exposed to various essential oils. This review was used to create a foundation for discerning areas of

viable treatment options for *Candida albicans* infections based upon published, evidence-based, scientific research, as well as any risk factors of *Candida* bloodstream infections that have been shown to be related to age, gender, race, and comorbidities.

Literature Search Strategy

Articles were chosen using the CINAHL and MEDLINE simultaneous database search. Inclusion parameters included peer-reviewed articles from 2000 to the present, full-text available, and written in English. Exclusions included abstract only, articles not peer reviewed, articles in another language, and articles that had limited number of samples or patients tested (fewer than five). Although the initial intent was to limit the research articles to the past 5 years, some of the primary investigations and research were published prior to 2011. Initial keywords used were *Candida* and *essential oils*. This search brought results for 489 articles. There were many articles from research performed outside of the United States and with oils other than the three used for this research project. A review of the first 10 articles provided four articles for review. Another search with the keywords *Candida, thyme*, and *essential oil* yielded 22 articles, but when the date was adjusted to the last 5 years, there were only two articles, and neither were selected. The next search for *Candida* and *oregano* from the last 5 years resulted in one article that related to the research topic. The additional search for *Candida* and *lemongrass* also revealed one article that was relevant to the research. Although multiple articles with *Candida* and essential oils have been published in the past 5 years and even more in the past 10 years, there were few articles of research published using any of the three essential oils used in this study. Essential oils tested

against *Candida* are common; however, as traditional treatment methods become less viable, alternative solutions could become more acceptable for common treatment practice. The remaining articles were found while working in a large teaching hospital and in the reference sections of other articles.

Articles were chosen based upon topics that included inhibition of *Candida* virulence factors, research on how antifungal medications target yeast cell walls, investigations of essential oils and their antifungal properties, synergy of essential oils and antimicrobials, and interaction of *Candida* virulence factors with the host response. Additional articles relating to potential risk factors for *Candida* bloodstream infections in adults were also reviewed. Articles were excluded for review if the organisms tested were only quality control organisms and did not include patient acquired organisms, if the methods section was too limited to determine testing validity, or if the articles were not peer reviewed.

Theoretical Framework

Theoretical frameworks included in this chapter include theories such as the immuno-epidemiological theory of host interaction with antimicrobial medicine and infectious microorganisms. Adaptive genetic theory was also used in studies where the researchers were investigating mutations and the ability for the organism to respond to stressors to promote organism survival (Ene et al., 2012). In addition, researchers have developed multiple theories that provide a framework for experimental studies, such as the germ theory from Pasteur that is based upon the knowledge that microorganisms cause infectious diseases; Koch built on the germ theory, but further elaborated to

identify the correct organism that causes a disease, a set of criteria must be met in order to conclude that the organism causes the disease; and the metabolic control theory where a set of genes in a linear processing chain inside the microorganism can be used to predict epistasis in antibiotic resistance (MacLean, 2010).

Although previous researchers may have different frameworks for the foundation of the study, these theories can all be related to the epidemiologic triangle. The epidemiologic triangle is a model that is based on the understanding that disease does not occur randomly throughout a population; rather, certain members of this population may have an increased risk for a disease that does not just randomly occur throughout the population (Soumpasis et al., 2015). The triangle can also be useful to identify increased risk factors for development of diseases in these subpopulations (CDC, n.d.). This type of causation model is composed of a susceptible host, an external agent, and the environment in which the host and the agent are in contact with each other (Orr, 2005). Contact within the host's environment provides the mode of transmission of the infectious agent into the susceptible host (CDC, n.d.). Assessment of this interaction is necessary to develop public health control measures for infectious diseases (CDC, n.d.).

This triangle of interaction has been used to research modes of transmission of Clostridium difficile that traditionally had been found only in patients who had been hospitalized but is now commonly found in community settings. The way to interrupt this disease process is to decrease the likelihood of the organism from being transferred into new environments from infected or carrier hosts (Gupta & Khanna, 2014). This type of transmission interruption could also be used to study ways to prevent disease

formation from *Candida albicans* in susceptible hosts. Interventions that interrupt the environmental factors associated with the risks of otitis media, such as nutrition, air quality, and housing could decrease the incidence of otitis media in children in the Northern areas of Canada (Orr, 2005). Interventions that prevent host access of *Candida albicans* by creating an environment that does not support growth or development of virulence factors would also disrupt the potential for disease. Soumpasis et al. (2015) used the epidemiologic triangle to evaluate the use of extended wear contact lenses, the host's hygiene, and the risks associated with the development of opportunistic corneal infections with *Pseudomonas aeruginosa*. Soumpasis et al. reported that there is significant overlap between the three components of the triangle, and intervention targets should be dependent upon whether the focus is epidemiologic investigations, risk assessments, or therapeutic treatments. From an epidemiological perspective, the focus of an epidemiologic triangle model is to target disease transmission. This includes factors in the host that increase susceptibility to disease, primary factors that promote the organism to cause disease, and proposed measures to control or reduce the incidence of disease (Soumpasis et al., 2015).

Infections with *Candida* are thought to be caused by the organisms that naturally reside on or in the host. Prevention of the formation of virulence factors in *Candida albicans* could reduce the risk of acquiring an infection from residing organisms (CDC, 2013). Other areas thought to act as a reservoir for *Candida* are fomites and hands of health care workers (Hota, 2004). *Candida albicans* has been shown to survive on metal and glass surfaces for 3 days and on cotton and cotton-polyester blend fabrics for at least

14 days; on human finger pads, 20% of the viable organisms remained detectable an hour after initial inoculation (Traore, Springthorpe, & Sattar, 2002). The environment for *Candida albicans* can be on or in the host itself or merely on the inanimate environments that the host has contact with regularly. Breaking the transference of the organism by disrupting the ability to survive in the host environment could disrupt the transmission of disease and prevent infections. The way to interrupt this disease process is to decrease the likelihood of the organism from being transferred into new environments from infected or carrier hosts (Gupta & Khanna, 2014). This type of transmission interruption could be used to study ways to prevent disease formation from *Candida albicans* in susceptible hosts. Interventions that prevent host access of *Candida albicans* by creating an environment that does not support growth or development of virulence factors would also disrupt the potential for disease.

Using a framework of the epidemiologic triangle for the research provided a foundation of an immunocompromised host, which can be placed into the susceptible host vertex, *Candida albicans*, which is placed at the agent vertex, and the environment which is at the third vertex of the triangle. In this study, I investigated potential means to disrupt the triangular interaction by breaking one of the sides of the triangle that would prevent the disease formation. In addition, the component relationships that enables disease to occur was also used for interpretation of the results from the study. This potential to inhibit virulence factors could prevent the agent from entering and causing disease in the host. Another potential place for disruption could involve prevention of the agent from replicating and surviving in either the host or the environment. I studied ways

to decrease *Candida albicans's* ability to cause disease by examining the prevention of virulence factor development and yeast cell inhibition or death as well as risk associated with the covariates age, gender, race, and comorbidities.

Risk Factors Associated with Bloodstream Infections

Yang et al. (2014) performed a retrospective analysis of the epidemiology of 121 *Candida* bloodstream infections and determined that advanced age and neutropenia were independent risk factors for mortality within 28 days of initial occurrence of *Candida* bloodstream infections. Over 95% of all patients with hospital acquired *Candida* sepsis between 2008 and 2012 had at least one comorbidity, and 56% of these patients had two or more comorbidities (Yang et al., 2014). Although internal medicine wards had higher 28-day mortality rates than surgical wards or intensive care units (ICU), Yang et al. did not further investigate this angle for infection but did comment that there was poor compliance to antifungal therapy in some of the wards. Yang et al. also noted that appropriate antifungal treatment reduced the likelihood of patient death within the first 28 days of infection. Yang et al.'s research sample size and institution were comparable to this study's sample size and type of hospital; however, the differences that may occur in United States hospitals have not been studied. Inclusion of patient covariates such as age, gender, race, and comorbidities could add knowledge to the body of literature about risk factors for *Candida albicans* bloodstream infections.

Kaye et al. (2011) examined 830 patients with bloodstream infections and compared them to 830 patients without bloodstream infections in the age group 69 to79 years; the predictors of bloodstream infections by any organism were male gender,

obesity, and a central venous catheter present upon admission. Other predictors included gastronomy, surgery, and previous hospital exposure (Kaye et al., 2011). The predominant organism isolated through this study was Methicillin-resistant *Staphylococcus aureus* (MRSA), and additional interventions were suggested that would target elderly males (Kaye et al., 2011). Although this research was used for overall bloodstream infections, not just *Candida albicans* infections, it can be used as a foundation to investigate whether the risk for *Candida* bloodstream infections are similar to other types of bloodstream infection risks.

Shigemura et al. (2014) performed a retrospective study of 110 *Candida* bloodstream infections over a 5-year period. Shigemura et al. determined that cardiovascular disease and having a recent surgery increased the risk of *Candida albicans* infections; however, chemotherapy and cancer increased the risk of *Candida non-albicans* species. In this study the majority (71%) of patients with *Candida albicans* bloodstream infections were male, but 62% of the septic patients were under the age of 70 (Shigemura et al., 2014). Hu et al. (2014) examined data of 294 patients and evaluated the potential risk factors for catheter-related *Candida* bloodstream infections (CRCBSI). The risk factors were determined to be age and low body weight (Hu et al., 2014). However, in this study, *Candida parapsilosis* caused more CRCBSI and *Candida albicans* caused more non-CRCBSI (Hu et al., 2014). Males accounted for the majority of these bloodstream infections with 75% of the CRCBSI and 68% of the non-CRCBSI (Hu et al., 2014). Although the central venous catheter (CVC) placement rate was

highest (89.7%) in patients with CRCBSI, patients with non-CRCBSI had a CVC placement rate of 81.9% (Hu et al., 2014).

Central-venous catheter placement was also found in 93% of patients with *Candida* bloodstream infections in a prospective study of 157 patients (Rajendran et al., 2016). Age was associated with increased mortality, and 47% of the 30-day mortalities were in patients with *Candida albicans* bloodstream infections (Rajendran et al., 2016). There was a higher mortality rate associated with *Candida albicans* than with *Candida glabrata* infections (Rajendran et al., 2016). In addition, there was a higher mortality rate for patients whose *Candida albicans* isolates were shown in laboratory testing to be high biofilm formers than isolates that were shown to be low biofilm formers (Rajendran et al., 2016). Removal of a CVC after the detection of a *Candida* bloodstream infection improved the clinical outcome of the patient (Rajendran et al., 2016). In a 6-month retrospective study of bloodstream infections from 2009 of 468 positive blood cultures, Chander, Singla, Sidhu, and Gombar (2013) showed that 5.79% were *Candida* isolates; however, in this study, the most common *Candida* recovered was *Candida tropicalis*. *Candida* bloodstream infections were associated with broad spectrum antibiotic use 91.6% of the time and associated with CVCs 83% of the time. These infections were nosocomial and occurred within 48 hours of admission (Chander et al., 2013). Almost 89% of candidemia were recovered from patients in the ICU (Chander et al., 2013). ICU patients usually have failed host defenses due to underlying disease or an immunocompromised state (Chander et al., 2013). Although these epidemiological scholars indicated a higher risk in males, other demographics and comorbidity risk

factors were not as consistent throughout the studies. This may be due to differences of predominant infections in various countries; however, additional studies used to examine risk factors for adult patients seen in the United States to provide additional means for reducing these factors in the high-risk patients. This type of study may provide insight on protection measures that could also be used in immunocompromised patients.

Immunocompromised Patients, Infections, and Prophylactic Treatments

Immuno-competent hosts can respond to the invasive hyphal and psuedohyphal forms of *Candida* through pathogen recognition receptors (Lewis et al., 2012). These receptors initiate adaptive immunity in the host that starts the inflammatory response to contain and destroy the invading organisms (Lewis et al., 2012). However, when an immunocompromised host becomes infected with *Candida*, the ability of the host's immune mechanisms to respond is diminished, and the potential for serious invasive infections increases (Lewis et al., 2012). The increase of at-risk populations with life-threatening fungal infections has also caused an increase in the fungus species that are considered pathogenic. The most common invasive fungal diseases are caused by *Aspergillus fumigatus* and *Candida albicans* (Alcazar-Fuoli & Mellado, 2014). Complications to successful treatment include the difficulty in correctly identifying a fungal infection, as well as the increasing resistance of these organisms to antifungal medications (Alcazar-Fuoli & Mellado, 2014). Although these types of infections can be difficult to manage due to the extended time it can take for antifungal medications to inhibit or kill the organism, there have been improvements in treatment methods and protocols (Alcazar-Fuoli & Mellado, 2014). However, acquired drug resistance in

Candida species is complex, and additional information on how the immune system responds to these resistant organisms may provide improved understanding on the interactions of virulence factors and drug resistance (Lewis et al., 2012). Providing alternative options that do not need host immune responses intact or treatments that do not depend on the same pathways into the yeast cells to disrupt virulence or cause cell death could enable better outcomes in high risk patients who are immunocompromised. Developing additional treatment options could minimize the impact of virulence and drug resistance in yeast cells.

Virulence and Resistance

Severity of *Candida* infections is correlated with higher virulence factors. Virulence factors in microorganisms can trigger immune responses in the host (Williams et al., 2013). When *Candida albicans* did not produce virulence factors, infections were generally less severe and less likely to be invasive (Bliss et al., 2012). Interruption of these virulence factors could improve patient outcomes (Bliss et al., 2012). Virulence factors include germ tube or pseudo-hyphae production that helps the organism to invade the mucosal lining and establish the organism to withstand competition from normal flora inhabiting the mucosal membrane (Bliss et al., 2012). This type of filamentous growth can be reversed back to unicellular forms based upon stress from the host environment, as well as the pH of the site of infection (Calderone & Fonzi, 2001). Other virulence factors that increase pathogenicity of *Candida albicans* include biomolecules known as adhesins that are found on the yeast cells wall that promotes binding to the host cells (Calderone & Fonzi, 2001). *Candida albicans* can also produce phosolipases and secreted aspartyl

proteases, which are enzymes that are needed for invasive disease development (Calderone & Fonzi, 2001). These types of virulence factors can increase mortality rates by making the infectious process more severe and increasing the yeast cell ability to evade host responses (Bliss et al., 2012).

Another key component for virulence is the production of biofilms. Biofilms are communities of organisms that have adhered to a biological or inert surface and formed an extra-cellular matrix that hold the multi-cellular community together (Lopez, Vlamakis, & Kolter, 2010). *Candida albicans* can be found in biofilms both as pseudo-hyphal and hyphal forms; this combination of forms is thought to contribute to the development of *Candida albicans* biofilms as well as conferring increased resistance to antifungal medications (Watamoto et al., 2009). *Candida albicans* biofilms have been shown to be up to 500 times more resistant to the polyene class of antifungals (such as amphotericin B) and the echinocandin antifungal drug, caspofungin, than high-density growth of planktonic (free floating, single cell type) cultures (Watamoto et al., 2009). This propensity for *Candida albicans* to produce biofilms increases the time that antifungal medications need to be taken and increases the chance that some of the organisms in the biofilm can develop resistance to the treatment and thus continue to replicate and survive in the host causing additional stress to the host systems (Watamoto et al., 2009). Antifungal medication classes have different methods of inhibition and actions to destroy yeast cells. *Candida albicans* survival mechanisms can inhibit these functions of the different antifungal drug mechanisms. Exploring components to add to the treatment of *Candida* infections could lower drug resistance and reduce treatment

failures through prevention of the organisms' resistance mechanisms. The known modes of action of the classes of antifungal medications are discussed below.

Amphotericin B has been shown to interact with the ergosterols in the yeast cell wall producing tiny channels that cause the potassium and magnesium to leak from the inside of the cell which then promotes the loss of the proton gradient of the cell wall (Watamoto et al., 2009). In addition, the disruption of the ergosterol production prohibits the organism from developing hyphal forms thus reducing its virulence capabilities (Lewis et al., 2012). *Candida albicans* biofilm researchers have suggested that the mechanism of maintaining membrane fluidity in mature biofilms relies less on ergosterol than younger biofilms and planktonic cultures which makes amphotericin B as well as the azole class of antifungal medications less effective for treatment of organisms from mature biofilms (Mukherjee, Chandra, Kuhn, & Ghannoum, 2003). This type of resistance mechanism that changes the permeability of the cell membrane can reduce the ability for amphotericin B and fluconazole to enter the *Candida* cells thus causing treatment failure (Mukherjee et al., 2003).

The echinocandin class of antifungal medications, including caspofungin, are fungicidal and inhibit the synthesis of a primary cell wall structural component, 1,3-β-D-glucan (Bachmann et al., 2002). Caspofungin has been shown to reduce the formation of *Candida albicans* biofilms as well as causing mutations and decreasing hyphal formations in established biofilms (Bachmann et al., 2002). However, researchers from a multicenter surveillance program have indicated that recent exposure to caspofungin in patients increased the likelihood of being infected with a *Candida* species with a higher

minimum inhibitory concentration (MIC) to caspofungin in laboratory testing including isolates of *Candida albicans* and *Candida tropicalis* which both generally have lower MIC levels for caspofungin (Lotholary et al., 2011). This increasing resistance to caspofungin, while still rare in *Candida albicans*, has caused researchers to focus on mechanisms in the organism that creates this resistance (Sanguinetti et al., 2015).

The azole class of antifungal drugs (which includes fluconazole) inhibit mycelial growth by interrupting ergosterol biosynthesis which is a major component in the production of the plasma membrane of the cell (Ha & White, 1999). Thus, the organism is more sensitive to azoles during the mycelial phase than the yeast phase due to the increased need of plasma membrane for hyphal growth during the mycelial phase (Ha & White, 1999). Azole resistance in *Candida albicans* isolates can be caused by several mechanisms with the primary cause of resistance being contributed to drug efflux pump. Overexpression with other mechanisms such as altering the plasma membrane, changes to the synthesis of ergosterol, and drug uptake all possibly contribute to the decrease in susceptibilities of other antifungal medications (Cernicka & Subik, 2006). Acquired amphotericin B resistance is thought to be caused by alterations in the production of ergosterol in the plasma membrane and by the organism's ability to produce catalases which minimize oxidative damage caused by the antifungal medication (Lewis et al., 2012). Widespread prophylactic use of fluconazole has contributed to changes in the epidemiology of *Candida* infections invoking the selection of increasingly more resistant strains causing infections (Sanguinetti et al., 2015). These types of acquired resistance can occur through gene mutations and altered gene expression (Sanguinetti et al., 2015).

Candida species resistance to echinocandins is generally associated with gene mutations of the 1,3-β-D-glucan synthase complex (Lewis et al., 2012). However, the ability of echinocandins to expose β-glucans hidden underneath mannoproteins can still induce an immune response which during treatment, may prevent the emergence of subpopulations that acquire resistance (Lewis et al., 2012). However, exposure to higher concentrations of echinocandins than the MIC level can cause *Candida albicans* to increase production of chitin and glucan which can cause an increase in tolerance to echinocandins which in turn could also inhibit the host's immune response (Lewis et al., 2012). Kurtz et al., had previously concluded that these mutant strains of *Candida albicans* that are resistant to echinocandins are however, less virulent due to impairment of germ tube production (1996). In addition, virulence can also be influenced by the utilization of different carbon sources by *Candida albicans* (Ene et al., 2012).

Alternate carbon sources such as lactic acid can affect the cell wall synthesis, hydrophobicity, virulence, and resistance to antifungal treatments (Ene et al., 2012). Lactic acid can be found in various niches in the human body including the gastrointestinal and urogenital tracts through the body's metabolic activity on ingested food and produced by bacteria commonly found in these areas (Ueno et al., 2011). In laboratory testing, *Candida albicans* isolates grown on lactate media had a thinner cell wall with a marked decrease of chitin and β-glucan when compared to isolates grown on a glucose enriched media (Ene et al., 2012). In addition, the lactate grown cell wall had a two-fold increase in porosity and showed a higher propensity to be hydrophobic than the glucose grown cells (Ene et al., 2012). The lactate grown *Candida albicans* also

exhibited a stronger adhesion to plastics than the isolates grown in the presence of glucose (Ene et al., 2012). This ability to adhere is a virulence trait that has a major role not only in biofilm production on medical devices but also contributes to tissue colonization (Calderone & Fonzi, 2001). In addition, lactate grown cells were more resistant to high salt concentrations and the cell walls were more rigid indicating an increased ability for the cells to penetrate the epithelial cells and establish pathogenic residency in the host (Ene et al., 2012).

With the reduction of β-glucan and chitin in the thinner cell wall, lactate grown *Candida albicans* were more resistant to caspofungin and amphotericin B (Ene et al., 2012). Cells grown with glucose were more sensitive to amphotericin B and had less tolerance to osmotic stress compared to lactate grown cells (Ene et al., 2012). Virulence factors can therefore also be affected by the carbon source (such as lactate or glucose) that is used by *Candida albicans*. The carbon utilization pathway has been shown in mouse models to be dependent upon the site of infection and which carbon source is in higher abundance (Ene et al., 2012). The immune system in an immunocompetent host contains additional antimicrobial fighting agents that can still respond to the invasion regardless of the location of the infection (Mehra et al., 2013). Host defenses cannot respond adequately to infections when the immune system has been inhibited. Healthy host defenses and integrative treatment options will be discussed in the next section.

Alternative Treatment Options and Host Defenses

Bacteria, fungus, and viruses can be directly killed through a component of the innate immunity known as antimicrobial peptides (AMPs) which attacks the organisms

through immunomodulation or inhibiting virulence factors (Mehra et al., 2013). AMPs can also synergistically interact with intact mucosal epithelial cells to prevent pathogenic invasions (Mehra et al., 2013). The antifungal peptides have different microbial targets than antifungal medications which has caused investigators to study laboratory production of these AMPs as an alternative method for treating fungal infections (Mehra et al., 2013). Topical treatments however appear to hold the most promising application, not systemic treatments therefore, other treatment options for systemic infections are still needed (Mehra et al., 2013). Other researchers have targeted the potential use of defensins which are a specific group of AMPs found in a large majority of eukaryotic cell types (Silva, Goncalves, & Santos, 2014). Defensins have antimicrobial properties but also can affect the functions of the immune system to protect the host from invading pathogens (Silva et al., 2014). Defensins can attack the microbial cell wall and disrupt the activation of virulence factors (Silva et al., 2014). While defensins can efficiently react to fungus and yeast, the serum half-life of these peptides is low and the treatment for invasive infections may also be limited (Silva et al., 2014). In an immunocompromised patient, the body cannot produce enough AMPs and the technology to increase the ability for the defensins to be viable for treatment in yeast systemic infections remains elusive therefore other options still need to be investigated. Even in immunocompromised patients however, the initial immune response starts with the epithelial cells.

Host epithelial cells respond differently to commensal *Candida* compared to infectious *Candida* (Williams et al., 2013). Mucosal epithelial cells comprise a first line

of defense against microorganisms and will respond differently to an organism based upon its production of virulence factors (Williams et al., 2013). Organisms will produce these virulence factors as a method of survival (Williams et al., 2013). Virulence factors trigger the host immune response and prevention of virulence factor production could prevent infections from occurring (Williams et al., 2013). Additional protective defenses in the mucosal tissue of the upper and lower respiratory tract include antioxidant capabilities that can reduce pulmonary inflammation. Enhancement of these protective mechanisms can also reduce tissue damage, immune responses, and the development of debilitating illnesses (Gao et al., 2011). Without an adequate immune response, however, protection from severe infections is limited and the risk of environmental exposure and subsequent infections caused by *Candida albicans* somehow needs to be reduced. Different methods of investigating treatment options using various plant derived oils have been studied.

Researchers utilized naturally occurring oils to strengthen the antioxidant and anti-inflammatory response in nasal mucosal epithelial cells of 12 healthy adults to evaluate a way to improve protection against infections in the lower airways (Gao et al., 2011). The combined mixture of aloe, orange, peppermint, coconut, and vitamin E, in a carrier oil of soy, reduced inflammation when volunteer test subjects were exposed to ozone (Gao et al., 2011). The ozone was intended to mimic endotoxin production when an organism invades the mucosal membrane. The authors extrapolated that the results could indicate that the combined oils could prevent infections from occurring by inhibiting pro-inflammatory actions of the immune system that occur from microbial

endotoxin production exposure (Gao et al., 2011). While this research provides an interesting foundation for additional studies, the test sample was small, and the healthy adults may respond differently with ozone exposure than immunocompromised patients that are at an increased risk of severe infections.

Other immune response mechanisms include the production of oxidative stress which has been shown to destroy microorganisms through the disintegration of the cell membrane (Khan et al., 2011). Production of oxidative stress using the phenylpropanoid chemical compounds, estragole, methyl eugenol, and eugenol that are found in essential oils, substantially destroyed *Candida albicans* in laboratory testing (Khan et al., 2011). This type of oxidative stress is independent of the host immune response and could be useful for patients that are immunosuppressed. While minimum inhibitory concentrations (MICs) of these compounds have previously been shown to disrupt membrane integrity and therefore cause fungal cell death, sub-MIC levels were also shown to severely damage the defense mechanisms of *Candida albicans* which ultimately also resulted in fungal cell death (Khan et al., 2011). The potential for naturally occurring chemical compounds found in plants to be used as antifungal treatments that are not dependent on an intact immune system and do not interact with the areas targeted by antifungal drugs provides additional areas for research to improve patient health outcomes and reduce mortality risks. Research involving interactions of various essential oils and fungus and yeast isolates will be discussed in the following section.

Essential Oil Treatments for Fungus and Yeast

Fenugreek (*Trigonella foenumgraecum*), cinnamon (*Cinnamomum verum*), papaya (*Carica papaya*), oregano, and garlic all contain phytochemical compounds known to have anti-mycotic activity (Varadarajan, Narasimhan, Malaisamy, & Duraipandian, 2015). Fenugreek, cinnamon, and papaya extracts were evaluated for activity against fluconazole resistant *Candida albicans* with zones of inhibition measured and the percentage of inhibition was then calculated (Varadarajan et al., 2015). The MICs were calculated per standard protocol and all 3 extracts were shown to have anti-mycotic activity with an MIC of 15.62 µg/ml (Varadarajan et al., 2015). Several chemical compounds found in these extracts are known to inhibit proteins and enzymes that are essential for cell wall survival of both bacteria and fungus (Varadarajan et al., 2015). Other essential oils such as black cumin, curry, ajwain, and betel leaf were tested against *Candida albicans*, *Candida tropicalis*, *Candida glabrata*, and *Candida parapsilosis* (Rath & Mohapatra, 2015). Ajwain and black cumin had the highest level of activity against the yeast by causing irreversible cell damage which led to subsequent cell death and the oils still maintained this activity after heat treatment and autoclaving (Rath & Mohapatra, 2015). Mechanisms of membrane inhibition are thought to be caused by the ability of the oils to make partitions in the cell membrane which then causes the cell components to leak out and the organism to die (Rath & Mohapatra, 2015). Various chemical compounds found naturally in essential oils are thought to be the agents responsible for the antimicrobial actions of essential oils (Bellete et al., 2012). While some of the research on essential oils against microbes was with indigenous species of

plants specific to the researchers' location, potential for widespread use could be limited. For this study, oregano, lemongrass, and thyme were chosen based upon not only previous research that has shown antifungal efficacy for all three oils, but also the widespread availability of the oils, which in turn could reduce the cost of adding the oils as treatment. Lemongrass has been shown to also have strong anti-inflammatory properties (Boukhatem, Ferhat, Kameli, Saidi, & Kebir, 2014), which could potentially improve a patient's well-being and comfort. Specific chemical compounds for the essential oils in this study will be discussed in the next section.

Chemical Composition and Mechanisms of Action of Lemongrass, Oregano, and Thyme Essential Oils

The monoterpene aldehyde, citral, which is comprised of neral and geranial, is found in the highest concentration in lemongrass (*Cymbopogon* species) essential oil (Shah et al., 2011). Other major components include the terpenes limonene and myrcene (Shah et al., 2011). Citral, thymol, carvacrol, and linalool are all oxygenated terpenes which have been shown to effectively kill various microbes (Korenblum et al., 2013). These oxygenated terpenes are thought to produce oxidative stress on the yeast causing cell death (Khan et al., 2011). The aldehydes are thought by some researchers to have more effective anti-microbial activity than the phenolic compounds such as thymol and carvacrol (Marin, Sanchis, & Ramos, 2011). However, other authors have indicated that the phenols thymol, carvacrol, and eugenol have the highest antimicrobial activity due to containing an acidic hydroxyl group (Kalemba & Kunicka, 2003). Aldehydes are also acidic by nature which could make it more difficult for *Candida albicans* to change the

environment to a basic pH to induce virulence factors (Shah et al., 2011; Vylkova & Lorenz, 2014). While there has been speculation that aflatoxins could be produced by certain fungus in relation to the increase in oxidative stress, the combination of phenols and aldehydes are thought to act synergistically and could provide alternatives that would be difficult for fungus and yeast to develop resistance or produce aflatoxins due to multiple fungi-toxic components (Molyneux, Mahoney, Kim, & Campbell, 2007; Sidhu, Chandra, & Behl, 2009).

In oregano (*Origanum vulgare*) essential oil, the major constituents are also oxygenated monoterpenes, mainly carvacrol and thymol (Teixeira et al., 2013). Pozzatti et.al, (2008) indicated that carvacrol along with thymol acts synergistically against *Candida albicans* and that fluconazole resistant strains appear to be highly susceptible to oregano. Phenolic and terpene components have also been shown in the host to produce strong inhibition to pro-inflammatory mediators in such as nitric oxide and reactive oxygen species (Leyva-Lopez et al., 2016). Nitric oxide (NO) and reactive oxygen species (ROS) can be utilized by the host to fight infections however, increased amounts of ROS can also destroy human cells (Brieger, Schiavone, Miller, & Krause, 2012). ROS tends to transfer an electron to oxygen thus forming a superoxide which in turn can react with NO to create a highly reactive peroxynitrite (Brieger et al., 2012). This peroxynitrite in turn has a high toxicity to human cells (Brieger et al., 2012). Reducing these biochemical reactions from over stimulation could also help the immune system react to yeast infections more efficiently with less host damage (Leyva-Lopez et al., 2016). Radical scavenging of essential oil compounds could also increase the synergistic

activity of combined essential oils (Tepe, Daferera, Sokmen, Polissiou, & Sokmen, 2004).

Like oregano, thyme (*Thymus vulagris L.*) essential oil also is primarily composed of the phenolic compounds, thymol and carvacrol (Agili, 2014). Thyme essential oil is acidic with a pH around a 3.5 (Agili, 2014). An acidic environment inhibits the ability of *Candida albicans* to switch to the more virulent hyphal phase (Vylkova & Lorenz, 2014). Thymols constitutes generally around 50% of the compounds in thyme and carvacrol makes close to 5% of the components (Agili, 2014). Thymol and carvacrol have shown synergistic anti-microbial effects when combined in laboratory experiments (Tepe et al., 2004). Researchers have speculated that the essential oil activity against microbes may be due to the ratio of the chemical components, not merely by the quantity found in the essential oil (Kalemba & Kunicka, 2003). This information provides a foundation to study the use of lemongrass, oregano, and thyme individually and combined for improved efficacy against *Candida albicans* isolates. Research on lemongrass, oregano, and thyme essential oil activity against fungus and yeast will be addressed in the following section.

Activity of Essential Oils Against Yeast and Fungus

While carvacrol can make up 90% of the compounds found in oregano, authors have indicated that purified constituents at equal concentrations are less effective against microbes than the original essential oil indicating that the components act synergistically even in small quantities (Turgis, Han, Caillet, & Lacroix, 2009). This could prevent the development of antifungal treatments with synthetically developed chemical compounds used alone. Other potential areas for use of essential oils include dentistry. Tissue

conditioner is used to promote healing around dental devices however, the conditioner can become a reservoir for microorganisms including *Candida* after extended use (Amornvit et al., 2014). Lemongrass essential oil and nystatin were each mixed with the tissue conditioner and tested against 10 clinical *Candida* isolates as well as a quality control strain (Amornvit et al., 2014). The lemongrass had a calculated MIC level of 0.635 µ/ml but to achieve effectiveness mixed in with the tissue conditioner, a 0.25% volume, calculated at 4-times the MIC level, was needed due to the viscosity of the tissue conditioner. Lemongrass was similar to nystatin in efficacy against the *Candida* strains tested (Amornvit et al., 2014).

Researchers also investigated the antifungal activities of lemongrass in the laboratory as well as its anti-inflammatory properties in mice (Boukhatem, Ferhat, Kameli, Saidi, & Kebir, 2014). The quick acting anti-inflammatory properties of lemongrass were demonstrated in mice through both topical and oral application (Boukhatem et al., 2014). The antifungal properties were assessed both in liquid and vapor phases (Boukhatem et al., 2014). While both phases inhibited fungal isolates, the vapor phase showed increased efficacy against the fungal isolates and *Candida albicans*. This may provide a basis for further studies using different methods of delivery of essential oil treatments. Lemongrass has been shown to disrupt biofilm formation in *Candida albicans* isolates that were known to be able to produce moderate to strong biofilms and the researchers were unable to increase tolerance to lemongrass by exposing the *Candida albicans* isolates to low levels of the essential oil (Sajjad Ahmad Khan & Ahmad, 2012). This lack of resistance to essential oils promotes the importance to

further study the use of essential oils against *Candida* since resistance to the antifungal medications continues to increase.

Thyme was also evaluated to examine antifungal activities against *Candida albicans* and *Aspergillus fumigatus* (Bellete et al., 2012). Thyme essential oil showed strong antifungal activity in low concentrations (Bellete et al., 2012). To confirm that this activity was due to chemical compounds in the oil and not just the use of any oil, vegetable oil and three additional essential oils (vetiver, wild orange, and argan) with known minimal antifungal activity were also tested. Increased dilutions of the organisms were still inhibited with the introduction of thyme (Bellete et al., 2012). Low concentrations of essential oils needed for antimicrobial activity and the volatile nature of essential oils provide additional areas for research (Bellete et al., 2012). Additional researchers compared the activity of cinnamon, lemon, eucalyptus, and thyme compared to activity of chloramphenicol against *Candida albicans*, *Tricophyton*, and *Microsporum*, and several bacterial strains (Nasir, Tafess, & Abate, 2015). Thyme had the largest zone of inhibition upon laboratory testing (Nasir et al., 2015).

Thyme has potential to be used in topical treatment of dermatophytes as well as other fungal, yeast, and bacterial isolates. Calculated MIC levels were shown to be lower for thyme than levels needed for chloramphenicol (Nasir et al., 2015). In addition, the zone of inhibition for thyme against the organisms tested was almost twice as large as the zone for chloramphenicol (Nasir et al., 2015). Other studies where researchers compared essential oil activity against *Candida* species in comparison to amphotericin B and fluconazole resulted in thyme essential oil having higher inhibitory activity against

Candida species than lemon or pennyroyal essential oil (Omran & Esmailzadeh, 2009). While amphotericin B had a larger zone of inhibition in this study than fluconazole, thyme exhibited a larger zone of inhibition than either antifungal medication tested (Omran & Esmailzadeh, 2009). Researchers do admit however, that zone size comparisons can be difficult to determine rates of effectiveness and further evaluations are warranted (Omran & Esmailzadeh, 2009). Time-to-kill assays proposed in this study may provide a more accurate assessment of essential oil activity against *Candida albicans*.

While the focus of multiple studies has been on antimicrobial activities of essential oils other researchers have examined mechanisms of how the essential oils cause damage and pathogen cell death. Rajkowska, Kunicka-Stycynska, Maroszynska, and Dabrowska (2014) evaluated the effect that tea tree and thyme essential oils had on biochemical and enzymatic processes of *Candida albicans* strains. Extensive loss of the ability to assimilate different carbon sources occurred in *Candida albicans* isolates after exposure to tea tree and thyme essential oils (Rajkowska et al., 2014). This disruption in enzymatic activity could decrease the pathogenesis of *Candida albicans* (Rajkowska et al., 2014). Thyme and tea tree essential oil have also been shown to not only reduce the activity of enzymes but the ability for *Candida* to uptake sugars was also inhibited or prevented (Rajkowska et al., 2014). Monoterpenes such as carvacrol and thymol are thought to disrupt ATP synthesis reducing regulation of other cellular functions (Custodio, Ribeiro, Silva, Machado, & Sousa, 2011). Other researchers have also concluded that *Candida* species do not appear to be able to develop resistance to essential

oils in laboratory testing (Budzynska et al., 2013). This inability to develop resistance could provide additional options for long-term prophylactic use of essential oils to prevent *Candida* infections in immunocompromised patients and potentially reduce the number of drug-resistant *Candida* in the host environment and therefore improve patient outcomes and reduce hospitalizations and mortality. In addition, various essential oils are quick-acting fungicides that have been shown to decrease the ability of *Candida albicans* to withstand oxidative stress from the immune system (Budzynska et al., 2013). This aspect could also benefit immune-competent patients. Other investigations have been used to evaluate the interactions between essential oils and antimicrobial treatments which will be discussed in the following section.

Effects of Combining Antimicrobials and Essential Oils

A total of 35 combinations of various essential oils and antibiotics were tested against three strains of *Escherichia coli* with four combinations showing high synergistic effects (Xi Yap, Lim, Hu, & Yiap, 2013). The combinations with the highest effects were piperacillin/cinnamon, piperacillin/lavender, piperacillin/peppermint, and meropenem/peppermint (Xi Yap et al., 2013). These combinations could allow for a reduction in the effective dose of the antibiotics needed which in turn, could decrease the adverse side-effects of antibiotic treatment (Xi Yap et al., 2013). There were also no antagonistic effects observed between the essential oils and antibiotics tested, which would indicate that essential oils do not lessen the effect of the antibiotics and the antibiotics do not interfere with the effect of the essential oils (Xi Yap et al., 2013). In addition, the effective combinations could substantially reduce MIC levels in organisms

that possess plasmid mediated resistance to the antibiotic (Xi Yap et al., 2013). Further investigations into interactions between antifungal medications and multiple essential oils have not been extensively studied. Increased resistance to antifungal medications as well as increased fungal infections due to *Candida albicans* has also created the need for researchers to find potential alternative treatment options (Sajjad Ahmad Khan et al., 2012).

Researchers screened 21 essential oils for activity against drug resistant strains of *Candida albicans* (Sajjad Ahmad Khan et al., 2012). Lemongrass exhibited strong antifungal activity and appeared to be more effective than fluconazole against *Candida* isolates tested (Sajjad Ahmad Khan et al., 2012). The eugenol compounds found in clove oil exhibited high levels of synergy with fluconazole and amphotericin B against drug resistant yeast strains (Sajjad Ahmad Khan et al., 2012). Eugenol is a phenylpropanoid and has similar properties to the phenols thymol and carvacrol which are found in oregano and thyme. Pozatti et al., (2008), concluded that oregano, thyme, ginger, and cinnamon all express antifungal activities on both fluconazole sensitive and resistant strains of *Candida* species. Oregano showed the highest activity against fluconazole resistant and susceptible strains of *Candida* which led the authors to suggest that the antifungal activity of oregano is not dependent upon changes in *Candida* that are associated with fluconazole resistance (Pozatti et al., 2008). Due to the higher activity of oregano compared to thyme, the authors concluded that carvacrol could be the main constituent responsible for the antifungal activity (Pozatti et al., 2008). In comparison, the fluconazole resistant *Candida albicans* and *Candida dubliniensis* were more sensitive

to thyme than the fluconazole sensitive counterparts which the authors concluded may be due to the combined activity of the chemical compounds found in the oils tested or that the resistance mechanisms could make the organisms more susceptible to the impact of the essential oils (Pozatti et al., 2008). Other studies on various thyme species have led researchers to conclude thyme essential oils showed a stronger synergy with fluconazole than amphotericin B against *Candida* species (Saad et al., 2010).

While tea tree oil and its chemical components were tested against 100 *Candida* isolates and shown to be effective against biofilm forming strains (Ramage et al., 2012), additional researchers investigating the activity of lemongrass essential oil against the formation and reduction of pre-formed *Candida* biofilms have also indicated that while these laboratory-created biofilms have shown increased resistance to antifungal drugs, the biofilms displayed no tolerance to lemongrass (Sajjad Ahmad Khan & Ahmad, 2012). Cancer patients are at increased risk of oral *Candida* infections due to the formation of these biofilms which then increases the risk of invasive infections (Ramage et al., 2012). Utilizing lemongrass to reduce oral biofilms could interrupt the reservoir for invasive infections of *Candida* and disrupt the epidemiologic triangle. Lemongrass was also shown to cause deformities in the 3-dimensional structures of the biofilms, even at sub-MIC levels (Sajjad Ahmad Khan & Ahmad, 2012). In addition, there was a 60% reduction of viable sessile cells of *Candida* within 12 hours of exposure to lemongrass but not with fluconazole or amphotericin B even after 48 hours (Sajjad Ahmad Khan & Ahmad, 2012). The authors concluded that the ability for lemongrass to prevent biofilm formation may provide alternatives to pre-treatment of devices as well as a reduction in

toxicity load to the patient (Sajjad Ahmad Khan & Ahmad, 2012). Reduction of toxicity of antifungal medications to improve patient outcomes has previously been discussed however, questions about potential of toxicity of essential oils to human cells will be discussed in the next section.

Evaluation of Host Cell Toxicity from Essential Oils

A potential concern for utilizing essential oils for treatment in patients is whether the essential oils are also toxic to human cells. Agili (2014) investigated the effects of thyme on three types of cancer cells lines as well as a non-tumor human cell line grown in the laboratory. While the thyme inhibited the tumor cell growth by 50%, the essential oil had no cytotoxic effect on the non-tumor cell line PLP2 even at concentrations higher than 450 µg/ml (Agili, 2014). Sinha, Jothiramajayam, Ghosh, & Mukherjee (2014) evaluated the potential toxic effects of lemongrass, palmarosa, citronella, and vetiver against human lymphocytes in cell cultures. The authors reported that while the essential oils tested were cytotoxic at 1000 µg/ml, this is over 100 times the amount needed to have an antifungal effect on yeast and fungus (Sinha et al., 2014). The MIC range for essential oils to kill yeast and fungus is normally between 0.2 and 10 µg/ml (Sinha et al., 2014). The authors concluded that lemongrass, palmorosa, citronella, and vetiver would be safe for human consumption at lower levels (Sinha et al., 2014). The essential oils in the Sinha et al. (2014) study were purchased through Sigma-Aldrich Chemical which may not provide high quality naturally derived plant oils and could therefore limit the toxicity results for the study.

Variances in study designs and essential oils tested were not always evaluated using standardized methods. For example, some researchers distilled the essential oils from dried plant material stored at various temperatures for various lengths of time (Agili, 2014; Bellete et al., 2012; Boukhatem et al.; 2014; Cleff et al.; 2010; Teixeira et al., 2013) and others purchased oils or chemical compounds from a vendor (Omran & Esmailzadeh, 2009; Pozatti et al.; 2008; Rajkowska et al.; 2014, Sajjad Ahmad Khan et al., 2012; Xi Yap et al., 2013). With the variety of distilling techniques and storage stability used in the reviewed research, there could be a potential to not be able to reproduce the results that were reported or for similar research to publish different results on the effectiveness of different essential oils against *Candida* isolates. To minimize result errors due to inconsistencies in the quality of the essential oils tested, the essential oils used for this research were all purchased from a known high-quality vendor, doTERRA International. Each batch of essential oils are tested using gas chromatography and mass spectrometry to evaluate the chemical compounds found in each oil. In addition, the oils are further tested using Fourier Transform Infrared Spectroscopy (FTIS) to ensure potency and consistency between each batch, chirality testing which verifies that there are no synthetic components found in the oils, heavy metal testing to ensure there are none present, and microbial testing to verify there is no contamination of bacteria, fungus, or viruses (doTERRA CPTG Certified Pure Therapeutic Grade Quality Testing website, 2016). The chemical components from each oil tested will be provided by the vendor to improve comparability with previous test results. The results of this study filled in the gap of utilizing combined essential oils as

well as combined essential oils with antifungal medications. This research also provided a foundation and direction for additional research. Further information concerning the methods employed in the reviewed articles are discussed in the next section.

Methodology Review

Increased drug resistance of fungus, yeast, and bacteria due to higher numbers of immunocompromised patients who are at high-risk for infection, have caused researchers to investigate potential alternative treatment options. *Candida albicans* is the second leading cause of fungal infections worldwide and this disease burden is likely underestimated due to misdiagnosis and lack of required reporting for all invasive fungal disease except for *Coccidioides* which causes Valley Fever (Brown et al., 2012). Most studies reviewed used a quasi-experimental design not an experimental design due to lack of randomizing the organisms studied (Amornvit et al., 2014; Bellete et al., 2012; Boukhatem et al., 2014; Gao et al., 2011; Khan et al., 2011; Lewis et al., 2012). Most of the research reviewed were performed primarily in laboratory-controlled settings. Several of the studies were case reports based on infectious disease outcomes when patients were infected with *Candida* (Alcazar-Fuoli & Mellado, 2014; Brown et al., 2012). Other research methods were a combination of case reports of patient outcomes and using organisms isolated from patient infections in experimental laboratory testing (Bliss et al., 2012). This section is used to review the methodology that was used to evaluate essential oils, antifungal medications and the interactions with various organisms.

Several of the studies performed used novel approaches to test interactions and some studies had very detailed methodology that could easily be expanded upon for future research (Omran & Esmailzadeh, 2009; Rajkowska et al., 2014; Sajjad Ahmad Khan & Ahmad, 2012). Some researchers attempted to address in-vivo issues using human cell lines and mouse models (Ramage et al., 2012; Sajjad Ahmad Khan et al., 2012). One study had human participants to study the ability of reducing nasal passage inflammation after ozone exposure using essential oils however, this study was small and used healthy individuals (Gao et al., 2011). All the experimental studies either had replicated sample testing multiple times or had large sample sizes to protect validity and reliability.

Evaluation of the potential covariates and risk factors were done using retrospective studies (Chander et al., 2013; Shigemura et al., 2014; Yang et al., 2014), a retrospective case-control study (Kaye et al., 2011), and prospective studies (Hu et al., 2014; Rajendran et al., 2016). The researchers in these studies reported on different risk factors or predictors but other information could also be acquired about some of the patient demographics however, race as a covariate was not discussed in any of the articles mentioned. Race has previously been shown to be a factor in other health risks (Frieden, 2011) therefore, the inclusion of race in evaluating risk factors for *Candida albicans* bloodstream infections may provide additional insight for prevention measures. The conclusion of this literature review will be discussed in the next section.

Conclusion

Increased numbers of long-term immunocompromised patients provide ample environments for opportunistic pathogens to infect patients and for well recognized pathogens to acquire higher levels of drug resistance. With limited anti-microbial medications available, acquired drug resistance especially in yeast such as *Candida* require new and different approaches to successfully treat these patients (Silva et al., 2014).

Novel options such as synthesis of naturally occurring antimicrobial proteins (AMPs) provide an exciting potential for treatment however, the short half-life of the proteins pose potential delivery issues in the patient as well as stability of the proteins to maintain efficacy while being stored or manufactured (Mehra et al., 2013). *Candida albicans* produce virulence factors that disrupt the mucosal epithelial surfaces which can allow for the yeast to create serious and life-threatening infections (Lewis et al., 2012). Preventing these virulence factors from forming could also improve patient outcomes and reduce the risk of serious infections (Bliss et al., 2012). Alternative options for treatment of *Candida* infections include the investigations of utilizing antimicrobial peptides (AMPs) for interrupting the disease process (Mehra et al., 2013). While AMPs show promise at being able to kill various microorganisms, the half-life is very limited in serum and these treatment options may not prove beneficial for invasive infections (Silva et al., 2014). One type of AMP, known as defensins, have also shown promise to reduce virulence in yeast and fungus and have been shown to be able to kill the organisms,

however, the ability to get the defensins to the infection site may prevent widespread use other than topical treatments (Silva et al., 2014).

Virulence factors in microorganisms can trigger immune responses (Williams et al., 2013). Pathogen recognition receptors (PRR) can initiate immune responses to invading yeast. Immune defenses can also include antioxidant activity in the mucosal tissues. Essential oils can increase antioxidants that are responsible for the reduction of pro-inflammatory toxins released by microorganisms that cause infections (Gao et al., 2011). Phenylpropanoid compounds found in various essential oils destroy *Candida* through oxidative stress and subsequent cell wall destruction in laboratory testing (Khan et al., 2011). Extracts of fenugreek, cinnamon, and papaya have all shown anti-mycotic activity by inhibiting proteins and enzymes needed for cell wall survival in bacteria and fungus (Varadarajan et al., 2015). In addition, other research has shown that tea tree oil can not only prevent biofilms caused by *Candida* isolates but also reduce inflammatory response in host tissue (Ramage et al., 2012). Black cumin, curry, ajwan, and betel leaf showed strong activity against various *Candida* species by causing irreversible cell damage which in turn caused cell components to leak out. These essential oils also maintained effectiveness after heat treatment and autoclaving (Rath & Mohapatra, 2015). While many essential oils have been evaluated as alternative treatment options, some are only available at a local level, are expensive to manufacture, or would not be available in larger amounts on a regular basis. Therefore, lemongrass, oregano, and thyme were chosen for further study against *Candida albicans* isolates.

Lemongrass essential oil treatment showed similar efficacy against *Candida* when compared to nystatin treatment of tissue conditioners that are used to promote healing after dental procedures (Amornvit et al., 2014). Additional studies were used to assess antifungal properties of lemongrass in both liquid and vapor phases as well as potential anti-inflammatory properties. The anti-inflammatory treatment was quick acting in mice and the vapor phase showed higher efficacy against *Candida albicans* and fungal isolates (Boukhatem et al., 2014).

Thyme essential oil has also been shown to have strong antifungal activity in low doses against *Candida* isolates (Bellete et al., 2012). In comparison of cinnamon, lemon, eucalyptus, and thyme to chloramphenicol against yeast colonies, thyme had increased zones of inhibition and lower MIC levels were needed for inhibition of the organisms tested (Nasir et al., 2015). Utilizing a combination of 35 various essential oils and antibiotics, additional researchers tested these combinations against resistant strains of *Escherichia coli (E. coli)*. Four of these combinations had high synergistic effects against *E. coli*. These combinations could reduce the amounts of antibiotics needed to treat infections (Xi Yap et al., 2013). This information could be used to develop additional studies for treatments against *Candida albicans* infections. Drug resistant *Candida albicans* were also tested against 21 essential oils. Lemongrass exhibited high antifungal activity and was more effective than fluconazole against these resistant strains in laboratory testing (Sajjad Ahmad Khan et al., 2012).

Essential oils have been used in remedies for centuries and researchers from all parts of the world have shown successful inhibition and microbial death with treatments

using a variety of essential oils against a multitude of organisms in laboratory testing. Essential oils such as oregano, lemongrass, and thyme have been shown to have inhibition and cidal capabilities against *Candida* isolates (Boukhatem et al., 2014). Added benefits of potential essential oil use for infections are the capabilities for the oils to reduce inflammation (Ramage et al., 2012).

While it may be difficult to study only essential oil interactions with *Candida* infections in human subjects due to a risk for the patient in deviating from standard of care, the addition of essential oils to standard antifungal treatment methods might prove to be a successful alternative. The pursuit of evaluating host interactions with essential oil treatments for infections may be enhanced by additional studies of synergistic interactions of antifungal medications and essential oils initially in the laboratory environment.

Chapter 3: Research Method

Introduction

The purpose of this study was to develop a system to examine the effectiveness of three individual essential oils (lemongrass, oregano, and thyme) used separately and in conjunction with each other in the presence of the antifungal medications fluconazole and caspofungin. These antifungal treatments are recommended for a patient with a *Candida albicans* systemic infection (Pappas et al., 2016). A quantitative method was used to evaluate the impact the use of these oils in combination with antifungal medications had on the ability to minimize virulence factors and to kill *Candida albicans* in-vitro.

Repeated and long-term use of antifungal medications has helped to develop increased resistance to these medications in *Candida* species (Mehra et al., 2013). This increased drug resistance has not only elevated the cost for treatment, but also has increased the risk of mortality from these infections. Limited treatment options and rising mortality rates in both adults and children infected with *Candida* necessitates new methods for treating these life-threatening infections (Zaoutis et al., 2005).

In this chapter, I will review the research design and rationale for using a quantitative method to examine the interactions of the essential oils lemongrass, oregano, and thyme with antifungal medications and the impact on known virulence factors of *Candida albicans*. The study participants, sampling, study variables, and measurements used will also be discussed in this chapter. This will be followed by the statistical analysis methods, as well as the potential ethical concerns of using data obtained from previously collected isolates.

Research Design and Rationale

I used a quantitative design to evaluate the impact these oils in combination with antifungal medications had on the ability to interfere with virulence factors of *Candida albicans*; the ability to cause death to the yeast cells; and to evaluate the impact of age, race, gender, and comorbidities on the risk of *Candida albicans* bloodstream infections. Quantitative experimental designs are used to test a cause and effect relationship with known variables (Frankfort-Nachmias & Nachmias, 2007). The research questions of whether essential oil treatment can have a positive impact on *Candida albicans* infections were best investigated by using a quantitative experimental design. This experiment was further designed to evaluate the covariates for confounding and risk factors. Using an experimental quantitative design also enables the researchers to replicate the study to verify the results or for additional researchers to repeat the study to determine whether the outcomes are similar (Frankfort-Nachmias & Nachmias, 2007).

For an organism to cause an infection, it must produce virulence factors and continue to replicate. Antifungal medications attack the organism by various means to kill the organism or prevent replication of the organism (CDC MASTER website, n.d.). This process can take an extended amount of time, and the organism may become resistant to the medication during that time (Bliss et al., 2012). Essential oils have been shown in previous laboratory studies to stop the organism from producing these virulence factors, as well as killing these infectious organisms (Cleff et al., 2010).

I investigated the effectiveness of essential oils against *Candida* infections, as well as the impact that the oils may have with the host's immune response, using a

deductive methodology to provide alternatives for these resistant organisms. Data were collected on whether the organism was inhibited or killed using repeat culturing methods for the organism, as well as investigating any statistical significance based upon the covariates.

The experimental design allowed for this research to be replicated with additional population groups or by other researchers (Frankfort-Nachmias & Nachmias, 2007). Use of this design can also provide a way to assure a high degree of validity due to the ability to culture each *Candida albicans* isolate before and after exposure to the essential oils and antifungal medications, as well as determining whether any of the covariates are related to increased yeast. Although current researchers provided promising in-vitro results of the efficacy of essential oils against *Candida* and its infectious properties, there are limited treatment options used for *Candida* infections when the organism has become resistant to multiple medications (Tyagi & Malik, 2010). With the continued increase in drug resistance, there have been few published studies aimed at validating the response in isolates collected from patients in populations being exposed to alternative treatment regimens. Scholars have investigated different essential oils and their effectiveness against *Candida* using various methods to monitor levels needed to inhibit or kill the yeast cells; however, there has been no standardized testing within a population of infected patients. This has made it difficult to determine the true effectiveness of essential oils against *Candida* infections.

The variables measured in the first five research questions of this study included the dependent variable of kill rates in *Candida albicans* isolates and eight levels of the

independent variable of treatment. These eight levels included treatment with the individual essential oils lemongrass, oregano, and thyme, individual treatment of the antifungal medications fluconazole and caspofungin, the combined treatment of the three essential oils, the combined treatment of the three essential oils with fluconazole, and the combined treatment of the three essential oils with caspofungin. The sixth research question had a dependent variable of inhibition of germ tube production with the independent variable of treatment with three levels. These three levels of treatment were the three combined essential oils, fluconazole, and caspofungin. The ability to measure these treatment outcomes also allowed me to ascertain whether there was a causal effect of the various treatments studied. The last research question had independent variables of age, gender, race, and comorbidities and the dependent variable of *Candida albicans* bloodstream infection.

Methodology

Population

The population for this study were adult patients (18-74 years of age) at a large teaching hospital who have had at least one positive blood culture with *Candida albicans* recovered within the past 7 years. Isolates recovered from blood cultures were cryo-preserved at -80º centigrade. Isolates were stored from the previous 7 years, and patient demographics were also available for this timeframe. Blood cultures with multiple organisms isolated were not included, and only the information from the initial isolate of *Candida albicans* from a patient's hospital stay was collected. Patients who had *Candida*

albicans isolated from a blood culture were included regardless of the length of stay. This population consisted of patients of various age, gender, race, and comorbidities.

Sampling and Sampling Procedures

The sampling techniques can be either a nonprobability method, which have a low generalizability, or a probability method, which can be generalized to the population with a higher degree (Burkholder, n.d.). In this study, the method used could be considered to have a low generalizability to the population at large but a high generalizability to the population receiving antifungal medications for *Candida* infections (Crosby, DiClemente, & Salazar, 2013). Using a sample strategy that can be generalized to the population at large would not be feasible due to the purpose of studying isolates recovered from bloodstream infections that may be immunocompromised patients, while the general population will include people who are immunocompetent and are less likely to have *Candida albicans* bloodstream infections. However, further studies could provide additional information outside of the hospitalized, potentially immunocompromised population.

A stratified sample was also used in this research to divide the sample frame into subpopulations. Variables for stratification include age, gender, and race (Crosby et al., 2013). For this study, the sampling population was all-aged adults, gender, race, and comorbidities seen at this large teaching hospital. The hospital internal review board (IRB) approved data collection for patient covariates to be obtained using access to the electronic health records (EHR) of patients who have previously been identified as having a *Candida albicans* bloodstream infection through a log sheet stored in the

microbiology department. The data were logged into a spread sheet with only age group, race, gender, and comorbidities. In defining comorbidities for epidemiologic studies, Yang et al. (2014) cited organ tumors, hematologic malignancy, diabetes, chronic pulmonary disease, renal failure, and cardiac disease as the primary comorbidities. Hu et al. (2014) reported that the main comorbidities included diabetes, solid tumor cancer, hematologic malignancy, cardiac, renal, or hepatic failures, as well as immunosuppression. Shigemura et al. (2014) listed diabetes, cancer, cardiovascular, renal, and liver diseases as the main comorbidities in reporting on 110 cases of *Candida* bloodstream infections. Although Kaye et al. (2011) also noted diabetes, metastatic malignancy, and chronic pulmonary or heart disease, HIV was also listed as a comorbidity in the 830 cases of blood stream infections from any type of organism. Based upon the reviewed epidemiologic reports, and the variety of patients who were seen at the hospital where the data were collected, the comorbidities that were evaluated included diabetes, kidney failure, organ transplant, stem cell transplant, solid organ tumor cancer, positive HIV status, hematological malignancy, substance abuse, abdominal surgery, severe burns, other types of surgery, soft tissue cancer, autoimmune disorder, and multiple organ failures. To compare covariates of a noninfected control group to the covariates of patients with *Candida albicans* blood stream infections, an aggregated sample of patient demographics and comorbidities were collected for the same period, and a randomized sample of 120 patients were pulled from the EHR using a research randomizer program (Research Randomizer, n.d.). There were no patient identifiers stored in the data spreadsheet for this research. To begin the process to de-identify

patients, the age groups were divided as 18-24, 25-34, 35-44, 45-54, 55-59, 60-64, and 65-74. Age groups were separated into divisions used by the United States Census Bureau (n.d.) because investigating epidemiology of bloodstream infections has referred to mean age, not age groups (Hu et al., 2014; Kaye et al., 2011; Shigemura et al., 2014; Yang et al., 2014). The hospital required a full IRB approval for data collected on patients 75 years and older therefore data were chosen to be collected on adults under the age restriction (University of Colorado Health [UCH], n.d.). Sample size affects the statistical significance (*p* value), and a larger sample size of 100 patients or more is preferred. The sample size was dependent upon the number of isolates recovered and frozen in the previous 9 years. Initial *Candida albicans* isolates were found by reviewing the positive blood culture patient log stored in the microbiology department's records. These log forms had minimal patient demographics, and the additional covariate information were accessed through the EPIC medical records system and documented without patient names on a spreadsheet that lists organisms as arbitrary numbers. The patient's data were initially sorted by age due to the normal decrease of the immune response of healthy, elderly patients (Wanke et al., 2000). The sample size was based upon the number of isolates of *Candida albicans* recovered and saved from 2009 through August 2017 that met the criteria including whether all the patient covariates were available. Immunocompromised patients who received bone marrow transplants had an 11.4% risk of developing invasive *Candida* infections (Goodrich et al., 1991). A G*Power analysis calculated that with a power $(1-\beta) = 1.0$ and an α err of 0.05, the total sample size would need to be 118 patients for an effect size of 0.25 (G*Power, n.d.).

This was calculated using an ANOVA repeated measures, within between factors test comparing eight groups and 10 measurements. This calculation was used to assess the first six research questions. Using a G*Power analysis for logistic regression, the total sample size for the patient demographics of 220, the power (1-β err prob) = 0.992.

Data Collection Procedure

The data collected from each patient listed age, gender, race, and comorbidities. Data was also collected on the growth response of *Candida albicans* in the presence of individual essential oils and the oils combined, and growth response of the organism to the combined use of essential oils and antifungal medications. Data collected from culture growth included the amount of growth at each time interval tested for each test treatment at 24 and 48 hours of incubation, and whether each treatment inhibited the germ tube production of each organism tested.

The patient data was de-identified and stored as isolate numbers only with gender, race, age range, and co-morbidities listed on a password protected portable drive. This information has been kept in a private office with limited access. The data will be compiled and presented at Infectious Disease rounds and at an annual conference on essential oil uses.

Instrumentation and Scale

To investigate the efficacy of treatment with essential oils in combination with prescribed antifungal medications on the time to kill *Candida albicans* recovered from hospitalized patients, an interval scale was used to determine the number of hours that the *Candida albicans* isolates were exposed to the various treatments to obtain cell death and

germ tube inhibition. An interval scale was used to measure the distance between variables but cannot be used to determine a ratio (Kimberlin & Winterstein, 2008). The interval scale for this study measured the time it took for the organism to be killed with the essential oil treatment compared to standard treatment, and the combined treatment. The level of measurement was chosen as a measure of the time until the organism was dead. A ratio level was used to compare the kill rates of the antifungal treatment with the essential oil treatment. A ratio scale was used to determine order, quantitate any differences between the values, and to calculate ratios (Kimberlin & Winterstein, 2008).

Testing a patient for cure from a *Candida* infection, is done by culturing specimens collected from the site of the patient's infection and quantitating any growth of *Candida* that occurs (Clinical Laboratory Standards Institute, n.d.). Essential oils have been shown to reduce virulence factors in *Candida* and to kill yeast and fungus in laboratory tests (Cleff et al., 2010). Essential oils have also been shown to increase the effectiveness of antibiotics when used in conjunction in-vivo (Langeveld, Veldhuizen, & Burt, 2013; Yap, Lim, Hu, & Yiap, 2013). These published research results would indicate that the test methods for this study were appropriate to measure whether essential oil are effective when used in addition to treatment protocols in laboratory testing of *Candida albicans* isolates.

To verify whether *Candida* grew or not after the treatment protocols in the different test variables, a colony count method using a modified Serum Inhibitory Test was used to quantitate how many colonies of the *Candida albicans* grew in culture when exposed to either the essential oil or the antifungal medication as well as the combined

treatment. Serum Inhibitory Titers have been used since the 1950's and provide a standardized method for verifying treatment with antimicrobials (Woolfrey et al., 1986). This method was used as a scale to evaluate the culturing techniques and the growth abilities of the organism after treatment exposures. The data points were plotted and compared to each test parameter and by the covariates to examine whether any other variables such as age, gender, race, or comorbidity affected the outcomes or risk of infection.

Exposure to treatments for kill times were based upon 1 hour, 4 hours, 8 hours, 12 hours, and 24-hour incubation. Each *Candida* isolate was brought to a 0.5 McFarland standard, or approximately 1.5×10^8 colony forming units per milliliter (cfu/ml), using a 5ml tryptic soy broth (TSB), acquired from Hardy Diagnostics. This was followed by the addition of the treatment level. The first protocol was done using each organism suspension in TSB inoculated with 10 µl of lemongrass essential oil and incubated at 35ºC for the time intervals. At each time interval, 10 µl of the inoculated TSB was plated onto Hardy Diagnostics Sabouraud Dextrose (SabDex) agar. The Sabdex plate was then incubated at 35ºC and examined for growth at 24 and 48 hours. The total colony count on each agar plate was recorded at the 24 and 48-hour time frames. The absence of *Candida* growth on the agar plate after 48 hours was considered organism death. This protocol was replicated each time with fresh organism suspensions and 10 µl of oregano essential oil; then a new suspension and 10 µl thyme; a new suspension of organism and 10 µl of an evenly combined mixture of lemongrass, oregano, and thyme (LTO) essential oils (10 µl of a mixture of 60 µl each of the three oils). This was then followed by fresh

organism suspension in TSB along with a standardized 25 µg disk of fluconazole and a fresh organism suspension with standardized 5 µg of caspofungin. Then fresh organism suspension with 10 µl of LTO plus the 25µg disk of fluconazole and lastly a fresh organism suspension with 10 µl of LTO plus 5 µg of caspofungin.

Disruption of the virulence factor of germ tube production was performed by inoculating a germ tube production media with each yeast isolate, incubating for 1-2 hours and then microscopically examining for germ tube production. This test was performed with just the media and the isolate to confirm that the organism produces germ tubes and then new germ tube media and each isolate with 5 µl of the LTO. This was followed by new germ tube media, the isolate, and 25 µg fluconazole disk as well as new germ tube media, the isolate, and 5 µg of caspofungin.

Establishing Reliability for the Measurement and Scale

Reliability of a test relates to whether the test can be replicated with similar results (Kimberlin & Winterstein, 2008). In this study, the ability to test and re-test for the same outcome on an individual isolate would be feasible due to being able to maintain *Candida* isolates for an extended length of time. Another option for reliability would be to repeat the same study with an additional group of isolates from a different facility; from a different time-frame; or from a different specimen source. Since all the isolates came from bloodstream infections, replicating the specimen source and culturing all the samples in the same way and incubating them for the same length of time provided an additional level of reliability to the test. To ensure thorough replication, the test methods were performed in the same way for each treatment session. The potential

causal effect of adding essential oils for treatment was verified by evaluating whether the isolates that received essential oils and antifungal medications were killed in less time or more often than when these isolates only were exposed to the antifungal medications.

Establishing Validity

For a measure to have content validity it should measure the variable that it was intended to measure (Kimberlin & Winterstein, 2008). Content validity is used to examine whether the test measures all the items the test should measure in relationship to the construct (Gabrenya Jr., 2003). The measurement of *Candida* growth in culture was verified using the Clinical Laboratory Standards Institute's (CLSI's) guidelines (2015) for standardized culturing of *Candida* species. The specimens collected for this study were all from a blood culture source. The face validity should be high for this study because the measurements for culture growth have been previously established as a test for kill rates and minimum inhibitory concentrations (CLSI, n.d.). The measurements were used to assess the construct that antifungal medications can be used to treat *Candida* infections and to assess whether the essential oils treatments provided the same or improved benefit (CDC: MASTER, n.d.). The content validity for sampling is used to estimate how much the measure represents the actual elements in the construct (Gabrenya Jr., 2003). The variables in this study were measured individually and well-established culture and sampling techniques were used to test for kill rates therefore, the sampling content validity was assumed to be strong.

The statistical evidence that a diagnostic instrument measures what it is supposed to measure is known as the empirical validity (Gabrenya Jr., 2003). The preliminary lack

of growth on culture media for *Candida albicans* would indicate that the treatment method is inhibiting the organism and not actually killing the organism. To ensure the validity of culture method, the specimens were plated on agar plates that enhance yeast growth and then incubated for 48 hours. The 10µl of treated broths were also inoculated into a new TSB tube to help facilitate inhibited organisms or low quantities of organisms into growing. This enrichment broth served as a vehicle to promote growth for organisms that may be weakened due to a treatment that is merely fungistatic and not fungicidal. These broth cultures were held for seven days to encourage the growth of weakened but still living yeast. After the seven days, 10 µl of the broths were inoculated onto SabDex agar then incubated for 48 hours and any colony growth was recorded.

Construct validity relates to the overarching quality of the research, the ability for the model to fit the data, and the ability to correlate results using the instrument (Alumran, Hou, Sun, Yousef, & Hurst, 2014). The observed results of how things work correlate with the theoretical pattern of how things work (Kimberlin & Winterstein, 2008). Antifungal medications work against yeast and fungus in similar ways that antibiotics work against bacteria (CDC, n.d.[1]). These medicines work in various ways including inhibiting replication of the organism or damaging the cell wall of the organism so that the immune system can work more effectively against the organism (CDC: MASTER, n.d.). However, immunocompromised patients lack the ability of the immune system to interact with antimicrobials effectively due to the compromised state of the immune system (Pellegrino et al., n.d.). To generalize this concept, it could be assumed that if the use of essential oils worked well to rid an immunocompromised patient of a

yeast infection, then it would work as well in people that have an intact immune system. Utilizing a standardized test method for the growth of the organism in conjunction with comparing both types of treatment as well as the combined treatment also increased the construct validity of this research.

The strengths for measuring growth of *Candida albicans* from patients with known infections are increased by using standardized methods for culture techniques that are used in microbiology laboratories around the world (CLSI, n.d.). These methods have been well documented and confirmed as the best methods that are available. The measurement of growth or no growth of *Candida* has been shown to be a valid and representative method for detecting *Candida* resistance to treatment regimens (CLSI, n.d.).

Addressing Potential Weaknesses and Threats to Validity

Potential weaknesses could be a misidentification of an organism that was previously identified as *Candida albicans* but was actually the phenotypically similar *Candida dublinensis*. To reduce the risk of reporting results on the wrong organism, the isolate identifications were confirmed with a matrix assisted laser desorption/ionization-time of flight (Maldi-TOF). Accurately measuring incubation time and growth results for each culture were tracked using a spread sheet to keep track of the time set up and time for each read. Threats to external validity were minimized due to the ability to accurately measure culture growth for the organisms. Rigor can be assured for validity of the study due to standardized culture testing using the same collection devices, the same media, and incubating the cultures for the same overall length of time.

Analysis

The study to determine the effectiveness of essential oil against *Candida albicans* infections is considered an experimental test due to the attempt to correlate the cause and effect relationship of adding essential oils to the standard treatment and whether this improves the curing of *Candida* infections. The testing of each isolate against both the antifungal treatments and the essential oil treatments to determine whether the *Candida* has survived treatment during the study is a norm-referenced test (NRT) because each culture was compared to the others in each individual test group as well as across the control and test groups ("Criterion and Standards Referenced Tests," 2007). This type of comparison also allowed for the results to be compared by groups based on age, gender, race, and comorbidities. The statistical software SPSS was used to perform statistical analyses.

The initial research hypothesis examined lemongrass essential oil compared to the antifungal medications, caspofungin and fluconazole on *Candida albicans* kill rates. The second research hypothesis examined oregano essential oil compared to caspofungin and fluconazole on *Candida albicans* kill rates. The third research hypothesis examined thyme essential oil compared to caspofungin and fluconazole on *Candida albicans* kill rates. It was hypothesized that the use of the essential oils would have a significant difference in kill rates compared to the standard antifungal medications. The fourth hypothesis was used to evaluate the combined lemongrass, oregano, and thyme essential oils compared to standard antifungal medications. It was hypothesized that the combined use of essential oils would have a significant difference in kill rate compared to

caspofungin and fluconazole. A two-way repeated measures ANOVA was used to evaluate what impact the individual oils had on the organism in culture when compared to only exposing the culture to antifungal medications. This was used to assess what impact each oil had on the treatment, what impact the combined use of the oils had on the treatment efficacy, how the main effects interacted, and what effect these interactions had on the *Candida* infection. The fifth hypothesis was used to investigate the combined use of lemongrass, oregano, and thyme essential oils in conjunction with caspofungin and fluconazole. It was hypothesized that there would be a significant difference in kill rates compared to the use of antifungal alone. Again, a two-way repeated measures ANOVA was used to compare the groups. The sixth research hypothesis was used to test how the combined use of lemongrass, oregano, and thyme essential oils compared to caspofungin and fluconazole to inhibit germ tube production. It was hypothesized that there would be a significant difference between the combined essential oils and the antifungal medications on germ tube production. This comparison was evaluated using a paired samples *t*-test. The final research hypothesis was used to assess the covariates of age, gender, race, and comorbidities as risk factors for *Candida albicans* bloodstream infections. A univariate analysis was used to determine the frequency of how many people in the study population belong to which age group then a binomial logistic regression and multinomial logistic regression were used to study the relationship between covariates and infections (Burkholder, n.d.).

Ethical Concerns

The hospital where the isolates were collected provided a template for informed consent that was submitted to the hospital's IRB prior to research approval. This template as well as the IRB assured that the patients' identities would be protected, the patients would not be discriminated against, and that the patients or their families would not be contacted in relation to this study (University of Colorado Health [UCH], n.d.). The data collected was entered into a UCH IRB approved computerized spreadsheet with the de-identified information stored on a password protected computer that is only accessible by myself and maintained in a secure area with limited access. The data has been maintained for the duration of the research and no patient identifiers were associated with the data. To reduce potential bias from the investigator on the efficacy of treatment, the specimens were blinded using a coded sheet and the de-identified patient demographics were not available while performing the experimental portion of the study.

Summary

Using a rigorous experimental study will enable healthcare providers to evaluate the research and determine whether this treatment addition is effective enough to be used for further research using volunteers as well as put into future recommendations as an improvement in best practices. This research was developed using an experimental design and evaluating the risk for *Candida albicans* bloodstream infections based on gender, race, age group, and co-morbidities. The effectiveness of treatment options was evaluated in attempts to expand into future research for improving clinical outcomes and

reducing expenses related to long-term treatment of critically ill patients with *Candida* infections.

This chapter outlined the selection of an experimental study design and the rationale to investigate risks associated with gender, race, age, and co-morbidities. The study variables, data access, and data collection were also addressed. Results from this study will be compiled in Chapter 4 and discussed in Chapter 5

Chapter 4: Results

Introduction

The purpose of this study was to evaluate the effect of lemongrass, oregano, and thyme essential oils *on Candida albicans*' ability to grow in the presence of each individual oil and the three oils combined in comparison to the antifungal treatments of fluconazole and caspofungin. In addition, *Candida albicans*' ability to produce germ tube virulence factors when exposed to the combined essential oils, caspofungin, and fluconazole was compared to the ability to produce germ tubes without any treatment. The gender, race, age group, and comorbidities of patients with *Candida albicans* bloodstream infections were also examined for potential risk factors in comparison to a control group that had blood cultures drawn during the same time-period but were negative for *Candida albicans* bloodstream infections. An experimental method was used to create and evaluate data for the first six research questions. A retrospective quantitative method was used to analyze secondary data collected from medical records of patients who had been hospitalized between January 2009 through August 1, 2017. Statistical analyses were performed to address the research questions on study variables and coding that were created during this study.

In this chapter, I describe the results from the following analyses: two-way repeated measures ANOVA to measure the dependent variable of *Candida albicans* kill rates over time when exposed to each study variable in the first five research questions; descriptive statistics for virulence factors and patient demographics; binomial logistic regression to examine patient demographics for risk factors; and multiple logistic

regression for potential confounders, such as age, gender, race, and comorbidities including kidney failure, organ tumor cancer, and multiple organ failures.

Chapter 4 is organized into four sections. In the first section, I describe the data collection, discrepancies from the initial plan in Chapter 3, the demographic and descriptive analyses of the data collected, external validity, and the results of the univariate analysis to justify the inclusion of the covariates in the evaluation of patient demographics. In the next section, I show the treatment methods, problems encountered, and how these problems were addressed. In the third section, I reported the analyses of the statistical testing that was performed, assumptions that were addressed, as well as the additional analysis used to address the examination of multiple comorbidities. I then summarize the information presented in this chapter and leads into the transition to discuss the results from the analyses in Chapter 5.

Data Collection

Descriptive statistical analyses were used to draw conclusions from the samples tested. SPSS Version 24 was used to code and examine frequencies of data, relationships between variables, and odds ratios for potential risk factors. Two-way repeated measures ANOVA, descriptive statistics, and binomial and multiple logistic regressions were used to evaluate the seven research questions and related hypotheses.

1. RQ1: How does the use of lemongrass essential oil compare to standard antifungal medications for *Candida albicans* kill rates in laboratory testing?

H_0^1: There is no significant difference in kill rates for the use of lemongrass essential oil compared to standard antifungal medications for *Candida albicans* in laboratory testing.

H_a^1: There is a significant difference in kill rates for the use of lemongrass essential oil compared to standard antifungal medications for *Candida albicans* in laboratory testing.

- Dependent variable: *Candida albicans* kill rates
- Independent variable: treatment at set time intervals (lemongrass exposure at 1,4,8,12, and 24 hours; caspofungin exposure at 1,4,8,12, and 24 hours; and fluconazole exposure at 1,4,8,12, and 24 hours).

2. RQ2: How does the use of oregano essential oil compare to standard antifungal medications for *Candida albicans* kill rates in laboratory testing?

H_0^2: There is no significant difference in kill rates for the use of oregano essential oil compared to standard antifungal medications for *Candida albicans* in laboratory testing.

H_a^2: There is a significant difference in kill rates for the use of oregano essential oil compared to standard antifungal medications for *Candida albicans* in laboratory testing.

- Dependent variable: *Candida albicans* kill rates
- Independent variable: treatment at set time intervals (oregano exposure at 1,4,8,12, and 24 hours; caspofungin exposure at 1,4,8,12, and 24 hours; and fluconazole exposure at 1,4,8,12, and 24 hours).

3. RQ3: How does the use of thyme essential oil compare to standard antifungal medications for *Candida albicans* kill rates in laboratory testing?

H_0^3: There is no significant difference in kill rates for the use of thyme essential oil compared to standard antifungal medications for *Candida albicans* in laboratory testing.

H_a^3: There is a significant difference in kill rates for the use of thyme essential oil compared to standard antifungal medications for *Candida albicans* in laboratory testing.

- Dependent variable: *Candida albicans* kill rates
- Independent variable: treatment at set time intervals (thyme exposure at 1,4,8,12, and 24 hours; caspofungin exposure at 1,4,8,12, and 24 hours; and fluconazole exposure at 1,4,8,12, and 24 hours).

4. RQ4: How does the combined use of lemongrass, oregano, and thyme essential oils compare to standard antifungal medications for *Candida albicans* kill rates in laboratory testing?

H_0^4: There is no significant difference in kill rates for the combined use of lemongrass, oregano, and thyme essential oils compared to standard antifungal medications for *Candida albicans* in laboratory testing.

H_a^4: There is a significant difference in kill rates for the combined use of lemongrass, oregano, and thyme essential oils compared to standard antifungal medications for *Candida albicans* in laboratory testing.

- Dependent variable: *Candida albicans* kill rates

- Independent variable: treatment at set time intervals (combined lemongrass, oregano, and thyme exposure at 1,4,8,12, and 24 hours; caspofungin exposure at 1,4,8,12, and 24 hours; and fluconazole exposure at 1,4,8,12, and 24 hours).

5. RQ5: How does the combined use of lemongrass, oregano, and thyme essential oils when used with standard antifungal treatments compare to standard antifungal medications for *Candida albicans* kill rates in laboratory testing?

   H_0^5: There is no significant difference in kill rates for the combined use of lemongrass, oregano, and thyme essential oils when used with standard antifungal medications compared to standard antifungal medications for *Candida albicans* in laboratory testing.

   H_a^5: There is a significant difference in kill rates for the combined use of lemongrass, oregano, and thyme essential oils when used with standard antifungal medications compared to standard antifungal medications for *Candida albicans* in laboratory testing.

   - Dependent variable: *Candida albicans* kill rates
   - Independent variable: treatment at set time intervals (combined lemongrass, oregano, and thyme with caspofungin exposure at 1,4,8,12, and 24 hours, and combined lemongrass, oregano, and thyme with fluconazole exposure at 1,4,8,12, and 24 hours).

6. RQ6: How does the combined use of lemongrass, oregano, and thyme essential oils compare to standard antifungal medications for disruption of the virulence factor of germ tube production of *Candida albicans* in laboratory testing?

H_0^6: There is no significant difference in the combined use of lemongrass, oregano, and thyme essential oils compared to standard antifungal medications for disruption of the virulence factor of germ tube production of *Candida albicans* in laboratory testing.

H_a^6: There is a significant difference in the combined use of lemongrass, oregano, and thyme essential oils compared to standard antifungal medications for disruption of the virulence factor of germ tube production of *Candida albicans* in laboratory testing.

- Dependent variable: germ tube production
- Independent variable: non-exposure; exposure to lemongrass, oregano, and thyme; exposure to caspofungin; and exposure to fluconazole.

7. RQ7: How do the covariates age, gender, race, or comorbidities contribute to the risk of *Candida albicans* bloodstream infections?

H_0^7: Age, gender, race, or comorbidities do not significantly contribute to an increased risk of *Candida albicans* bloodstream infections.

H_a^7: Age, gender, race, or comorbidities do significantly contribute to an increased risk of *Candida albicans* blood stream infections.

- Dependent variable: *Candida albicans* bloodstream infections
- Independent variable: age, race, gender, and comorbidities

Prior to analyzing the research questions, accuracy of coding the data in SPSS was verified, and I confirmed that appropriate statistical assumptions were met. Normality was determined and met for the variables in the first six research questions using a Q-Q plot of studentized residual for time-treatment combinations. Mauchly's test for sphericity indicated that the assumption for sphericity had been violated for the two-way interaction between time and treatment so a correction for bias was made using a Greenhouse-Geisser correction. Summary details of the variables and tests used to evaluate the hypotheses are displayed in Table 1.

Table 1 *Summary Details of Variables and Analyses used to Evaluate Hypotheses*

Hypotheses	Independent Variable	Dependent Variable	Analysis
1-5	*Candida albicans* kill rates	Treatment at time intervals	Repeated measures ANOVA
6	Germ tube production	LTO vs Caspofungin vs Fluconazole	Chi-square Test of Independence
7	CABSI	Age, race, gender, comorbidities	Binomial logistic regression

Note. CABSI: *Candida albicans* bloodstream infections
LTO: Lemongrass, thyme, oregano essential oils combined

Descriptive Statistics

The initial criteria for selecting study participants were patients hospitalized between January 2009 and August 1, 2017, in the age range of 18 through 74, who acquired *Candida albicans* bloodstream infections during their length of stay at the hospital. Once the number of patients during that time frame were gathered, the same number of patients during the time period with blood cultures collected during a hospital stay but negative for *Candida albicans* bloodstream infections were also collected. From

the 120 positive samples, the total number of *Candida albicans* isolates that were able to be recovered from the frozen archives were 117. Although this was one less than the G*Power analysis requirements of 118 for an effect size 0.25, using 117 still fit the analysis for a power (1-β) =1.0 and an α err of 0.05. For the patients with *Candida albicans* bloodstream infections during the collection time, a total of 107 patients were in the age range of 18-74. Analysis of 113 randomized hospitalized patients with blood cultures during the same period who did not have *Candida albicans* infections and were in the selected age range was also performed. Using a G*Power analysis for logistic regression with the total sample size for the patient demographics of 220, the power (1-β err prob) = 0.992. Due to time and space constraints, the experiments to investigate the first six research questions were divided into five groups. Initial tests were performed on 20 isolates with seven of the test levels (lemongrass, thyme, oregano, combined oils, fluconazole, and combined oils with fluconazole). On this first day, time to kill rates were collected at 1, 4, 8, 12 hours, and the following day at 24 hours. These inoculated plates were incubated at 35°C, and colony counts were recorded at 24 hours and then again at 48 hours to more accurately ensure organism death versus inhibition. On the second day, an additional 20 isolates were tested with these initial incubation times overlapping with the first set of isolates incubation period. With this many culture plates, after the initial 24 hours of incubation and examination for colony growth, the 24-48-hour culture plate incubation was performed at 25°C instead of 35°C. This lower incubation does not impact the colony growth, only the size of the colonies. All colony counts were examined using a lighted magnifying lens to ensure colonies were correctly counted.

Incubation of all the isolates in TSB were performed at 35°C for the duration of the experiment. Additional isolate groups were performed on subsequent weekends when adequate space and time were available. Due to delivery difficulties of the antifungal, caspofungin, all 117 isolates were tested against caspofungin and caspofungin with the combined essential oils on the same weekend.

The test for the virulence factor of germ tube production was conducted during these same time-frames. For the 117 isolates, 108 (92.3%) produced germ tubes in the germ tube test medium alone; when the combined essential oils of lemongrass, thyme, and oregano were added to germ tube test medium, 116 isolates did not produce germ tubes and the germ tubes of the remaining isolate were abnormally large and deformed. Adding caspofungin to the germ tube medium produced germ tubes in 110 of the isolates (94.0%). The addition of fluconazole to the germ tube medium also resulted in 110 of the isolates producing germ tubes. Adding caspofungin or fluconazole promoted germ tube production in two additional isolates that did not produce germ tubes in the initial germ tube medium only. These additional germ tube producing isolates were not the same isolates for both fluconazole and caspofungin but rather four separate isolates. Seven days following each set of experimental testing, each TSB broth was also sub-cultured again to SabDex and examined for growth after 48 hours incubation at 35°C. None of the isolates that had been exposed to any essential oil treatment in the TSB broth, including those combined with the antifungals, were able to grow after 48 hours on the SabDex medium. All the isolates that had been exposed to only caspofungin or fluconazole were still viable for culture after seven days of exposure to the antifungals. When data

collection was completed for the experimental portion of the study, patient demographics were collected.

While collecting patient demographics, any comorbidity listed upon admission was documented. After all the demographics were collected for the test and control groups, a binomial logistic regression was performed to investigate the effects of age, race, gender, and comorbidities on the likelihood of acquiring *Candida albicans* bloodstream infections after admission. There were 15 individual comorbidities with multiple patients having more than one comorbidity. The initial individual comorbidities examined were diabetes, HIV/AIDS, kidney/renal failure, solid organ tumor cancer, haemotologic malignancy, substance abuse, abdominal surgery, severe burns, organ transplant, stem cell transplant, other surgeries, soft tissue cancer, autoimmune disorder, multiple organ failure, and the rest were combined into an "other" category (see Table 2).

Table 2 *Patient Data Collection*

		CABSI – Yes	CABSI – No
Number of cases (n)		107	113
Age Group	18-24	5 (4.7)	5 (4.4)
	25-34	24 (22.4)	7 (6.2)
	35-44	16 (14.9)	19 (16.8)
	45-54	19 (17.8)	22 (19.5)
	55-59	11 (10.3)	19 (16.8)
	60-64	12 (11.2)	11 (9.7)
	65-74	20 (18.7)	30 (26.6)
Gender	Male	58 (54.2)	69 (61.1)

Table 2 continued		CABSI-Yes	CABSI-No
	Female	49 (45.8)	44 (38.9)
Race	White	69 (64.5)	62 (54.9)
	Black	10 (9.3)	23 (20.4)
	Hispanic	16 (15.0)	17 (15.0)
	Other/Unknown	12 (11.2)	11 (9.7)
Comorbidities	Diabetes	21 (19.6)	23 (20.4)
	Kidney Failure	41 (38.3)	25 (22.1)
	Solid Tumor Cancer	27 (25.2)	14 (12.4)
	Haemotologic Malig,	1 (0.9)	3 (2.6)
	Substance Abuse	8 (7.5)	8 (7.1)
	Abdominal Surgery	11 (10.3)	5 (4.4)
	Burns	4 (3.7)	2 (1.8)
	Organ Transplant	14 (13.1)	13 (11.5)
	HIV/AIDS	1 (0.9)	3 (2.6)
	Stem Cell Transplant	0 (0)	2 (1.8)
	Other Surgery	6 (5.6)	6 (5.3)
	Soft Tissue Cancer	4 (3.7)	0 (0)
	Autoimmune Disorder	5 (4.7)	0 (0)
	Multi Organ Failure	9 (8.4)	25 (22.1)

Note: CABSI: *Candida albicans* bloodstream infection

While this model correctly classified 75% of the cases and explained 43% (Nagelkerke R^2) of the variance in *Candida albicans* bloodstream infections, the effect of individual comorbidities as potential confounders was unclear. Data for the

comorbidities were then reclassified individually and a multinomial regression was performed to assess potential confounders.

There were 49 females out of 107 patients in the test group (45.8%) with a slightly lower amount of 44 females in the control group of 113 (38.9%). The most CABSI were in the age group 25-34 and the highest number for the control subjects was in the 65-74 age group (see Figure 3).

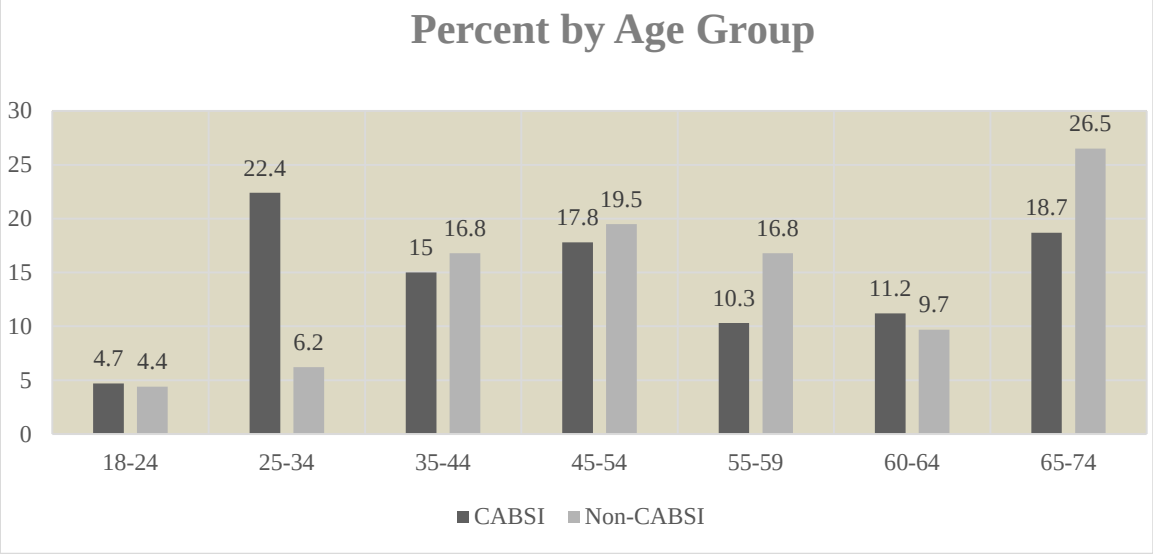


Figure 3. Percent of CABSI and non-CABSI by age group.

Race was categorized based upon hospital demographics of White, Black, Hispanic, or other/unknown. The other category includes records where race/ethnicity were entered as unknown and for other groups which were in small numbers where de-identification would be difficult. Most of the patients in the test and control groups seen at the hospital during this collection period were listed as White. Positive for CABSI group had 64.5% listed as White and the control group (non-CABSI) had 54.9% listed as White (see Figure 4).

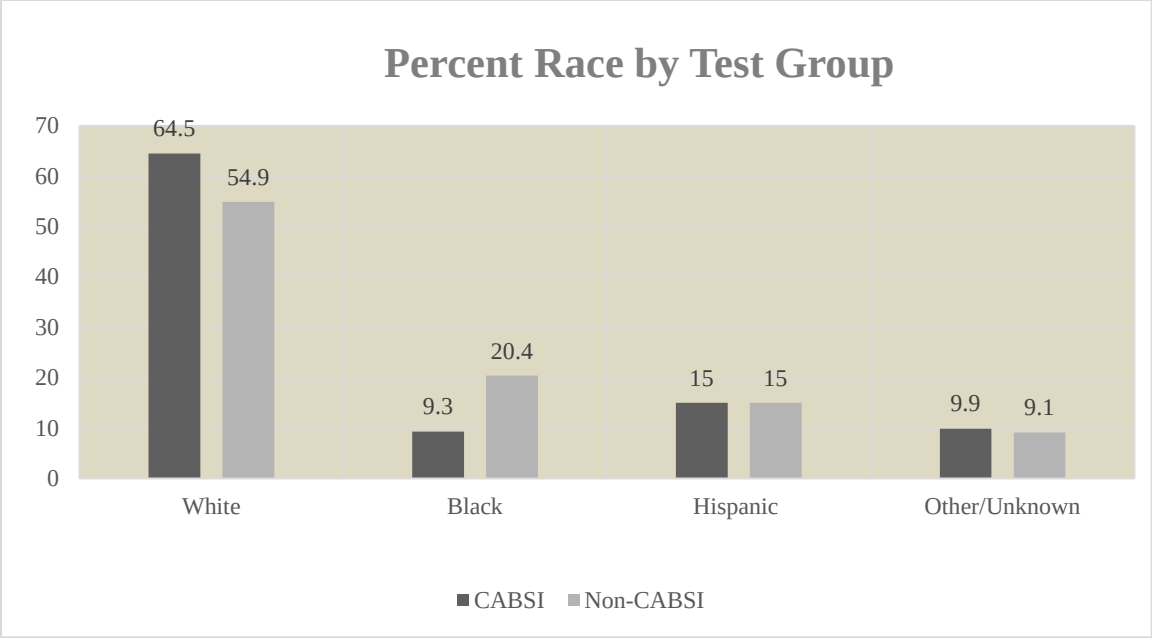


Figure 4. Percent of CABSI and non-CABSI by race.

Categorizing comorbidities as individual variables and calculating a Chi-square test for association for each comorbidity with CABSI showed that kidney failure, solid organ tumor cancer, autoimmune disorder, and multiple organ failure all had a statistically significant association with CABSI however, for the autoimmune disorder, not all expected cell frequencies were greater than 5 and therefore not included in the final analyses. A multiple logistic regression was then performed on the combined control and test groups for the comorbidities of kidney failure, solid organ tumor cancer, and multiple organ failure. Combining the control group with the test group to the analysis provided additional data points to determine whether any of these factors could be determined to be statistically significant potential risk factors.

Analyses of Tests for Hypotheses

Hypotheses 1-5 were evaluated using two-way repeated measures ANOVA to determine if any statistically significant differences existed between treatment types over a 24-hour time-frame. Hypothesis 6 was evaluated using a Chi-square test for independence and to examine hypothesis 7, a binomial logistic regression was utilized followed by a multinomial logistic regression to investigate potential confounders. Specifically, the dependent variable for hypotheses 1-5 was the kill rates for *Candida albicans* when exposed to the independent variables of treatment. Hypothesis 6 had a dependent variable of germ tube production and hypothesis 7 had a dependent variable of *Candida albi*cans bloodstream infection.

The independent variables for hypotheses 1-5 were treatment with lemongrass, oregano, thyme, combined essential oils, caspofungin, fluconazole, caspofungin with the combined essential oils, and fluconazole with the combined essential oils. The independent variables for hypothesis 6 were no treatment, treatments with combined essential oils, fluconazole, or caspofungin. The independent variables for hypothesis 7 included age, race, gender, and comorbidities. Initial comorbidities included 12 variables with only three with statistically significant results.

Results of Hypothesis 1

$H_0{}^1$: There is no significant difference in kill rates for the use of lemongrass essential oil compared to standard antifungal medications for *Candida albicans* in laboratory testing.

H_a^1: There is a significant difference in kill rates for the use of lemongrass essential oil compared to standard antifungal medications for *Candida albicans* in laboratory testing.

Using SPSS 24, a two-way repeated measures ANOVA was conducted to determine whether statistically significant differences existed between treatment with lemongrass, caspofungin, and fluconazole over five time-measurements. Results of mean colony counts after a 24-hour plate incubation, indicated that at each exposure interval, there was a lower mean colony count for lemongrass compared to exposure to caspofungin and fluconazole. After an 8-hour exposure to lemongrass, the colony count mean dropped to 0, the caspofungin colony count continued to lower, but after an 8-hour exposure to fluconazole, the mean colony count increased above the 4- hour exposure. Twelve- hour exposure to lemongrass again resulted with a mean of 0 colonies, colony counts for caspofungin rose slightly, and fluconazole mean colony counts decreased again. At the end of 24-hour exposure times, colony counts for exposure to lemongrass remained at 0, caspofungin rose slightly, and colony counts for exposure to fluconazole increased substantially (see Figure 5).

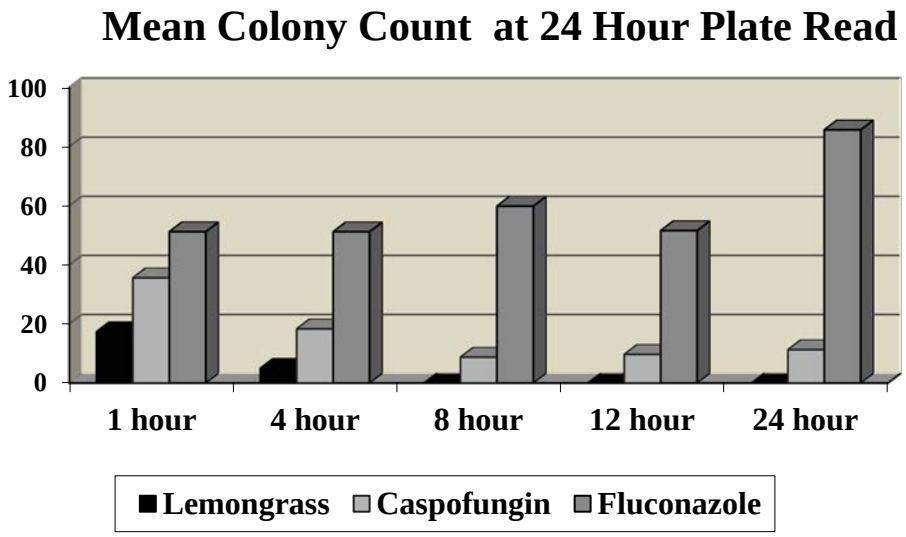


Figure 5. Mean colony count of lemongrass, caspofungin, and fluconazole at 24hr.

The agar plates for these colony counts were then re-incubated for another 24 hours. Results of mean colony counts after the 48-hour plate incubation, indicated that at each exposure interval, there was a lower mean colony count for lemongrass compared to exposure to caspofungin and fluconazole. For the 8-hour exposure to lemongrass, the colony count mean stayed at zero. The caspofungin colony count was also lower, but the 8-hour exposure to fluconazole, agar plate at 48 hours incubations was above the 4-hour exposure count similar to the results after 24 hours incubation. Twelve- hour exposure to lemongrass again resulted with a mean of 0 colonies, colony counts for caspofungin rose slightly, and fluconazole mean colony counts decreased again. At the end of 24-hour exposure times, colony counts for exposure to lemongrass remained at 0, caspofungin

rose slightly, and colony counts for exposure to fluconazole increased substantially (see Figure 6).

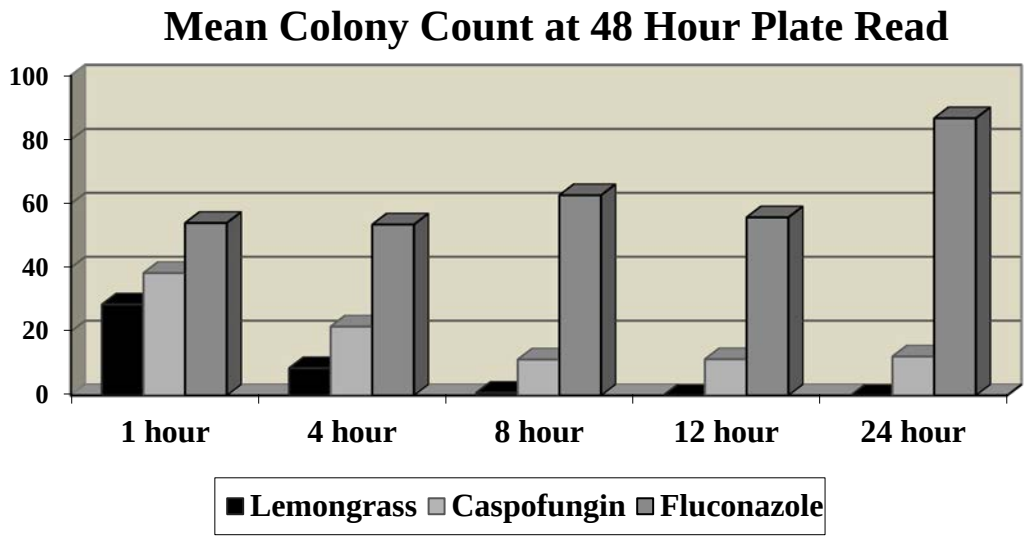


Figure 6. Mean colony count of lemongrass, caspofungin, and fluconazole at 48hr.

Mean colony count results for 1-hour, 4-hour, 8-hour, 12-hour, and 24-hour exposure to lemongrass, caspofungin, and fluconazole at 24-hour plate incubation and 48-hour plate incubation are listed in Table 3.

Table 3 *Mean Colony Count After Exposure to Lemongrass, Caspofungin, and Fluconazole*

Treatment Exposure Time	Lemongrass		Caspofungin		Fluconazole	
	24hr plate incubation	48hr plate incubation	24hr plate incubation	48hr plate incubation	24hr plate incubation	48hr plate incubation
1 hour	18.17	28.48	36.66	38.31	50.59	53.97
4 hour	5.22	8.54	19.13	21.56	50.95	53.47
8 hour	0	0	9.21	11.25	58.77	62.65
12 hour	0	0	9.71	11.35	51.58	55.73
24 hour	0	0	11.60	12.19	85.26	86.73

Results for lemongrass exposure after the increased plate incubation time showed an increase in mean colony counts for the 1-hour and 4-hour exposure times. This

increase in mean colony counts could indicate that at these lower exposure times the organisms are being inhibited and are not completely killed. However, the 8-hour, 12-hour, and 24-hour exposures to lemongrass maintained 0 mean colony counts which would indicate that after sufficient exposure to lemongrass, the organisms are no longer viable.

Caspofungin also had higher colony counts after 48-hours plate incubation, verifying the antifungal inhibition properties. The mean colony counts for caspofungin at 24-hours and 48-hours plate incubation followed the same pattern of decreasing colony count at 4-hours and 8-hours but beginning to increase again after 12 hours of exposure. Caspofungin is considered to be fungicidal however, the organisms were not killed in the time-frame of these experiments. Without additional antifungal being added, the organisms were able to overcome the inhibition action of caspofungin and continue to proliferate.

Fluconazole had higher mean colony counts at the 24-hour and 48-hour plate incubations. Interestingly, the mean colony counts for *Candida albicans* with fluconazole exposure decreased at the 4-hour and 12-hour exposure but not at the 8-hour exposure and then had a substantial increase after 24 hours of exposure. The increase in the mean colony counts at 8-hours of exposure were duplicated at the 24-hour and 48-hour plate incubations. Clearly fluconazole is shown to begin to inhibit the organisms, however this spike at 8 hours and jump at 24 hours may indicate that the medication may not be able to sustain inhibition after 4 hours and drug dosing could become very important with treatment options. Overall lemongrass had a lower mean colony count

than both caspofungin or fluconazole for all exposure times at the 24-hour and 48-hour plate incubations.

The mean kill rates of lemongrass essential oil were statistically significant over time, $F(1.617, 187.191)= 40.865$, $p<.0005$. Test of within subject effect using a Greenhouse-Geisser correction for the time and treatment interaction effect is .000 ($p<.0005$). There was a statistically significant interaction between lemongrass treatment and time (see Table 4).

Table 4 *Tests of With-In Subjects of Lemongrass Treatment Over Time*

Source	df	F	Sig.
Lemongrass	1.617	40.865	<.001
Error (Lemongrass)	187.191		

Over the time that *Candida albicans* isolates were exposed to lemongrass the ability for the organisms to survive and proliferate decreased until at 8-hours of exposure, the organisms were no longer viable. Using a pairwise comparison to measure lemongrass exposure to caspofungin exposure, there was a colony count mean difference of -12.582, 95% *CI* [-17.429 to -7.735], $p<.0001$ for lemongrass exposure compared to caspofungin exposure. For the pairwise comparison of lemongrass exposure to fluconazole exposure, the mean colony count difference was -54.50, 95% *CI* [-62.108 to -47.392], $p<.0001$ (see Table 5).

Table 5 *Pairwise Comparison Between Treatments*

(I) to (J) treatment	Mean Difference (I-J)	Std. Error	Sig.	95% CI
Lemongrass to Caspofungin	-12.582	1.514	<.001	-17.429, -7.735
Lemongrass to Fluconazole	-54.750	2.299	<.001	-62.108, -47.392

Lemongrass exposure had a statistically significant lower mean colony count after exposure when compared to caspofungin and fluconazole. Therefore, the null hypothesis can be rejected in favor of the alternate hypothesis.

Results of Hypothesis 2

H_0^2: There is no significant difference in kill rates for the use of oregano essential oil compared to standard antifungal medications for *Candida albicans* in laboratory testing.

H_a^2: There is a significant difference in kill rates for the use of oregano essential oil compared to standard antifungal medications for *Candida albicans* in laboratory testing.

A two-way repeated measures ANOVA was conducted to determine whether statistically significant differences existed between treatment with oregano, caspofungin, and fluconazole over five time-measurements. Results indicated that at each time exposure to oregano, there was a mean colony count of 0. Exposure to caspofungin and fluconazole were discussed under Hypothesis 1 results. Results of mean colony counts after a 24-hour plate incubation, indicated that at each exposure interval, there was an

obvious lower mean colony count for oregano compared to exposure to caspofungin and fluconazole (see Figure 7).

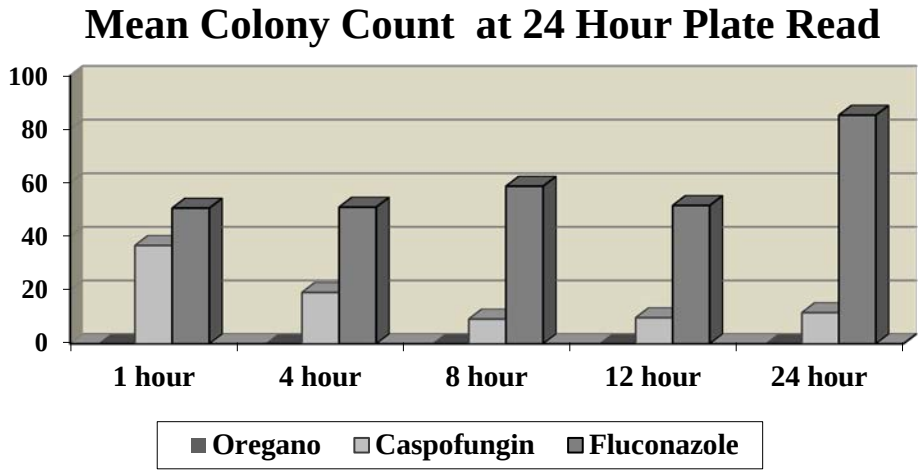


Figure 7. Mean colony count of oregano, caspofungin, and fluconazole at 24hr.

The agar plates for these colony counts were then re-incubated for another 24 hours. The 48-hour growth for colony counts after 1-hour exposure to oregano increased slightly to .05. All other time exposures with oregano had a mean colony count of 0 which was consistent with the 24-hour plate incubation. Caspofungin and fluconazole exposures were previously discussed in Hypothesis 1 results. (see Figure 8).

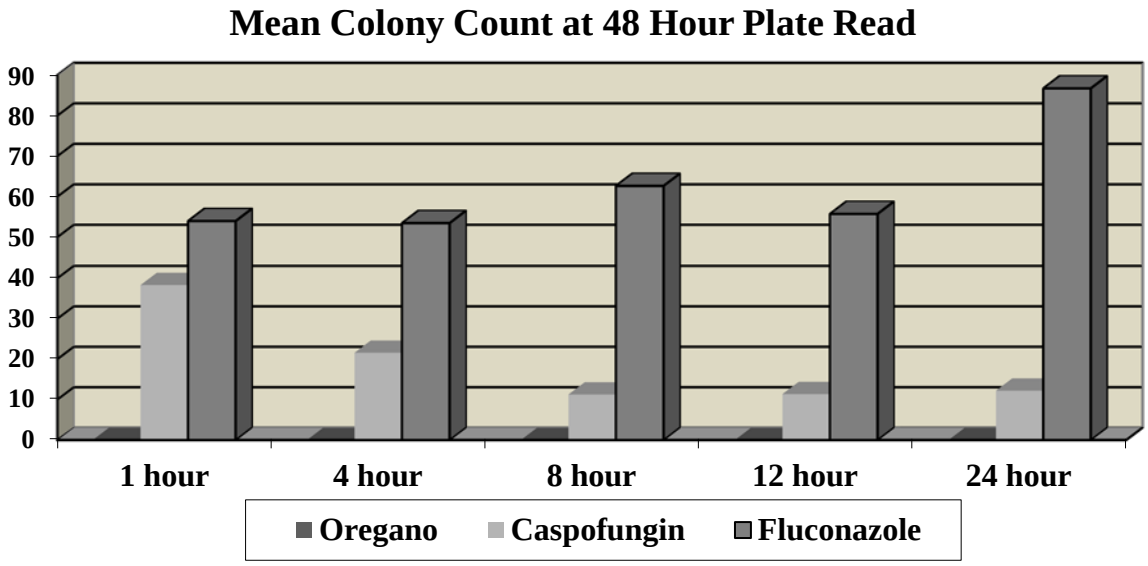


Figure 8. Mean colony count of oregano, caspofungin, and fluconazole at 48hr.

As indicated in Table 6, overall, oregano had a lower mean colony count than both caspofungin or fluconazole for all exposure times at the 24-hour and 48-hour plate incubations.

Table 6 *Mean Colony Count After Exposure to Oregano, Caspofungin, and Fluconazole*

Treatment Exposure Time	Oregano		Caspofungin		Fluconazole	
	24hr plate incubation	48hr plate incubation	24hr plate incubation	48hr plate incubation	24hr plate incubation	48hr plate incubation
1 hour	0	0	36.66	38.31	50.59	53.97
4 hour	0	0	19.13	21.56	50.95	53.47
8 hour	0	0	9.21	11.25	58.77	62.65
12 hour	0	0	9.71	11.35	51.58	55.73
24 hour	0	0	11.60	12.19	85.26	86.73

The mean kill rates of oregano essential oil were not statistically significant over time, $F(1, 116)= 1.813$, $p=.181$. This is due to the lack of colony growth throughout the exposure to oregano. While statistically this exposure over time for oregano is not

significant, the fact that oregano killed off the *Candida albicans* isolates in such a short exposure time provides an area to determine with future research whether this in-vitro phenomenon would replicate in-vivo.

Using a pairwise comparison to measure oregano exposure to caspofungin exposure, there was a colony count mean difference of –17.261, 95% *CI* [-21.499 to -13.023], $p<.0001$ for oregano exposure compared to caspofungin exposure. For the pairwise comparison of oregano exposure to fluconazole exposure, the mean colony count difference was -59.429, 95% *CI* [-67.025 to-51.832], $p<.0001$ (see table 7).

Table 7 *Pairwise Comparison Between Treatments*

(I) to (J) treatment	Mean Difference (I-J)	Std. Error	Sig.	95% CI
Oregano to Caspofungin	-17.261	1.324	<.001	-21.499, -13.023
Oregano to Fluconazole	-59.429	2.373	<.001	-67.025, -51.832

Oregano exposure had a statistically significant lower mean colony count after exposure when compared to caspofungin and fluconazole. Therefore, the null hypothesis can be rejected in favor of the alternate hypothesis.

Results of Hypothesis 3

H_0^3: There is no significant difference in kill rates for the use of thyme essential oil compared to standard antifungal medications for *Candida albicans* in laboratory testing.

H_a^3: There is a significant difference in kill rates for the use of thyme essential oil compared to standard antifungal medications for *Candida albicans* in laboratory testing.

A two-way repeated measures ANOVA was conducted to determine whether statistically significant differences existed between treatment with thyme, caspofungin, and fluconazole over five time-measurements. Mean colony count results for 1-hour, 4-hour, 8-hour, 12-hour, and 24-hour exposure to thyme, caspofungin, and fluconazole at 24-hour plate incubation and 48-hour plate incubation are listed in Table 8. Results indicated that at 8, 12 and 24-hour time exposure to thyme, there was a mean colony count of 0. Exposure to caspofungin and fluconazole were discussed under Hypothesis 1 results. Results of mean colony counts after a 24-hour plate incubation, indicated that at each exposure interval, there was a lower mean colony count for thyme compared to exposure to caspofungin and fluconazole (see Figure 9).

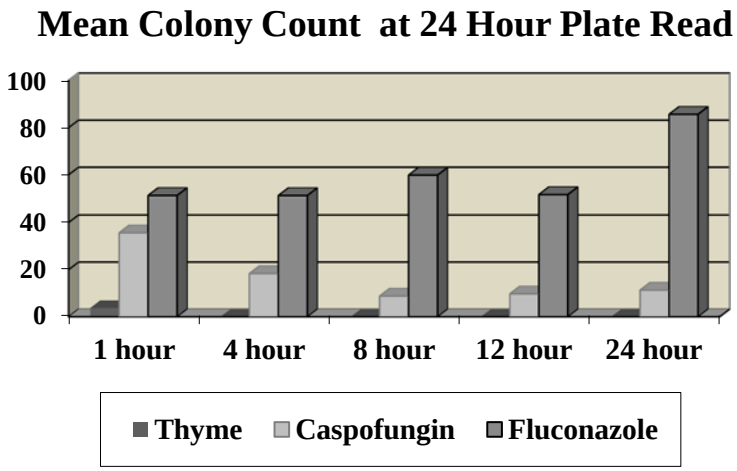


Figure 9. Mean colony count of thyme, caspofungin, and fluconazole at 24hr.

The agar plates for these colony counts were then re-incubated for another 24 hours. Results for thyme exposure after the increased plate incubation time showed a slight increase in mean colony counts for the 1-hour and 4-hour exposure times. This increase in mean colony counts could indicate that at these lower exposure times the

organisms are being inhibited and are not completely killed. However, the 8-hour, 12-hour, and 24-hour exposures to thyme maintained 0 mean colony counts which would indicate that after sufficient exposure to thyme, the organisms are no longer viable. (see Figure 10).

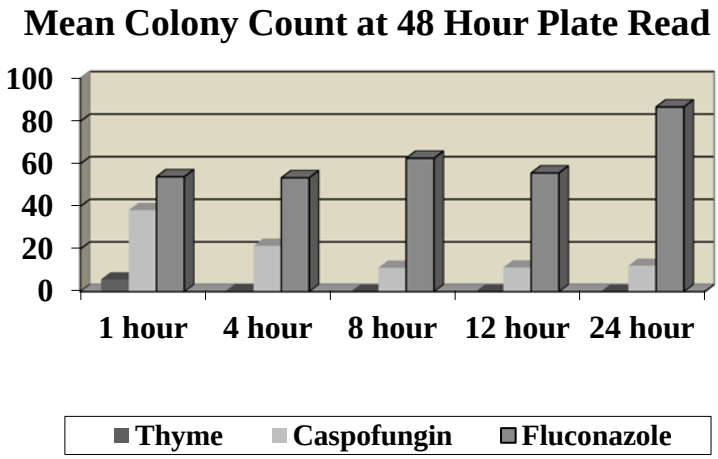


Figure 10. Mean colony count of thyme, caspofungin, and fluconazole at 48hr.

Overall, thyme also had a lower mean colony count than both caspofungin or fluconazole for all exposure times at the 24-hour and 48-hour plate incubations. Caspofungin and fluconazole mean colony counts were previously discussed under Hypothesis 1 results. Thyme mean colony counts were below 1 colony forming unit after the 48-hour plate incubation at the 4-hour exposure, this would indicate that similar to oregano, further testing into in-vivo reactions could provide additional avenues for research (see Table 8).

Table 8 *Mean Colony Count After Exposure to Thyme, Caspofungin, and Fluconazole*

Treatment Exposure Time	Thyme		Caspofungin		Fluconazole	
	24hr plate incubation	48hr plate incubation	24hr plate incubation	48hr plate incubation	24hr plate incubation	48hr plate incubation
1 hour	3.73	5.75	36.66	38.31	50.59	53.97
4 hour	0	0.40	19.13	21.56	50.95	53.47
8 hour	0	0	9.21	11.25	58.77	62.65
12 hour	0	0	9.71	11.35	51.58	55.73
24 hour	0	0	11.60	12.19	85.26	86.73

The mean kill rates of thyme essential oil were statistically significant over time, $F(1.108, 128.571)= 18.339$, $p<.0001$. Test of within subject effect using a Greenhouse-Geisser correction for the time and treatment interaction effect is .000 ($p<.0005$). There was a statistically significant interaction between thyme treatment and time (see Table 9).

Table 9 *Tests of With-In Subjects of Thyme Treatment Over time*

Source	df	F	Sig.
Thyme	1.108	18.339	<.001
Error (Thyme)	128.571		

Over the time that *Candida albicans* isolates were exposed to thyme the ability for the organisms to survive and proliferate decreased until the organisms were no longer viable. Using a pairwise comparison to measure thyme exposure to caspofungin exposure, there was a colony count mean difference of –16.482, 95% *CI* [-20.631 to -12.333], $p<.0001$ for thyme exposure compared to caspofungin exposure. For the pairwise comparison of thyme exposure to fluconazole exposure, the mean colony count difference was -58.650, 95% *CI* [-66.286 to -51.014], $p<.0001$ (see Table 10).

Table 10 *Pairwise Comparison Between Treatments*

(I) to (J) treatment	Mean Difference (I-J)	Std. Error	Sig.	95% CI
Thyme to Caspofungin	-16.482	1.296	<.001	-20.631, -12.333
Thyme to Fluconazole	-58.650	2.386	<.001	-66.286, -51.014

Thyme had a statistically significant lower mean colony count after exposure when compared to caspofungin and fluconazole. Therefore, the null hypothesis can be rejected in favor of the alternate hypothesis.

Results of Hypothesis 4

H_0^4: There is no significant difference in kill rates for the combined use of lemongrass, oregano, and thyme essential oils compared to standard antifungal medications for *Candida albicans* in laboratory testing.

H_a^4: There is a significant difference in kill rates for the combined use of lemongrass, oregano, and thyme essential oils compared to standard antifungal medications for *Candida albicans* in laboratory testing.

A two-way repeated measures ANOVA was conducted to determine whether statistically significant differences existed between treatment of the combined use of lemongrass, oregano, and thyme (LTO), with caspofungin, and fluconazole over five time-measurements. Results indicated that at 1-hour, 4-hour, and 8-hour exposure to LTO, there was colony growth. However, at the 12 and 24-hour exposure the mean colony count for LTO was zero. Exposure to caspofungin and fluconazole were previously discussed under Hypothesis 1 results (see Figure 11).

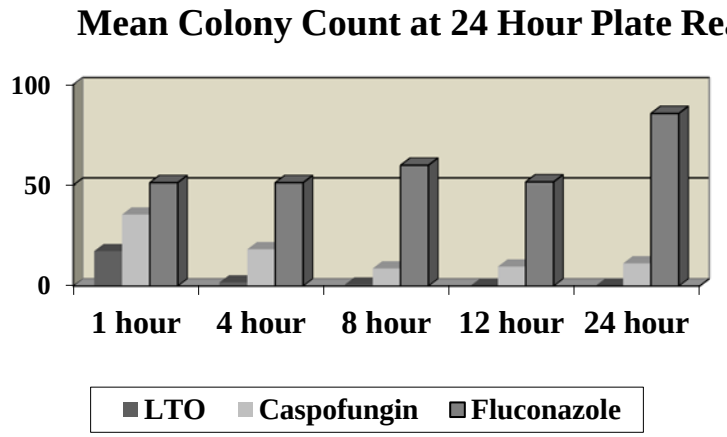


Figure 11. Mean colony counts for lemongrass, oregano, and thyme (LTO), caspofungin, and fluconazole at 24hr.

The agar plates for these colony counts were then re-incubated for another 24 hours. The 48-hour growth for colony counts after 1-hour, 4-hour, and 8-hour exposure to LTO increased slightly. Caspofungin and fluconazole results after the 48-hour plate incubation were previously discussed (see Figure 12).

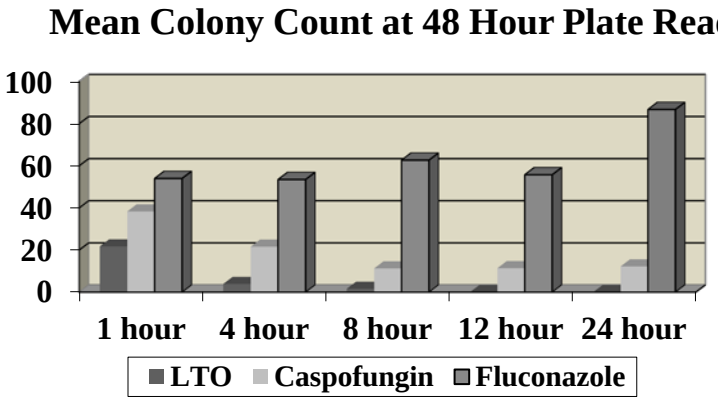


Figure 12. Mean colony counts for lemongrass, oregano, and thyme (LTO), caspofungin, and fluconazole at 48hr.

Overall, LTO had a lower mean colony count than both caspofungin or fluconazole for all exposure times at the 24-hour and 48-hour plate incubations.

Combining the three essential oils seems to have a slightly antagonistic effect on organism death seen with higher mean colony counts at 1-hour, 4-hour and 8-hour exposure. However, at the 12-hour and 24-hour exposure times the effect appears equal to testing each oil individually (see Table 11).

Table 11 *Mean Colony Count After Exposure to LTO, Caspofungin, and Fluconazole*

Treatment	LTO		Caspofungin		Fluconazole	
Exposure Time	24hr plate incubation	48hr plate incubation	24hr plate incubation	48hr plate incubation	24hr plate incubation	48hr plate incubation
1 hour	18.63	21.71	36.66	38.31	50.59	53.97
4 hour	1.83	3.87	19.13	21.56	50.95	53.47
8 hour	0.74	1.57	9.21	11.25	58.77	62.65
12 hour	0	0	9.71	11.35	51.58	55.73
24 hour	0	0	11.60	12.19	85.26	86.73

Note: LTO: Combined Lemongrass, Oregano, and Thyme

The mean kill rates of LTO combined essential oils were statistically significant over time, $F(1.609, 186.643) = 79.476$, $p < .0001$ (see Table 12).

Table 12 *Tests of With-In Subjects of LTO Treatment Over Time*

Source	df	F	Sig.
LTO	1.609	79.476	<.001
Error (LTO)	186.643		

Note: LTO: Combined Lemongrass, Oregano, and Thyme

Using a pairwise comparison to measure LTO exposure to caspofungin exposure, there was a colony count mean difference of –12.995, 95% *CI* [-17.903 to -8.086], $p < .0001$. For the pairwise comparison of LTO exposure to fluconazole exposure, the

mean colony count difference was-55.163, 95% *CI* [-62.454 to -47.871], $p<.0001$ (see Table 13).

Table 13 *Pairwise Comparison Between Treatments*

(I) to (J) treatment	Mean difference (I-J)	Std. Error	Sig.	95% *CI*
LTO to Caspofungin	-12.995	1.533	<.001	-17.903, -8.086
LTO to Fluconazole	-55.163	2.278	<.001	-62.454, -47.871

LTO had a statistically significant lower mean colony count after exposure when compared to caspofungin and fluconazole. Therefore, the null hypothesis can be rejected in favor of the alternate hypothesis.

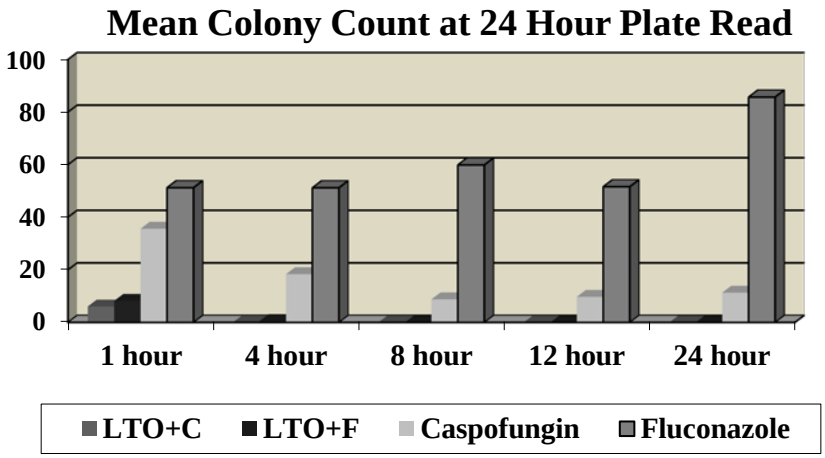
Results of Hypothesis 5

H_0^5: There is no significant difference in kill rates for the combined use of lemongrass, oregano, and thyme essential oils when used with standard antifungal medications compared to standard antifungal medications for *Candida albicans* in laboratory testing.

H_a^5: There is a significant difference in kill rates for the combined use of lemongrass, oregano, and thyme essential oils when used with standard antifungal medications compared to standard antifungal medications for *Candida albicans* in laboratory testing.

A two-way repeated measures ANOVA was conducted to determine whether statistically significant differences existed between treatment of the use of LTO combined with caspofungin and the use of LTO combined with fluconazole, compared to caspofungin, and fluconazole alone over five time-measurements. I found that at 1-hour

exposure to LTO plus caspofungin (LTO+C) and LTO plus fluconazole (LTO+F), mean colony counts were similar to each other but markedly lower than caspofungin or fluconazole alone. Caspofungin and fluconazole mean colony counts were discussed under Hypothesis 1 results at length. The results at 4 hours for both LTO+C exposure LTO+F exposure dropped to mean colony counts below 1. After 8-hour exposure and 12- hour exposure to LTO+C, the mean colony counts stayed close to zero but at 24-hour exposure, the mean colony count rose slightly to 0.51. At 8, 12, and 24-hour exposure to LTO+F, mean colony counts dropped to zero (see Figure 13).

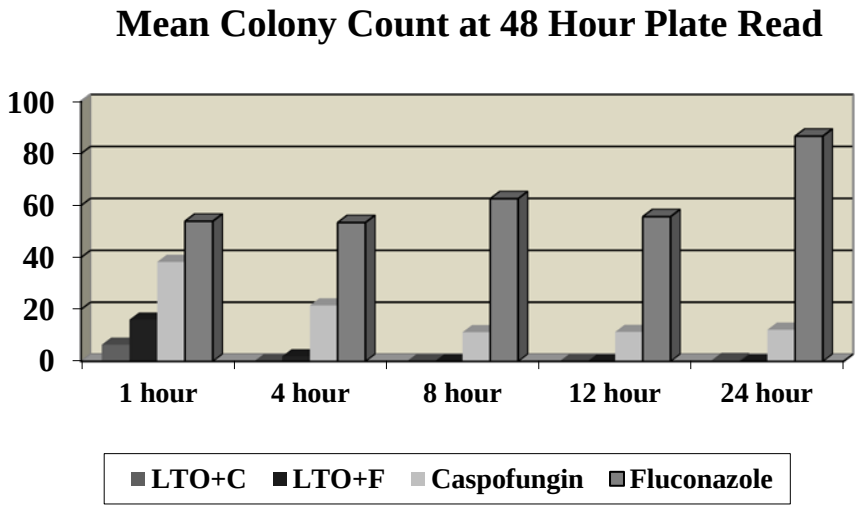


Note: LTO+C: Lemongrass, Oregano, and Thyme combined with Caspofungin
LTO+F: Lemongrass, Oregano, and Thyme combined with Fluconazole

Figure 13. Mean colony counts of LTO+C, LTO+F, caspofungin, and fluconazole at 24 hr.

The agar plates for these colony counts were then re-incubated for another 24 hours. The 48-hour growth for colony counts after 1-hour exposure to LTO+C increased slightly while the LTO+F mean colony count almost doubled. Slight increases after the

48-hour plate incubation were seen at the 4-hour and 8-hour exposure for LTO+C, while maintaining the same mean colony count at 12-hour exposure and raising slightly at 24-hour exposure but still at less than 1 mean colony count. The LTO+F after the 48-hour plate incubation had almost 2 colonies for the mean count but dropped to 0.5 at the 8-hour exposure time and subsequent exposure hours of 12 and 24 maintained a 0 mean colony count (see Figure 14).



Note: LTO+C: Lemongrass, Oregano, and Thyme combined with Caspofungin
LTO+F: Lemongrass, Oregano, and Thyme combined with Fluconazole

Figure 14. Mean colony counts of LTO+C, LTO+F, caspofungin, and fluconazole at 48hr.

Both combinations seemed to be more effective after the 1-hour exposure than LTO alone or caspofungin or fluconazole alone. However, when isolates were exposed to LTO combined with caspofungin there was a less than 1 mean colony count at 4-hours of exposure and this was maintained at subsequent exposure times. A 0 mean colony count was not achieved with LTO+C like it was at 12 and 24-hour exposure with LTO alone (see Table 14).

Table 14 *Mean Colony Count After Exposure to LTO+C, LTO +F, Caspofungin, and Fluconazole*

Treatment Exposure Time	LTO+C		LTO+F		Caspofungin		Fluconazole	
	24hr P.I.	48hr P.I.	24hr P.I.	48hr P.I.	24hr P.I.	48hr P.I.	24hr P.I.	48hr P.I.
1 hour	6.03	7.51	8.18	16.10	36.66	38.31	50.59	53.97
4 hour	.07	.10	.53	1.96	19.13	21.56	50.95	53.47
8 hour	.04	.07	0	.05	9.21	11.25	58.77	62.65
12 hour	.05	.05	0	0	9.71	11.35	51.58	55.73
24 hour	.51	.54	0	0	11.60	12.19	85.26	86.73

Note: P.I.: Plate Incubation. LTO+C: Lemongrass, Oregano, Thyme and Caspofungin
LTO+F: Lemongrass, Oregano, Thyme and Fluconazole

The mean kill rates of LTO+C were statistically significant over time, $F(1.068, 123.887) = 33.079$, $p<.0001$. For LTO+F the mean kill rates were statistically significant over time, F (1.399, 160.871) = 68.639, $p<.0001$ (see Table 15). Indicating that in laboratory testing, combining LTO with either caspofungin or fluconazole increased the cellular death of *Candida albicans* isolates over exposure time.

Table 15 *Tests of With-In Subjects of LTO+C and LTO+F Treatment Over Time*

Source	Df	F	Sig,
LTO + Caspofungin	1.068	33.079	<.001
Error (LTO + Caspo)	123.887		
LTO + Fluconazole	1.399	68.639	<.001
Error (LTO + Fluc)	160.871		

Using a pairwise comparison to measure LTO+C exposure to caspofungin exposure, there was a colony count mean difference of –15.913, 95% *CI* [-20.148 to -11.677], $p<.0001$. For the pairwise comparison of LTO+C exposure to fluconazole exposure, the mean colony count difference was -58.080, 95% *CI* [-65.663 to -50.498],

p<.0001. Using the pairwise comparison for LTO+F to caspofungin exposure, there was a colony count mean difference of -15.502, 95% *CI* [-20.034 to -10.970], *p*<.001. For the pairwise comparison of LTO+F to fluconazole exposure, there was a mean colony count difference of -57.670, 95% *CI* [-64.812 to -50.527], *p*<.001 (see Table 16).

Table 16 *Pairwise Comparison Between Treatments*

(I) to (J) treatment	Mean Difference (I-J)	Std. Error	Sig.	95% CI
LTO+C to Caspofungin	-15.913	1.323	<.001	-20.148, -11.677
LTO+C to Fluconazole	-58.080	2.369	<.001	-65.663, -50.498
LTO+F to Caspofungin	-15.502	1.416	<.001	-20.034, -10.970
LTO+F to Fluconazole	-57.670	2.231	<.001	-64.812, -50.527

Note: LTO+C: Lemongrass, Oregano, and Thyme + Caspofungin
LTO+F: Lemongrass, Oregano, and Thyme + Fluconazole

LTO+C and LTO+F both had a statistically significant lower mean colony count after exposure when compared to caspofungin and fluconazole. Therefore, the null hypothesis can be rejected in favor of the alternate hypothesis.

Results of Hypothesis 6

H_0^6: There is no significant difference in the combined use of lemongrass, oregano, and thyme essential oils compared to standard antifungal medications for disruption of the virulence factor of germ tube production of *Candida albicans* in laboratory testing.

H_a^6: There is a significant difference in the combined use of lemongrass, oregano, and thyme essential oils compared to standard antifungal medications for disruption of the virulence factor of germ tube production of *Candida albicans* in laboratory testing.

To evaluate whether LTO exposure resulted in a significant decrease of germ tube production when compared to caspofungin and fluconazole exposure, a paired samples *t*-

test was used. The frequency of germ tube production with no exposures was 92.3% of the isolates produced germ tubes. Indicating that 7.7% of the *Candida albicans* isolates tested were not able to produce germ tubes in when exposed to media that enhances germ tube production. When exposed to LTO only one isolate produced germ tubes that were abnormally large and deformed this means that 99.1% of the isolates when exposed to LTO were unable to produce germ tubes. When the isolates were exposed to caspofungin, 94.0% of the isolates were still able to produce germ tubes. For exposure to fluconazole, there were also still 94.0% of the isolates able to produce germ tubes. Germ tube testing with each treatment mode was performed simultaneously with the organism and only germ tube media as a control to ensure which organisms were able to produce germ tubes. By performing the testing at the same time, ensured that the organism had not lost the ability to produce germ tubes if the testing had been done with different subcultures of isolates at different times. Including the control group also verified that the organisms were still able to produce germ tubes since testing the caspofungin had to occur at a later date than the LTO and fluconazole testing. The manufacturer also indicates that there should be no more than six cells per 400x field to prevent false negative results. Each testing vial was verified to not be over inoculated (data not shown).

Comparing the ability for the organisms to naturally produce germ tubes in specialized medium (GTM) with exposure to LTO in the specialized medium, a paired samples *t*-test was performed to determine whether there was a significant difference first between GTM and LTO with GTM. The *t*-statistic was significant at the 0.5 critical

alpha level, $t(116) = 30.512$, $p < .0001$. Comparing LTO exposure to caspofungin exposure for germ tube production, the paired samples *t*-test indicated that the *t*-statistic was significant at the 0.5 critical alpha level, $t(116) = -33.495$, $p < .0001$. Comparison of LTO and fluconazole exposure was also performed using a paired samples *t*-test that was significant at the 0.5 critical alpha level, with the same results of $t(116) = -33.495$, $p < .0001$ (see Table 17). Therefore, the null hypothesis can be rejected, and the alternate can be accepted.

Table 17 *Comparison of Germ Tube Production Using Paired Samples t-Test*

Paired Differences	*t*	df	Sig. (2-tailed)	Mean	95% CI (Lower, Upper)
GTM to LTO	30.512	116	.000	.906	.847, .965
LTO to Caspo	-33.495	116	.000	-.923	-.978, -.868
LTO to Fluc	-33.495	116	.000	-.923	-.978, -.868

Results of Hypothesis 7

H_0^7: Age, gender, race, or comorbidities do not significantly contribute to an increased risk of *Candida albicans* bloodstream infections.

H_a^7: Age, gender, race, or comorbidities do significantly contribute to an increased risk of *Candida albicans* blood stream infections.

To determine whether age, gender, race or comorbidities could be risk factors for CABSI, a binomial logistic regression was performed to determine the probability of being in the category of CABSI given the independent variables. Test for linearity was not needed as all the independent variables are nominal and not continuous (Laerd Statistics, 2015). The logistic regression model was statistically significant, $\chi^2(28) = 86.304$, $p < .0001$. The model explained 43% (Nagelkerke R^2) of the variance in CABSI

and correctly classified 75% of the cases. Sensitivity was 78%, specificity was 87%, the positive predictive value was 75%, and the negative predictive value was also 75%. In this model with all the comorbidities coded under one heading the only statistically significant variable was the age group 25-34. To further investigate the factors associated with the comorbidities, each comorbidity was separated into individual variables. A Chi-square goodness of fit test was performed to determine the highest level of prediction. With age as a covariate, the model predicted 69.1% of the cases with a Pearson goodness of fit of 115.295(106), p=.253. Using gender as a covariate, the model predicted 70% of the cases correctly with a Pearson goodness of fit 112.424 (101), p=2.47.

Race as a covariate correctly predicted 69.5% of the cases with a Pearson goodness of fit 112.418 (103), p=2.47. Utilizing comorbidities separately, kidney failure as a covariate predicted 70% of the cases with Pearson goodness of fit at 112.24(101), p=.206. Solid organ tumor cancer, autoimmune disorder, or multiple organ failure as individual covariates all predicted the cases at the same level of 67.3% with a Pearson goodness of fit of 42.305(13), p<.0001. Multiple combinations of covariates did not improve the predictability of correct cases (data not shown).

Next a Chi-square test for association was done to determine which comorbidities were significantly associated with CABSI. There was a statistically significant association between CABSI and kidney failure, $\chi^2(1) = 6.863$, p=.009. Other statistically significant associations with CABSI and a comorbidity included solid organ tumor, $\chi^2 = 5.980$ (1), p=.014, and multiple organ failure $\chi^2 = 7.909$ (1), p=.005. Autoimmune disorder was also statistically significant but did not meet the criteria for each cell having

greater than 5 cases for a Chi-square test for association and thus it was not included in further analysis. To consider the overall effect of statistically significant nominal variables, a likelihood ratio test was evaluated in SPSS. With no covariates, age, kidney failure, solid organ tumor cancer, and multiple organ failure were all statistically significant (see Table 18).

Table 18 *Likelihood Ratio Test (No Covariates)*

Effect	Chi-square	df	Sig.
Age	8.774	1	.003
Kidney Failure	9.584	1	.002
Solid Organ Tumor Cancer	7.835	1	.005
Multiple Organ Failure	7.237	1	.007

Using age as a covariate the variables kidney failure, solid organ tumor cancer, and multiple organ failure were all still statistically significant (see Table 19).

Table 19 *Likelihood Ratio Test (Covariate of Age)*

Effect	Chi-square	df	Sig.
Kidney Failure	10.614	1	<.001
Solid Organ Tumor Cancer	8.904	1	.003
Multiple Organ Failure	8.068	1	.005
Age	18.795	6	.005

A binary logistic analysis was used next since the dependent variable has two outcomes (yes or no). Using age as the only independent variable correctly predicted 56.4% of the CABSI cases with Exp (β)= .845, p=.022. Kidney failure as the only

independent variable predicted 58.6% of the case with Exp(β) = 2.187, *p*=.009. Using solid organ tumor cancer as the only independent variable predicted 57.3% of the cases with Exp(β) = 2.387, *p*.016. Multiple organ as the only independent variable predicted 55.9% of the cases correctly with Exp(β) = .323, *p*=.007. Including all four of these independent variables increased the correct predictability of the model to 69.1% which was the highest percent for all possible combinations. With this model, age has an Exp(β) = .762, *p*=.001. Kidney failure calculated as Exp(β) = 3.136, *p*=.006, solid organ tumor cancer was similar with Exp (β) = 3.010, *p*=.006. Multiple organ failure had Exp(β) = .306, *p*=.008. Further exploration using a multinomial logistic regression produced similar results. For the risk of CABSI the age group of 24-35, kidney failure, solid organ tumor cancer, and multiple organ failure were all still statistically significant (see Table 20).

Table 20 *Risk of CABSI Using Multinomial Logistic Regression*

	Exp (B)	Sig.
Age (25-34)	9.52	<.0001
Kidney Failure	.71	<.0001
Solid Organ Tumor Cancer	.71	<.003
Multiple Organ Failure	2.80	.005

It is important to note that for multiple organ failure, β= -1.129 which indicates that there was a decrease in odds of having CABSI with multiple organ failure. This model correctly predicted 69.5% of the cases which was slightly higher than the predicted value using a binary analysis.

Summary

Information discovered from this study may allow for practical measures to be taken to disrupt the epidemiologic triangle and prevent *Candida albicans* bloodstream infections in hospitalized patients. Essential oils such as lemongrass, oregano, and thyme have shown to be statistically superior at killing *Candida albicans* in laboratory testing. This information can be used in areas of cleaning as well as patient care. The reduction of the virulence factor germ tube production when exposed to LTO provides areas to improve patient outcomes with potential decrease in virulence of *Candida albicans* that the patient may be exposed to in the environment. Data analysis of risk factors provide areas to target protocols that could reduce patient risk for acquiring *Candida albicans* bloodstream infections while in the hospital. In Chapter 4 I presented procedures used for this data analyses as well as the analyses for the seven hypotheses for this study.

Of the seven hypotheses examined, all seven null hypotheses were rejected. A significant difference in kill rates existed for each essential oil, the combined essential oils LTO, as well as LTO plus caspofungin or fluconazole when compared to caspofungin or fluconazole alone. LTO significantly reduced the germ tube production of the *Candida albicans* isolates tested when compared to reduction of germ tube production using caspofungin or fluconazole. Risk factors that were statistically significant for *Candida albicans* bloodstream infections included age 25-34, kidney/renal failure, solid organ tumor cancer, and multiple organ failure. While these risk factors cannot be controlled, knowing that a patient has one or more of these risk factors could allow for additional contact precautions, extra room cleaning, or astute attention to proper

hand hygiene for each person entering these patient rooms. In Chapter 5 I will present details of the interpretations of the findings of this study, the associated findings, as well as strengths and limitations of the study. In addition, future recommendations for research and social implications will be addressed.

Chapter 5: Discussion, Conclusions, and Recommendations

Introduction

The purpose of this study was to investigate the impact of lemongrass, oregano, and thyme essential oils individually and combined on *Candida albicans* ability to reproduce in comparison to the antifungal medications, caspofungin and fluconazole individually, and with the combined essential oils. Additional studies were performed to determine whether the combined essential oils, caspofungin, or fluconazole inhibited the virulence factor of germ tube production in *Candida albicans* isolates. The patient demographics collected from patients with *Candida albicans* bloodstream infections were compared to a control group of patients with blood cultures collected during the same collection period without *Candida albicans* bloodstream infections to determine whether age, race, gender, or comorbidities contributed to risk factors for these bloodstream infections.

This study was quantitative in nature with experimental data collected for the first six research questions and secondary data collected on patient demographics to evaluate the seventh research question. Patients who acquire *Candida* bloodstream infections during hospital stays have been shown in previous research to have a 30-day mortality rate between 31 to 61% (Diekema, Arbefeville, Boyken, Kroeger, & Pfaller, 2012). Calculating 30-day mortality rates for this study was beyond the scope of this research. Future studies incorporating essential oils in different modalities and comparing mortality rates of pre and post-incorporation would provide data on whether long-term implementation of essential oils would be beneficial to patient outcomes. I found that

lemongrass, oregano, thyme, and the oils blended together had statistically significant higher kill rates on *Candida albicans.* isolates than caspofungin and fluconazole. In addition, combining the blended oils with caspofungin or fluconazole also statistically significantly improved the levels of kill rates compared to using caspofungin or fluconazole alone. Germ tube virulence factors were also inhibited by the combined treatment of lemongrass, oregano, and thyme. There was no statistically significant inhibition of germ tube production with either caspofungin or fluconazole. Risk factors that were statistically significant in contributing to *Candida albicans* infections included the age group of 23-34 and the comorbidities of kidney failure, solid organ tumor cancer, and multiple organ failures. These findings will be discussed in more detail in the following section. I will also discuss limitations of the study, recommendations for future research, and implications for social change.

Interpretation of the Findings

Lemongrass, thyme, and the three combined essential oils showed statistically significant impact on the kill rates of the *Candida albicans* isolates tested over time. Oregano did not have a statistically significant impact over time due to the high kill rates after 1 hour. After allowing for a 48-hour growth period on culture plates to determine whether the effect on the colonies were merely inhibition and not cell death, exposure to lemongrass after 12 hours resulted in a mean colony growth of zero. The colonies were no longer viable after being exposed to lemongrass for 12-hours. Oregano and thyme both had a mean colony count of zero after only 4 hours of exposure of the isolates to the respective essential oil. Combining the lemongrass, oregano, and thyme resulted in a

similar result as lemongrass alone with the zero-growth rate of colonies after 12 hours. The impact of lemongrass had previously been reported to have similar activity to nystatin orally to treat oral yeast infections by Amornvit (2014). Although nystatin cannot be used for invasive infections, lemongrass appeared in this study to have a higher efficacy of yeast cellular death than either caspofungin or fluconazole which are used to treat systemic fungal and yeast infections. Oregano and thyme in this study yielded similar results that have previously been reported. Bellete et al. (2012) reported that low concentrations of thyme essential oil were shown to be effective at inhibiting *Candida* species. Omran and Esmailzadeh (2009) supported the evidence drawn from this study that thyme has a higher level of inhibition than fluconazole; however, zones of inhibition were used instead of verifying whether the organism is in fact killed when exposed to thyme essential oil. I found that the organisms were killed when exposed to thyme and not just inhibited. Pozzatti et al. (2008) indicated that *Candida albicans* isolates that were resistant to fluconazole were highly susceptible to oregano essential oils. I found that all *Candida albicans* isolates were highly susceptible to exposure to oregano after only 1 hour. Although this study supported previous research that indicated the essential oils lemongrass, oregano, and thyme were effective at inhibiting *Candida albicans*, it also showed that the oils were effective at killing the isolates in less than 24-hours. Although neither of the antifungals tested during this study were able to obtain a mean colony count of zero, caspofungin had the lowest mean colony count of 8.76 after an 8-hour exposure before an increase in mean colony counts at 12-hours. Fluconazole had the

lowest of its colony counts at the 4-hour exposure time of 53.47 but then the colony counts increased after further exposure.

After the initial inoculation of the essential oils or antifungals, no additional treatments were added to the organisms growing in TSB. The essential oils continued to kill the *Candida albicans* isolates, while the antifungals were able to inhibit temporarily, but without additional treatments the organisms were able to overcome exposure and continued to proliferate. Combining the essential oils and antifungals did not prevent the essential oils from killing the isolates; however, the combination of LTO and fluconazole had a mean rate of 0 which was slightly lower after 8 hours of exposure compared to LTO and caspofungin, which had a mean colony count of 0.05 after 8 hours. The addition of the combined essential oils did not inhibit the action of fluconazole or caspofungin to the *Candida albicans* isolates, but rather provided an additional mechanism to kill the organisms instead of merely inhibiting the growth of the organisms. Although the use of the combined LTO did not completely kill after 1 hour of exposure, it did still show statistically significant inhibition of the virulence factor of germ tube production in laboratory testing.

Testing for germ tube production while simultaneously incubating isolates with either LTO, caspofungin, or fluconazole also validated potential uses of lemongrass, oregano, and thyme essential oils. The combined LTO essential oils inhibition of germ tube production was statistically significant at the 0.05 critical alpha level. This corresponds to previous research by Gaspar De Toledo et al. (2016) who indicated that lemongrass inhibited hyphal formation as well as biofilm formation. Thyme was shown

to inhibit virulence factors, such as proteinase, haemolysin, and biofilm production by Khan, Ahmad, Cameotra, and Botha, (2014). Not only was germ tube production was prevented, but biofilm development was diminished when used against *Candida albicans* (Rodrigues, Rodrigues, Silva, & Henriques, 2017). Exposure to caspofungin and fluconazole did not decrease germ tube production. Two isolates with exposure to caspofungin and two different isolates exposed to fluconazole created germ tubes when exposed to the antifungals but not when these four isolates were incubated only in the germ tube medium. Fluconazole was unable to disrupt virulence factors in *Candida albicans* (Khan et al., 2014). This phenomenon for lemongrass, oregano, and thyme to disrupt virulence factors poses an interesting aspect for further research. Additional research into potential risk factors rounded out this study of *Candida albicans* bloodstream infections.

The potential risk factors for CABSI that were examined included, race, gender, age, and comorbidities. Previous research by Yang et al. (2014) indicated that advanced age and neutropenia were risk factors for *Candida* sepsis, however, for this research group, the statistical significance for age as a risk factor was in a lower age group of 25-34. In this study, gender and race were not statistically significant as had previously been reported for all types of bloodstream infections by Kaye et al. (2011). A study by Diekema et al. (2012) examining data from 1983 through 2007 reported that the mean age in years ranged from 38 to 45.8 depending on the years of data collected for health care associated *Candida* infections but the research did not list the age range of the data collected over the 24- year time frame. This study by Diekema et al., (2012)

unfortunately did not compare a control group to evaluate whether age was a risk factor. It may also be relevant to note that the closer age-range from the Diekema et al., (2012) study was from data collected in Iowa and the Yang et al., (2014) study was in Shanghai. Additional nationwide research may shed light on whether age range is statistically significant for risk factors of *Candida albicans* bloodstream infection and whether the age range is consistently younger than reported in other countries. More in-depth analysis of other factors for the 25-34 age group may provide additional insight for preventative measures in the future. The relationship between the comorbidities of renal failure, solid organ cancer, and multiple organ failure with CABSI were all statistically significant; however, patients with multiple organ failure were at a decreased risk of CABSI whereas renal failure and solid organ tumor cancer were positively related to acquiring CABSI while hospitalized. Recognizing these potential risk factors can provide areas to target for interventions to reduce the risk of *Candida albicans* bloodstream infections. Data collected by Diekema et al., (2012) for 108 cases from 2004-2007 had surgery as the most patients with healthcare acquired *Candida* infections. Again, without a comparison group, it is unknown whether this data reported by Diekema et al., (2012) was statistically significant. In the study by Yang et al. (2014), neutropenia was the most common comorbidity but there was no control group to compare for statistical significance. While Kaye et al. (2011) indicated that obesity, surgery, and previous hospital exposure were risk factors, the study only evaluated patients that were in the age range of 69-79 and they examined all bloodstream infections, not just *Candida albicans*. Sigemura et al. (2014) reported that 62% of patients with *Candida* bloodstream

infections occurred in the Japanese study were under the age of 70 with cardiovascular disease and surgery the more common comorbidities. Similar to the age group risk factors, additional national research may indicate whether certain comorbidities are significant risk factors for patients in the United States or perhaps in regions of the United States.

Using the theoretical framework of the epidemiological triangle for this study provided a foundation for potential areas of prevention and improving patient outcomes. Recognizing potential risk factors such as age and comorbidities provides areas of intervention including additional patient precautions for patients with these risk factors, prudent hand washing by visitors and care providers, as well as studying the effects of developing interventions such as diffusing the essential oils in patient rooms to potentially disrupt the organism's ability to survive in the host environment or topically applying the essential oils to prevent colonization of *Candida* on skin surfaces. Further studies in disrupting the epidemiological triangle would be warranted to verify these areas as opportunities to prevent infections.

Limitations of the Study

Limitations to this study include the ability to apply this research to the general population at large. This study examined the patient demographics and characteristics of *Candida albicans* isolates from a large adult teaching hospital which may not apply to smaller community hospital patients and the isolates recovered from these smaller facilities. The adult age range of 18-74 may also limit the generalizability to the general population outside of this age range as pediatrics and those over the age of 74 were not

studied. Other potential limitations include the lack of further investigation into why the age range of 25-34 was a risk factor. In addition, the ability to gather demographics and isolates from other institutions was beyond the scope of this study. The experimental data collected was from previously frozen isolates and this could have resulted in organisms that were not as robust compared to the initial isolation of these organisms. Controls and comparisons were built in to minimize this potential limitation. Furthermore, how *Candida albicans* would react to the eight interventions while actively infecting a patient was not determined. Any discrepancy between in-vitro and in-vivo results due to host response to colonization versus invasive *Candida albicans* infections could not be determined in this study. These limitations; however, also provide areas for continued research.

Recommendations

The ability to provide a large sample size to study at a large teaching hospital provided opportunities for future research. Areas for recommended further study include whether diffusion of these essential oils in patient rooms could decrease the number of *Candida albicans* infections as well as other *Candida* infections. In addition, further investigations as to whether other *Candida* species such as *Candida glabrata* react in similar manners when exposed to these essential oils compared to *Candida albicans* could also prove to be beneficial. *Candida albicans* isolates had a disruption of germ tube production when exposed to lemongrass, oregano, and thyme. Whether this is a permanent disruption or whether the organism, if still viable, would be able to make germ tubes when grown without the essential oils would require additional research. While

there has been research involving the effect of lemongrass, oregano and thyme on *Candida* isolates (Amornvit et al., 2014; Bellete et al., 2012; Boukhatem, Ferhat, Kameli, Saidi, & Kebir, 2014; Nasir, Tafess, & Abate, 2015; Omran & Esmailzadeh, 2009; Pozatti et al., 2008), the possibility of studying not only the effects of the oils on *Candida* species, but also on how the patient responds to the oils being diffused or applied topically remains to be determined again with future research. This study provided additional information using a larger sample size as well as investigation into possible combination therapy treatments. I will discuss further implications in the following section.

Implications

Based on this research, further implications for positive social change include the ability to improve the public's health by limiting potential transmission of multi-drug resistant yeast to others in the general population. This would be due to fewer *Candida* isolates surviving long-term treatment with antifungal medications and therefore reducing the likelihood of developing resistance to these drugs. With less multi-drug resistant isolates developing, fewer of these isolates would be in circulation (CDC, 2011). Information collected from this study could improve outcomes by targeting risk factors for prevention and utilizing the essential oils from this research to disrupt the epidemiological triangle and prevent infections from occurring while higher risk patients are hospitalized. Reducing hospital acquired infections caused by *Candida* and other organisms reduces the cost for the hospital and the patient (CDC, 2013). Decreasing hospital acquired infections decreases the mortality rate due to these infections.

Improved patient outcomes in clinics and hospital not only impacts the financial cost but also the associated emotional and physical costs related to long-term illnesses on both the family and patient,

The information collected from this study could provide a foundation for future research involving other microbes and the effects of the interactions with essential oils to reduce infection rates. Reduction of hospital acquired *Candida* infections could decrease mortality rates and improve patient outcomes both while hospitalized and after discharge by decreasing the likelihood of colonization and thus decreasing the potential risk of infections of these patients. Reduction of these hospital acquired infections could also decrease lengths of stay, decrease hospital charges to the patient, and decrease overall hospital expenses.

Providing opportunities to change the environment of the patient's room while hospitalized would enable a reduction in the risk of infections while also giving patients and their care givers the possibility to continue this mode of disruption for *Candida* survival outside the hospital setting thus enabling self-care once the patient was discharged. Additional research into potential modalities of treatment with essential oils (aromatic, topical, or internal) in conjunction with standard medical treatment could open additional areas for improving patient outcomes. Introduction of aromatic methods into a hospital setting could be beneficial to the hospital as well as the patient. Targeting at-risk patients to receive additional essential oil treatments whether it is simply diffusing antifungal essential oils in the room during their stay or providing essential oils as adjuncts to every room cleaning, reduction of hospital acquired infections could be

monitored and the aromatic treatment modality could be validated as a new standard of care. This in turn could lead to expansion into the additional treatment modalities in a controlled research setting to monitor for improvement of patient outcomes. Improving patient outcomes has a positive effect on the people, their families, and the communities they live in. This can be achieved by decreasing patient length of stays in hospitals, which in turn enables them to return to their communities, families, and way of life sooner. This can also create ways to enable preventative care methods that prevent additional hospitalizations, which then reduces financial burdens for the hospital, the patient and those caring for them.

Conclusion

This study was used to examine a multitude of data that was created and collected in the laboratory setting as well as investigating risk factors for CABSI at a large teaching hospital with various patient demographics and comorbidities. Lemongrass, oregano, thyme, and the three essential oils combined all showed statistically significant improvement over caspofungin and fluconazole to not only inhibit *Candida albicans* from growing but to also kill it within a brief time period. The ability for these oils to rapidly kill *Candida albicans* not only decreases the likelihood of the development of drug resistance but also minimizes the chance that the organism would contaminate the environment and be spread to other areas and patients. Prevention of the germ tube production virulence factor provides an exciting area for additional research on preventing not only severe *Candida albicans* infections but less serious but troublesome yeast infections as well. Targeting at-risk patients with prevention measures enables the

providers to concentrate on other aspects of admission without introducing an additional life-threatening infection that needs to be aggressively and rapidly treated (CDC, 2011). Further studies are needed to better understand the interactions of host cells, invading organisms, and how essential oils interact in-vivo. While synthetic antimicrobial options are very limited, and organisms have often developed resistance prior to the treatment being approved for use on the public (CDC, 2011), essential oils provide a means to potentially prevent infections and reduce resistance.

Anyone that has had a yeast infection regardless of the severity could potentially benefit from this research for viable alternative treatment options. Hospitals and clinics could benefit from this information by being able to reduce costs and improve patient outcomes (Bliss et al., 2012). The ability to provide new ways for patients to engage in treatment regimens helps to empower patients and their families by providing opportunities to actively participate in the treatment plan which in turn can improve outcomes and patient satisfaction. Research and inception of new treatment modalities are rapidly needed in the United States to reduce mortality rates due to preventable infections (CDC, 2011). The addition of essential oils into standard of care warrants serious consideration and research such as this study continue to provide proof of validity of these treatment potentials as well as means for implementation.

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