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Substrate Utilization of the Emerging Fungal Pathogen, Candida auris, and the Antifungal Activity of Select Essential Oils

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Substrate Utilization of the Emerging Fungal Pathogen, *Candida auris*, and the Antifungal Activity of Select Essential Oils

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Thesis presented for the Master of Science in Integrative Biology

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Abstract

Candida auris is an emerging fungal pathogen that commonly causes nosocomial blood infections in the immunocompromised. Three factors make this emerging pathogen a global threat. First, it is frequently misidentified by commonly used diagnostic platforms. Second, it is able to survive for weeks on fomites. Third, it is almost always drug resistant, sometimes to all three classes of antifungal drugs used to treat *Candida* infections. The objectives of this study are three-fold. First, two existing methods, population estimation using absorbance-based standard curves and methylene blue viability staining, were investigated as to application in determining *Candida auris* cell population size and viability, respectively. Both the spectrophotometric study and methylene blue staining were successfully applied to *C. auris* concentrations. A standard curve plotting absorbance to concentration were constructed for several organisms for standardizing inoculum for subsequent assays. Second, a description of the basic metabolic capabilities of *Candida auris* to assimilate a variety of chemicals as a sole source of carbon or nitrogen was determined and compared to related yeasts. *Candida auris* displayed a unique pattern of carbon and nitrogen assimilation as compared to the other, related species. This included several carbon sources that may have future utility in a diagnostic media. Several isolates of *C. auris* were also examined using the Biolog YT plate for yeast identification, which operates under a similar principle. Although the organism is absent from database and thus misidentified as one of two organisms in all cases, a significant amount of carbon utilization data was added to the results of the previous study. Third, the antifungal activities of select essential oils were tested against *C. auris*. This was followed by testing the interaction of the three most effective oils with four commonly used antifungal drugs. Several of the essential oils displayed the ability to inhibit the growth or even kill *C. auris*, *Candida lusitaniae*, and *Saccharomyces cerevisiae* when in direct contact. The three most effective oils were those of lemongrass, clove bud and cinnamon bark. These three oils even retained some antifungal activity in vapor-phase. These were also the oils used in combination with fluconazole, amphotericin B, flucytosine and micafungin. While cinnamon bark oil displayed little interaction with the drugs, lemongrass oil displayed positive or neutral interactions with all four drugs, while clove bud oil had mixed results. The combination of clove bud oil and amphotericin B resulted in an antagonistic outcome, whereas it showed no improved effect when combined with micafungin but displayed positive interactions with fluconazole and flucytosine.

Introduction

Drug Resistant Pathogens

Drug-resistant pathogens are becoming an increasing problem [1]. Pathogens once easily treated are becoming increasingly more difficult to treat due to multi-drug resistance, leading to an increased incidence of debilitating and often fatal infections. Adding to this problem, the rise in conditions compromising the immune system are promoting infection by opportunistic pathogens [2]. Immune compromising conditions are caused by a variety of factors, including primary genetic disorders, acquired secondary diseases, and human interventions that suppress the immune system, like chemotherapy [3, 4]. All these conditions open the possibility of infection by organisms previously thought to be harmless, including many fungi. One report shows a threefold increase in deaths from invasive fungal infections between 1981 and 1996 [2]. Current estimates place the global incidence of several invasive fungal infections at 100,000 cases or more each year (Table 1) [5].

Table 1: Global incidence of invasive fungal infections as of 2017 [5].

Infection	Annual Incidence	Rate per 100,000 people
Invasive Candidiasis	~750,000	10.00
Invasive Aspergillosis	~300,000	4.00
Pneumocystis jirovecii pneumonia	~500,000	6.67
Cryptococcosis in AIDS	~223,000	2.97
Mucormycosis	>10,000	0.13
Disseminated histoplasmosis	~100,000	1.33

Among these fungal opportunists are species like *Candida albicans* and *Aspergillus fumigatus* [2]. These organisms are ubiquitous in the environment and some are even normal commensals, or organisms that harmlessly colonize various parts of the body [6, 7]. Individuals are often at risk of acquiring these organisms from their surroundings or when they come into contact with asymptomatic

carriers, in addition to those showing clear signs of infection. For immunocompromised individuals, the environment and colonized individuals are dangerous reservoirs for fungal pathogens. When exposed, healthy individuals' immune systems are able to prevent clinical infection by these organisms. In this case, the organism is either completely cleared or maintains a presence as a commensal. While some of these infections are non-life-threating, some can escalate to systemic infections in the immunocompromised when their immune systems cannot eliminate the infection [8]. If the organisms can gain a foothold, they can then grow unabated, leading to serious complications and often death if not treated promptly [2]. Many times, these infections occur in healthcare settings where both vulnerable people and potential pathogens are concentrated in high densities. Those who do seek treatment often receive antifungal drugs and have a much higher chance of recovery. Even with treatment, these infections can be deadly. Multiple studies report mortality rates as high as 50% in patients treated for invasive fungal infections [9, 10].

Antifungal Drugs

To date, only three classes of antifungals have been developed that are considered safe and widely used to treat invasive fungal infections. Echinocandins (eg. micafungin) inhibit fungal cell membrane formation by disrupting synthesis of the structural component 1,3-β-d glucan [11]. Azoles (eg. fluconazole) inhibit the biosynthesis of the cell membrane component ergosterol while polyenes (eg. amphotericin B) bind to membrane ergosterol to induce pores and cause cell death through leakage of cytosolic components [11]. In addition to these, a nucleoside analog, flucytosine (5-fluorocytosine or 5-FC), is sometimes used in combination with other drugs as a treatment, and acts by inhibiting synthesis of pyrimidine and their incorporation into larger nucleic acids [11, 12]. Two metrics that are frequently utilized to assess the effectiveness of antifungals are minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC). The MIC is the minimum concentration required to totally inhibit the growth of a starting inoculum. The MFC is the minimum concentration required to totally kill the starting inoculum. Despite the presence of multiple treatment options, resistant organisms exist for all three classes of these drugs [13, 14]. Researchers have discovered many modes of resistance, discussed briefly below and in-depth elsewhere [15]. This presents a need for additional therapeutic options for these deadly infections.

Although research to develop effective treatment options is ongoing, this progress is slow for several reasons. Development is very expensive due to the extensive testing required by the Food and

Drug Administration in the United States, among other agencies [16]. These requirements are present to ensure the safety of the drugs before administering them to the public. Development of antifungal drugs is often more complicated than when developing antibiotics to treat bacterial infections. Much of this problem is rooted in the genetic and cellular similarity between fungal pathogens and their hosts. As fungi and mammals are both eukaryotic, several cell structures are conserved in both groups of organisms. Important cellular structures synonymous with the antifungal drug targets are present and can be damaged by the antifungals, and therefore selective toxicity against the pathogen without causing harm to the host is problematic. As such, the drugs used to treat fungal infections are often harmful to the patient, especially at the elevated doses needed to treat resistant strains [17]. This similarity limits the availability of drug targets when developing new drugs, exacerbating the problem. Even though they are declared safe to use, they often have significant side-effects that may be unacceptable to certain patients [17]. Another issue is a lack of a singular codified method for determining the effectiveness of an antimicrobial. Since multiple methods are in common use, inconsistent results are often obtained. When the methods are not properly defined, other researchers can misinterpret these results. Combined, these problems not only lead to the slow development of new treatment options, but also place a large burden of cost on the patients in need of these drugs. Thus, there exist a need for cheap and effective treatment options to supplement the existing methods.

Antifungal Resistance

Several mechanisms of antifungal drug resistance exist. These can be innate to the organism or acquired through gene transfer. When a pathogen is innately resistant to an antifungal and a potential host is repeatedly treated with that antifungal, a selective pressure is placed that allows the drug-resistant organism to outperform the other organism competing for the host [18]. This allows the drug-resistant organism to proliferate and possibly transfer the resistance genes to non-resistant organisms. In acquired resistance, the drug-resistant fungus acquires the genetic material containing the resistant genes. While there is some evidence of horizontal gene transfer, it is little understood in yeasts and happens rarely [19]. The most common mode of gene transfer is through mating with other members of its species [20].

Regardless of where the resistance comes from, many mechanisms exist by which this resistance takes effect. These mechanisms are generally recognized to rely on one of several strategies. One strategy is to alter the target of the drug [15]. By altering the target, the drug cannot bind effectively and

is unable to affect the organism. Another strategy involves creating a workaround of the drug's target. If the drug blocks one metabolic pathway, the second strategy allows the organism to bypass the pathway by using an alternative one [15]. A common strategy is to effectively lower the bioavailability of the antifungal. Efflux pumps transport drugs out of the cell and prevent a dangerous concentration from building up [15]. Some organisms overexpress the number of drug targets so that the target's function is not impeded despite the presence of the drug [15]. Another tactic is to isolate the drug, by methods such as vesicles or biofilm capture, so that it is spatially separated from its target and cannot affect it [15]. A final mechanism is present for drugs that require metabolization to become active. In this instance, the process that converts the pro-drug to the active drug becomes less efficient or is lost altogether, thus leaving the drug in its non-active state [15]. All these mechanisms prove to be effective strategies for increasing an organism's resistance to antifungal drugs.

For clarification, resistance does not necessarily mean that a drug will unsuccessfully treat an organism. The term describes when the level of the drug that a particular strain requires to achieve inhibition of growth is higher than what is considered typical for the species [15]. These increased dosages may lead to increased host toxicity and further complicate the patient's health-state and thus can prevent their effective usage. This is problematic because when a healthcare provider detects a pathogen causing disease in an individual, the provider will prescribe a dose that will inhibit a standard strain of the organism. When the fungal organisms are resistance to these drugs, they can still be used, but the dosage must be increased to achieve efficacy [15]. Hence, the dosage prescribed may be too low and greatly decrease the likelihood of a positive clinical outcome.

Candida Species

Candida are a genus of yeast in the division Ascomycota within the same family as *Saccharomyces cerevisiae* or common brewer's yeast [21]. These species are commonly human commensals and can be opportunistic pathogens that account for a large portion of nosocomial, or hospital-acquired, infections [22]. Candidemia, or blood infection by *Candida* spp., is a much more serious instance of candida infection, with *Candida albicans* historically being the major cause [22]. *Candida albicans* displays an exceptional functional plasticity thanks to its many morphotypes [20]. Thanks to this functional plasticity, *Candida albicans* can colonize many areas of the body [20]. Two of its morphotypes, the yeast form and the hyphal form, are believed to be required for its pathogenicity. Studies examining mutants lacking either morphotype found them to be avirulent [23, 24]. In addition,

biofilm formation and the secretion of hydrolytic enzymes are crucial to the pathogenesis of *C. albicans* [25]. While *C. albicans* has claimed many lives over the years, it is successfully treated in many cases and rarely displays a significant number of drug-resistant strains. The greatest resistance appears against flucytosine, where only 3% of all strains where reported resistant [26]. In recent years, however, there has been an increase in the number of other *Candida* species causing candidemia, such as *Candida glabrata, Candida parapsilosis* and *Candida tropicalis* [27]. The Center for Disease Control and Prevention (CDC) currently offers a panel of drug-resistant isolates for each of these species, known as the drug-resistant *Candida* panel (Appendix I) [28]. Many of these species are innately resistant to some of the antifungal drugs that are normally effective at combatting candidemia [18, 29]. The treatment of fungal infections with antifungal drugs has placed selective pressure that may be contributing to the rise in incidence of infection by drug-resistant species of yeast [18]. When *C. albicans* is successfully eliminated with treatment and the immunocompromised host remains in a vulnerable state, other *Candida* species often fill the void, leading to reinfection. This problem is further compounded by the appearance of acquired antifungal drug resistance in both *C. albicans* and other *Candida* species [30]. One study reported between 20% and 25% of *C. albicans* isolated from two hospitals were resistant to at least one antifungal, and of these, 42% and 64% were resistant to more than one antifungal, respectively [30]. This resistance included azoles, echinocandins, polyenes, and flucytosine. [29]. The above scenario illustrates another important point. Even though only a small percentage of *C. albicans* isolates are resistant, those strains tend to be concentrated in places like hospitals. This is likely a result of the high levels of antifungal drugs in use selecting for resistant organisms and the high density of vulnerable individuals.

Of these other *Candida* species, infection by *Candida glabrata* is the most prevalent [25]. This organism is interesting due to its unique genetic state when compared to many other *Candida* species. *C. glabrata* is well-known to be fastidious, having lost many of the genes required to utilize a wide range of substrates [25]. In addition to this, the organism appears to have undergone amplification of genes specific to its ability to act as a pathogen and survive inside a host, such as those related to cell adhesion [25]. *Candida tropicalis* is another common cause of candidemia, especially in tropical climates. This species shows many of the same morphotypes as *Candida albicans*, although their exact role in its virulence is less well understood [25]. However, one study does show that *C. tropicalis* can only effectively colonize the oral surface in its hyphal form [31]. Closely related to *C. tropicalis*, *Candida parapsilosis* doesn't display the same levels of morphotype variability as *C. tropicalis* or *C. albicans*, but it is still virulent in vulnerable hosts. It does still manifest a hyphal form, although this is not required for

invasion of the oral epithelium [31]. *C. parapsilosis* relies heavily on hydrolytic enzymes and biofilm formation to successfully cause disease in its host [25].

Candida auris

One such organism that exemplifies the issue of emerging drug-resistant fungal pathogens is *Candida auris*. It was originally isolated from the inner ear of a patient at a Japanese hospital in 2009 [32]. Since its initial isolation, it has becoming quite well known as a new "super bug". Several unique strains from four geographically distinct clades have been identified in many clinical cases worldwide (Table 2) [33]. These clades are purported to have emerged simultaneously and their identity has been confirmed by whole genome sequencing which has identified thousands of single nucleotide polymorphisms [33]. The Center for Disease Control and Prevention (CDC) currently offers an isolate panel for testing of *C. auris,* that contains strains from all four clades from different parts of the world as well as several related species (Appendix 1). The type strain from the original isolation is isolate number 0381 and is the only member of its clade on the panel. In most cases, the organism is found causing nosocomial candidemia that is often lethal [34]. Recent media attention is causing a rapid increase in attention for the organism, but there is a serious lack of information due to the recent emergence as a pathogen. Three traits of this organism are currently thought to be responsible for its lethality and rapid spread. These are its ability to persist, its tendency to be misidentified and the prevalence of drug-resistant strains.

Table 2: *C. auris* isolates by clade.

Isolate #	Clade
0381	East Asia, Clade II
0382	South Asia, Clade I
0383	Africa, Clade III
0384	Africa, Clade III
0385	South America, Clade IV
0386	South America, Clade IV
0387	South Asia, Clade I
0388	South Asia, Clade I
0389	South Asia, Clade I

Emergence and Persistence

Candida auris is becoming well known for its ability to persist in conditions where related yeasts cannot survive. The original description of *Candida auris* shows that the organism can thrive in temperatures as high as 40°C, where most other species grow well only at lower temperatures [32]. A recent study suggests that this tolerance may be related to increasing global temperatures [35]. The author suggests that the organism originated as a plant saprophyte until its thermal tolerance reached levels compatible with mammalian and avian body temperatures. The author also offers a potential

transmission from wetland birds to rural humans and then to urban humans (Figure 3) [35]. Another claims that *C. auris* remains viable for as long as 14 days on soiled healthcare surfaces [36]. The same study reports enzymatic activity for *C. auris* persisted for as long as 28 days post-inoculation [36]. The persistence of this organism is enhanced by its transmissibility from surface to host and back. Examinations of several early hospital outbreaks detected *C. auris* colonization a variety of surfaces in contact with patients, including medical instruments, bedsheets and several others [37]. A logical step to preventing its spread is to follow strict disinfection protocols, which are still in development. However, this is complicated by the yeast's apparently high tolerance to commonly used disinfectants, including the ubiquitously used ethanol [38]. To make matters worse, the Environmental Protection Agency (EPA) has only recently established guidelines for testing potential disinfectants with *C. auris*, and the Center for Disease Control and Prevention (CDC) has suggested the use of chemicals approved for use with the endospore-producing bacterium *Clostridium difficile* to eliminate *C. auris* from surfaces [39].

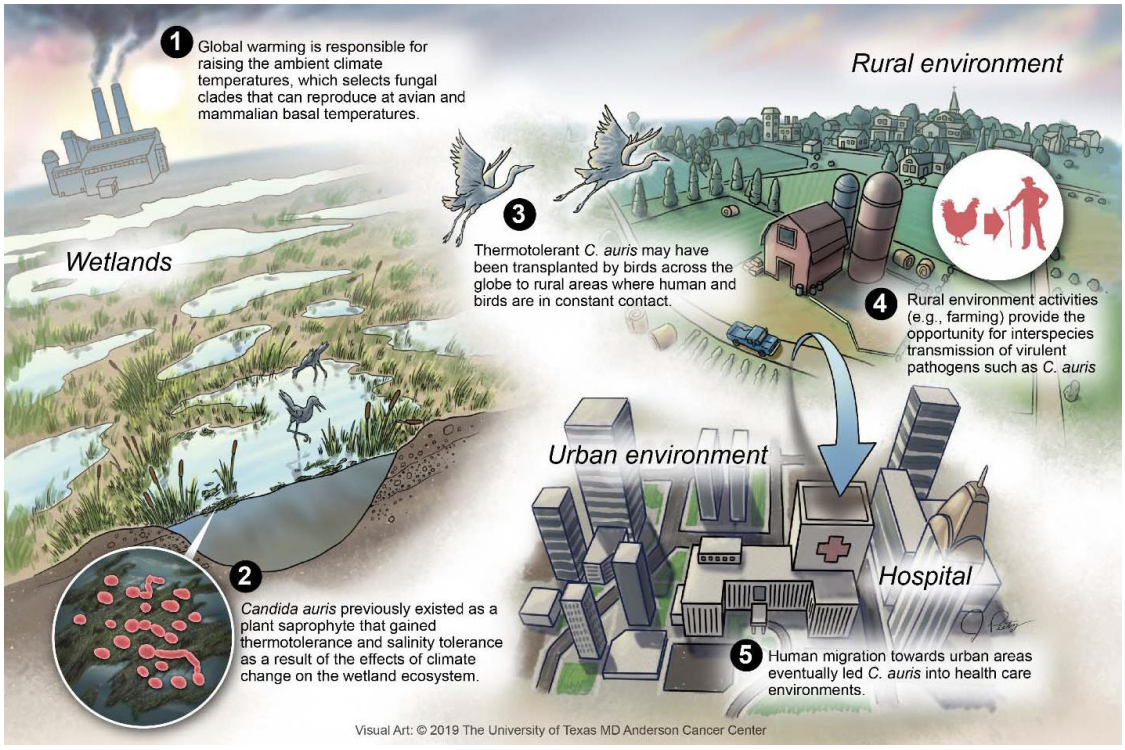

Figure 1: Proposed emergence of *Candida auris* [35].

Misdiagnosis

The second factor that makes *Candida auris* so threatening is that it is frequently misdiagnosed by commonly used diagnostic platforms (Table 3). *Candida auris* has a general appearance like that of many other yeasts when examined on basic growth mediums and under a microscope (Figure 2 and Figure 3) [40, 41]. One interesting morphological characteristic of the organism is that it has yet to be observed forming hyphae or pseudohyphae [42]. Thus, more sophisticated identification methods are required identify *Candida auris*. The majority of these diagnostic methods identify an organism based on

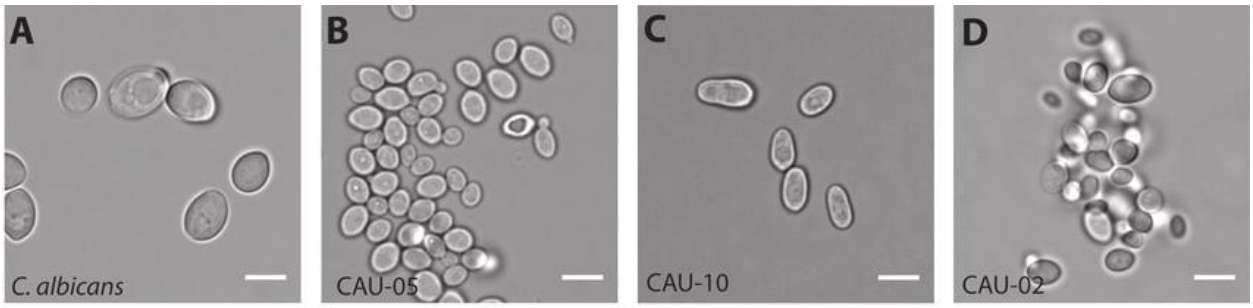

Figure 2: Microscopic comparison of *Candida albicans* (A) and *Candida auris* isolates 0385 (B), 0390 (C) and 0382 (D); adapted from [41].

Figure 3: *Candia auris* on CHROMAgar. The appearances of several *Candida* species on CHROMagar are shown in the photograph to the right [43]. On the left is *Candida auris* on CHROMagar, which resembles *Candida glabrata* [42].

its ability to grow using certain substrates or by the chemical reactions that take place as it does so. An example of the latter case is CHROMagar *Candida*. This growth medium-based diagnostic test is able to differentially identify many common *Candida* species based on a color change caused by the organism's

enzymatic activity (Figure 3) [43]. When attempting to identify *Candida auris* on CHROMagar *Candida*, the organism bears a color very similar to *Candida glabrata* and can easily be misidentified (Figure 3) [42].

The concept of identification by substrate utilization has been expanded by several biotechnology companies, such as Biolog and BioMérieux. These companies have developed tests that can identify a species by cross-referencing its assimilation pattern of various substrates with a database comprised of the typical pattern for most isolates of the organism. Because *C. auris* is a novel pathogen, it has yet to be added to many of these databases, including the aforementioned Biolog database. The diagnosis in these and other platforms also presents as a related species in most cases (Table 1) [34]. One notable exception occurs when using the MALDI Biotyper. On this platform, *Candida auris* is identified as one of two bacterial species. This is a serious error, because a prescribing clinician would prescribe antibacterial drugs that would have no effect on the fungal pathogen.

Table 3: Misdiagnosis of *Candida auris* using different identification platforms.

Identification Platform	**Diagnosis of *Candida auris***
API 20C	*Rhodotorula glutinis*
	Candida sake
API Candida	*Candida famata*
BD Phoenix Yeast Identification System	*Candida haemulonii*
	Candida catenulate
MALDI Biotyper	*Neisseria meningitides*
	Pseudomonas rhizosphaerae
MicroScan	*Candida famata*
	Candida lusitaniae
	Candida guilliermondii
	Candida parapsilosis
	Candida albicans
	Candida tropicalis
RapID Yeast Plus	*Candida parapsilosis*
Vitek	*Candida haemulonii*
	Candida lusitaniae
	Candida famata
Vitek 2 YST	*Candida haemulonii*
	Candida duobushaemulonii
Vitek MS	*Candida albicans*
	Candida haemulonii
Adapted from [34,42]	

Even when diagnosed as a related species, the treatment process is complicated due to differences in treatment procedures between the related species and the likelihood of a positive clinical outcome is decreased. Fortunately, new methods of identification that correctly identify isolates as *C. auris* are being developed and validated. The most effective methods utilize quantitative polymerase chain reaction (qPCR) to detect representative DNA sequences in the ribosomal RNA and internal transcribed spacer regions [44, 45]. These methods can provide a rapid identification and, with the right primers, can diagnose other *Candida* species as well. However, qPCR is a specialized method that requires training and equipment that increases the cost of identification. The specialized nature of this method presents issues in resource-deficient communities where the organism often flourishes. Thus, the gold standard for identifying organisms such as *Candida auris* will likely remain an approach using culture-based media. Through the use of specialized media and occasionally a few simple biochemical tests, an organism can be identified with low cost. However, the examination of the metabolic capabilities of *C. auris* to assimilate a variety of compounds and how this compares to related species is needed to address a major knowledge gap about the basic properties of this little-understood organism and pave the way to produce diagnostic and selective media. The species most closely related to *Candida auris* are *Candida haemulonii*, *Candida duobushaemulonii,* and *Candida lusitaniae,* none of which are frequent cause of disease [46]. A simple phylogenetic tree generated using the Taxonomy Browser and Tree Viewer of the National Center for Biotechnology Information's website displays the taxonomic relationship between the isolates of both the *Candida auris* and the drug-resistant *Candida* panels (Figure 4) [47, 48].

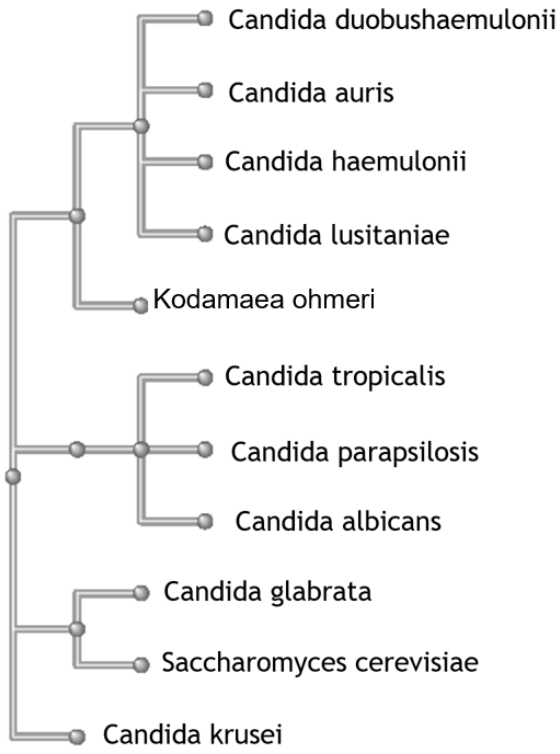

Figure 4: Simple phylogenetic tree showing the relationship of the organisms from the CDC *Candida auris* and drug-resistant *Candida* panels; created using [47, 48].

Resistance to Antifungal Drugs

The third trait of *Candida auris* that causes concern is its propensity to be drug resistant. Most strains are resistant to at least one antifungal drug, usually fluconazole [49]. Many strains display multi-drug resistance, and some have been found to be resistant to all three major classes of antifungal agents [49]. This trait is not isolated to one geographic region. The *Candida auris* panel mentioned previously contains isolates of each clade resistant to at least one class of antifungal [28]. Much uncertainty exists about the origin, spread, and epidemiology of *C. auris*, especially considering that the current opinion is that the separate clades emerged as pathogens simultaneously [33]. Regardless of the source, the production of new and effective treatment methods is critical to control this organism.

Methylene Blue

As part of the race towards effective methods for control of *C. auris*, assessing viability of cell cultures in a timely fashion is important. A stain that differentiates viable cells would provide a tool to rapidly assess the load of viable cells on those surfaces. This would extend to both persistence of the cells in an untreated environment and surface disinfection testing. Currently, viability assessments are made using the serial dilution and plate count methods. While these methods are effective at determining viability, it is time and resource intensive. Due to incubation times, viability based on plate counts cannot be determined in less than 48 hours. The use of a hemocytometer allows for quick and accurate estimation of total cell count, but without additional tools, it is impossible to differentiate between viable and nonviable cells. In the field of brewing, methylene blue staining is used with hemocytometer counts to quantify the percentage of viable *Saccharomyces cerevisiae* and other organisms of an inoculum source for adjusting to appropriate inoculum [50]. In metabolically active cells, the stain is metabolized to a colorless compound and the cells retain their hyaline (unstained)appearance under light microscopy. In metabolically inactive cells, the stain remains, and the cells appear blue. It is reasonable to assume that this technique could be extended to the pathogenic *C. auris*, possibly allowing a combination of rapid cell counts and viability determination.

Essential Oils

Plants have long been utilized to treat a variety of diseases, infectious or otherwise. As plant cells are fixed in place and thus circulating immune cells are not an option for protection from pathogens, other defense mechanisms must be present to combat pathogens [51]. One of these

mechanisms is the production of antimicrobial compounds, many of which are of low molecular weight, volatile and bear a pronounced odor [51]. The volatile nature and odor have drawn human interest in extracting these compounds for use as air fresheners and other scented products. Essential oils are produced by separating these volatile compounds from plant matter primarily by steam distillation, although extraction by cold pressing is sometimes used. After the liquid condenses, the water insoluble compounds separate and are collected. A review of the chemistry and biological activities of essential oils is published elsewhere [52].

Antimicrobial Activity

Essential oils are also a combination of some of the chemical weaponry utilized by plants. The volatile compounds easily diffuse from the damaged plant tissue and bear a strong odor, many of which deter predatory animals from consuming the plant [53]. The aversion to the smell is actually a response that organisms have developed because the odor indicates the presence of compounds that are toxic to other organisms [54]. The toxic properties of these chemicals extend beyond animal predators. Many of these oils also have antimicrobial properties against viruses, bacteria, and fungi [55, 56, 57, 58]. This makes the oils an attractive and mostly untapped reservoir for antimicrobial treatments. This interest is enhanced as they are also markedly less expensive than clinical drugs for the end-user. One issue in their application as therapeutic medicine is in their indiscriminate toxicity [59]. Just as they can kill microbes, they can prove damaging to human cells in elevated doses. Thus, many are considered “hot” oils in that they must be diluted to avoid reactions and cell damage when used topically [59]. Another issue is that many factors, such as plant growth conditions and manufacturing procedures, can alter the balance of the various chemicals present in the oils and make it more difficult to standardize them [52]. However, there is yet little evidence that these differences significantly impact the effectiveness. Other factors also increase the value of these oils as potential treatment options. Due to the volatile nature of essential oils, they are often used in aromatherapy and some reports claim that they retain their antimicrobial properties when volatilized [58, 60].

Synergism

Combination treatment is not a new concept. Drugs have been used in combination to treat difficult infections for almost as long as drug-resistance has been a problem [61]. A classic example is

using augmentin to treat penicillin-resistant bacteria [62]. Some of these bacteria produce an enzyme that breaks down the active region of the beta-lactam antibiotics, to which penicillin and cephalosporin belong. Augmentin contains penicillin and a salt of clavulanic acid. The acid salt is present because it competitively inhibits the beta-lactamase (enzyme conferring resistance) and allows penicillin to function. An example of combination treatment used on a fungal pathogen is the combination of amphotericin B and flucytosine against the yeast pathogen *Cryptococcus neoformans.* One study shows an enhanced killing of all isolates tested when the two drugs are used in combination [63]. A comprehensive review describing combination therapies in use and guidelines for the validation and use of combination treatments is published elsewhere [61]. Recent evidence also suggests that essential oils can function in a synergistic association with antimicrobial drugs, including antifungals [57, 64, 65]. Synergism is the name given to the property of some chemicals to enhance the effect of another drug when used in combination. This is different than additivity, where the effect is the sum of its parts. In synergism, the effects are actually enhanced. For example, assume two hypothetical drugs each required two grams per milliliter to elicit a three-log reduction of a pathogen when used alone. If these two drugs were additive, the same three-log reduction can be achieved using a mixture containing one gram, or half the dose of each, per milliliter of each. If the two drugs were synergistic, on the other hand, the same mixture might elicit a 4-log reduction of the pathogen instead. This is a desirable outcome because, as previously mentioned, antifungal drugs are expensive and can cause side effects that can be severe at the elevated doses needed to treat infections by resistant organisms. This phenomenon can restore the utility of antibiotics that have mostly been abandoned due to the widespread resistance to them. Given the current situation where antibiotics are being rendered increasingly ineffective due to drug-resistant organisms, the benefits of exploring essential oils are at least two-fold. For one, they may provide alternative treatment options for these resistant pathogens. Secondly, they can restore and enhance the utility of other treatments that have been benched due to ineffectiveness.

There are problems when evaluating combinations for synergistic associations, however. First, chemicals don't always complement each other. Just as some combinations display synergism, others will display indifference or even act against each other. In the case of indifference, the treatment will require the full dose of both chemicals, which, if used, can present the side effects for both chemicals. In the latter case, which is known as antagonism, the drugs inhibit each other, causing an increased overall dose required to treat the infection [61]. The second issue is the evaluation of the interaction between

the drugs. Several methods are currently used, and the description and interpretation of the results can differ between each [66]. Thus, a clear description of the method used to acquire and interpret results is mandatory so that other researchers can build upon the data generated. The third issue is that combinations sometimes vary in effectiveness based on the timing of the administration of each drug. An example is the combination of amphotericin B and fluconazole [61]. When fluconazole is introduced first, it depletes the ergosterol targets of amphotericin B and thus decreases the overall effectiveness of the combination. When amphotericin B is added first, however, it creates pores in the cellular envelope that allows the later-added fluconazole greater access to its targets. The final issue is present for all drug treatments but is still important to consider. Even though a combination may display a certain interaction and effectiveness *in vitro*, further investigation is needed to determine if these properties will be retained *in vivo*.

Research Objectives

The investigations conducted here are multifaceted and sought to address several of the issues mentioned above. All of the work focused on a core group of isolates. All but one of these isolates were obtained from the CDC, described in Appendix I. The other isolate was a strain of *Candida albicans* that was obtained from the United States Department of Agriculture's Northern Regional Research Laboratory (NRRL) collection. This isolate's details are also listed in Appendix I.

This study was organized into several experimental objectives. The first objective of this study was to streamline a process for the preparation of a standardized inoculum. Second, the work sought to test the efficacy of methylene blue as a viability staining agent on *C. auris*. Third, the basic metabolic capability of *C. auris* to assimilate various chemicals as a sole source of carbon or nitrogen was examined. Fourth, *C. auris* was examined for a distinct pattern of fermentation and assimilation using the appropriate Biolog product. Fifth, the antimicrobial activity of select essential oils against *C. auris* was examined. Sixth, the ability of these essential oils to display antimicrobial activity in vapor phase was briefly explored. Seventh, the interaction between the most effective oils and select antifungal drugs was examined using *C. auris* as well as *Candida lusitaniae* and *Saccharomyces cerevisiae.* The overall goal was to increase and understanding of the novel fungal pathogen and produce data and methods that could lead to diagnostic methods and treatment options to combat this emerging global threat.

Methods

Culture preparation and maintenance

Two panels of drug resistant *Candida* species were acquired from the CDC (Appendix I). The first was the Drug Resistant *Candida* Panel (species other than *C. albicans*) and the second was the *Candida auris* Panel, consisting of 32 and 20 strains, respectively. The isolates were received as glycerol stocks, which were inoculated into malt extract broth (MEB) and onto malt extract agar (MEA). All cultures were incubated at 37°C for two days. Samples from colonies grown on MEA, of each organism, were used to create stocks in 30% glycerol for long-term storage at -80°C. The MEA plates were retained for future use and preserved by wrapping in parafilm and refrigerating at 4°C. New MEA plates were inoculated at least once a month by quadrant-streaking from a colony of an older plate. After a fifth-generation plate was made, a fresh series was created from the glycerol stocks, which was performed to maintain the isolate's wild-type characteristics.

Absorbance-Population Determination

To allow for rapid determination of population size using optical density at 600 nm (OD600), a standard curve and function of these two values was constructed for each species present in either panel. Isolates 0385, 0389, 0391, 0393, 0396, 0397, 0398, 0399, 0325, 0344, 0345, and NRRL-Y12983 were chosen to represent one isolate of each species. Two isolates of *Candida auris* were chosen, however, to verify that that the standard curve would remain consistent between isolates of the same species. To construct the curves, an overnight culture of each isolate was grown in MEB at 30°C with shaking at 250 RPM. The following day, the cultures were diluted to a population that appeared countable using a hemocytometer. This dilution was considered the starting population. Further dilutions were created to yield 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, and 90% of the starting population. These nine dilutions and the starting population encompassed the samples tested. Triplicate aliquots of each of these samples were transferred to cuvettes and the OD600 was measured for each using the spectrophotometer. Then, triplicate aliquots of 10 µL of each dilution were added to the hemocytometer and counted under the light microscope. The mean of the triplicate of both the OD600 values and the cells counts was calculated and the OD600 was plotted against the cell counts for each organism using Excel™. The equation for the trendline and the R^2 were then generated using the same software.

Working Stock Preparation

To obtain standardized starting populations with a more rapid preparation for all experiments, glycerol working stock were prepared at standardized population sizes. For each isolate used, an overnight culture was grown at 30°C with shaking. The following day, the population was standardized to 1.0 – 2.0 x 10^6 cells mL^{-1} based on the standard curve previously determined. Then, 1.0 mL of the culture was mixed with an equal volume of 60% glycerol to yield a 2.0 ml working stock in 30% glycerol. These were stored at -80°C and were discarded if not used within a month to ensure viability was retained.

Methylene Blue Validation

C. auris (AR0389) of the CDC *Candida auris* panel was used as the challenge organism. *S. cerevisiae* (AR0399) from the same panel was used as the control organism. Overnight cultures of *S. cerevisiae* and *C. auris* were grown at 37°C with 250 RPM shaking in MEB. One half of the volume, or 10 mL, of each culture was placed in boiling water for 10 minutes while the other half was held at room temperature to generate four samples. An aliquot of 100 µL from each sample was mixed with an equal volume of 0.02% methylene blue and incubated for 5 minutes at room temperature. Triplicate aliquots of the stained samples were loaded into a hemocytometer, and each was microscopically observed and counted to determine the percentage of viable cells. Viable cells were identified by the retention of their normal hyaline character. To then validate the viable counts estimated by the hemocytometer with methylene blue, the experiment was repeated with the addition of the following step. Samples from the treatment groups were diluted to less than 10^3 cells mL^{-1}, viable or otherwise, using the hemocytometer counts to determine the relevant dilution factor. From the diluted samples, 0.1 ml of each were spread across the surface of separate MEA plates in triplicate and incubated for 48 hours at 37°C. The hemocytometer and plate counts were compared to determine the accuracy of the staining method to quantify viable cells. A two-tailed Student's t-test assuming unequal variance was used to determine if any statistically significant difference was present between samples.

Carbon and Nitrogen Source Testing

Carbon Source Utilization

To determine the capability of *C. auris* and the related *Candida spp.* to utilize various substrates as their sole carbon source, growth experiments were performed in minimal media containing only the tested substrate as a carbon source. The base media was yeast nitrogen base without amino acids (YNB). This was supplemented with the following carbon sources separately at a concentration of 2% by volume: D-glucose, D-galactose, L-arabinose, D-xylose, maltose, lactose, sucrose, raffinose, maltodextrin, glycerol, dulcitol, mannitol, and dodecane (Table 4). Dodecane was adjusted to 1% by volume due to the compound containing nearly twice the carbon per unit weight, whereas the others had the same levels of carbon by weight. The isolates used encompassed the entire *Candida auris* panel and included isolates 0325, 0333, 0340, 0344 and 0345 for the Drug-Resistant *Candida* panel as well as the NRRL Y-12983 isolate of *Candida albicans*. A working stock of each isolate used was obtained from the -80°C freezer, thawed and centrifuged. The excess glycerol solution was then decanted, and the organism was washed and resuspended in 1.0 mL of conidia harvesting solution (CHS). Each well of a 96-well plate was filled with 190 µL of YNB supplemented with one of the carbon sources and was inoculated with 10 µL (approximately 10^4 cells) of the prepared working stock culture. Negative controls for each carbon source using CHS in place of inoculum were included. Each carbon source was tested in triplicate for each isolate. The plates were sealed using parafilm to prevent evaporation and incubated at 30°C. At 0, 24, 48 and 72 hours, the plate was agitated to homogenize the contents of each well and the OD600 of each well was read.

Nitrogen Source Utilization

A modified carbon-source utilization method was used to determine the capability of utilizing sole nitrogen sources. The base media used was yeast carbon base (YCB) instead of yeast nitrogen base, supplemented with one of the following nitrogen sources: ammonium acetate, sodium nitrate, sodium nitrite, urea, L-arginine, L-valine, glycine, L-proline, D-valine, uracil, thymine, thiamin or pyridoxin. These were standardized to contain an equivalent amount of nitrogen as 0.01% ammonium acetate (Table 4). Growth parameters and procedures otherwise followed the Carbon Source Utilization protocol described above.

Table 4: Carbon and Nitrogen Sources.

Carbon Source	Percent (v/v)	Nitrogen Source	Percent (v/v)
D-glucose	2%	ammonium acetate	0.01%
D-galactose	2%	sodium nitrate	0.01%
maltose	2%	sodium nitrite	0.01%
sucrose	2%	L-valine	0.004%
lactose	2%	glycine	0.006%
L-arabinose	2%	L-proline	0.02%
D-raffinose	2%	L-arginine	0.01%
D-xylose	2%	D-valine	0.02%
glycerol	2%	urea	0.02%
mannitol	2%	thiamin	0.01%
maltodextrin	2%	pyridoxin	0.01%
dodecane	1%	thymine	0.01%
dulcitol	2%	uracil	0.03%

Substrate Utilization using Biolog YT Plates

The Biolog YT plate was used to examine how *C. auris* was identified by the Biolog system and how the organism utilized the substrates contained by the plate (Appendix II). The first objective was to determine the accuracy of the system in identifying known organisms. To do this, overnight cultures of *Candia auris* (AR0385), *Candida lustaniae* (AR0398) and *Saccharomyces cerevisiae* (AR0399) were grown on Biolog Universal Yeast (BUY) Agar. The following day, the colonies from the plates were suspended in sterile deionized water and standardized to within 2% of the transmittance of the Biolog YT standard using the Biolog turbidimeter. One hundred µL of one culture was transferred to each well of the YT plate. Triplicate plates were set up for each organism. These were incubated for 48 hours at 30°C. Following incubation, the plates were read using the Biolog Microstation and examined using the Biolog Microlog software. The remaining isolates of *Candida auris* were each tested once using the procedure above.

Antifungal properties of Essential Oils

Antifungal Activity Testing

The antifungal activity of several essential oils was tested using a modified microdilution method. The selected essential oils are listed in Appendix III with the maximum concentration safe for dermal use, where available [59]. Two isolates of *Candida auris* (AR0381 and AR0385) were used, as well as one isolate each of *C. lusitaniae* (AR0388) and *S. cerevisiae* (AR0399). The base media was yeast nitrogen base supplemented with 2% glucose (YNBG). 380 µL of YNBG supplemented with 1% of the tested essential oil was added to the first well of a column on a 96-well plate. The remaining wells of the column were filled with 190 µL of YNBG with 1% dimethyl sulfoxide (DMSO). Microdilutions were performed by transferring 190 µL of the top well's contents to the second well and mixing. One hundred ninety µL of the second was transferred to the third well and mixed. This was repeated for the entire column of eight wells, with 190 µL of the contents of the last well being discarded to ensure an equal volume in each. This process replaced the diluted essential oil with DMSO to ensure an equal concentration of media was present in each well. After the microdilutions were prepared, each well was inoculated with 10 µL of a thawed working stock, for a starting population size of 1.0 – 2.0 x 10^4 cells/well. The final working volume of each well was 200 µL. The plates were sealed using breathable cover films and incubated for 72 hours at 30^{o}C. Following incubation, the plates were agitated to homogenize the contents of each well and the OD600 was read using a plate reader. Each microdilution series was performed in triplicate. The mean was taken for the triplicate of each concentration. Complete inhibition was determined by a mean OD600 value of less than 0.5. The MIC was assigned to the minimum concentration that inhibited the growth of the starting population.

The contents of the inhibited wells were then transferred to 1.0 mL of Letheen neutralizing broth. Letheen broth functions both to neutralize the antimicrobial activity of the essential oil and as a growth medium. The broth cultures were then incubated for 72 hours at 30°C with shaking. Following incubation, the tubes were observed for any visual signs of growth. The MFC was determined as the minimum concentration that displayed no growth at this phase. Negative controls were included for the essential oil, DMSO, YNBG and the Letheen broth. A positive control containing DMSO and Letheen broth was included.

Antifungal Activity of Essential Oils in Vapor-Phase

Each essential oil was tested for antifungal activity in vapor-phase in a sealed airspace. Working stocks of *C. auris* (0385) were diluted to yield population sizes of 50-100 cells per 10 µL. Then, 10 µL of this culture was transferred to four 60 mm Petri dishes containing MEA and spread over the agar. Three of these plates were then placed with the lids removed in an empty 150 mm Petri dish. A small cup made of aluminum foil was placed at the center of the large Petri dish and filled with 100 µL of the tested essential oil. The lid of the 150 mm Petri dish was immediately replaced, and the plate was sealed with parafilm to create an enclosed airspace. The fourth 60 mm plate was independently sealed with parafilm and used as a viability control. All plates were incubated for 72 hours at 30°C. Following incubation, the 60mm plates were observed for growth and inhibition was determined when no growth was observed. Lids were then replaced on the inhibited plates, sealed with parafilm and returned to incubation for an additional 72 hours. Following the second incubation period, the plates were again checked for growth. Lethality was determined when the plates showed no obvious signs of growth at this phase. This experiment was then repeated using 10 µL of the essential oils that displayed fungicidal activity at 100 µL and using 1 µL of the essential oil diluted in 99 µL of DMSO for the oils that displayed fungicidal activity at 10 µL.

Synergism Testing

A modified checkerboard method was used to examine the interaction between the essential oils displaying the lowest MIC's and select antifungal drugs. The oils tested were lemongrass, clove bud and cinnamon bark. The antifungals used were amphotericin B, flucytosine, fluconazole, and micafungin. One strain each of *C. auris* (0381)*, C. lusitaniae* (0398)*,* and *S. cerevisiae* (AR0399) was used as challenge organisms. Stock solutions of the antifungals were prepared by dissolving the drug in pure DMSO to yield concentrations not exceeding the manufacturer's recommended solubility limit. Working solutions were prepared for each organism by diluting the stock solutions in sterile deionized water to produce concentrations equal to 320 times the published MIC for the organism. Then, a separate solution was mixed using YNBG with 1% of a combination of DMSO and the tested essential oil.

The volume of essential oil used was calculated to yield a final solution containing 16 times the previously determined MIC for the organism. Three hundred sixty uL of this solution was added to the first well of the first column on the 96-well plate. One hundred ninety µL was added to the remaining

wells of the column and to the first well of column 9. The remaining solution containing YNBG, essential oil, and DMSO was then diluted with an equal volume of YNBG plus 1% DMSO to yield a new solution with half the concentration of essential oil. 360 µL of this solution was added to the first well of the second column and 190 µL was added the remaining wells of the column and the second well of column nine. The process of diluting the essential oil mixture and adding it to the plate was repeated for the next six columns of the 96-well plate. Three hundred sixty µL of the YNBG plus 1% DMSO was added to the top well of the tenth column and 190 µL was added to the remaining wells of the column.

After the essential oil microdilutions were performed, 20 µL of the antifungal working solution was added to the top well of each column, except for columns nine, eleven and twelve. Then, 190 µL of the contents of the top well of the first column was transferred to the second well and mixed. 190 µL of the second well was transferred to the third and mixed. This was continued for the remainder of the wells in the column, with 190 µL of the contents of the last well being discarded to ensure an equal volume was present in all wells. These microdilutions were repeated for columns two through eight, as well as column ten. The final plate contained combinations of every tested concentration of essential oil and antifungal as well as each concentration of essential oil and antifungal in isolation in columns nine and ten, respectively (Table 5).

After the antifungal microdilutions were complete, 10 µL containing approximately 1.0 - 2.0 x 10^4 cells of the working stock culture was added to each test well. Triplicate plates were sealed using breathable cover films and incubated for 72 hours at 30°C. Following incubation, the plates were agitated to homogenize the contents of each well and the OD600 was observed and recorded for each well. The mean of the triplicate results was calculated, and complete inhibition was determined when the mean of the OD600 readings for a particular combination was less than 0.5. The fractional inhibitory concentration index (FICI) of the checkerboard assays was calculated from the fraction inhibitory concentrations (FICs) as shown in Table 6 [64]. The test well used to determine the MIC in combination was the one located most centrally along the inhibition interface. The interpretation of the interaction is also as previously described (Table 6) [67].

Table 5: General layout of the checkerboard plates. EO: essential oil MIC, AF: antifungal MIC, DMSO: dimethyl sulfoxide.

16xEO 16xAF	8xEO 16xAF	4xEO 16xAF	2xEO 16xAF	1xEO 16xAF	0.5xEO 16xAF	0.25xEO 16xAF	0.125xEO 16xAF	16xEO	16xAF	Negative Media Control	Negative Media Control
16xEO 8xAF	8xEO 8xAF	4xEO 8xAF	2xEO 8xAF	1xEO 8xAF	0.5xEO 8xAF	0.25xEO 8xAF	0.125xEO 8xAF	8xEO	8xAF	Negative DMSO Control	Negative DMSO Control
16xEO 4xAF	8xEO 4xAF	4xEO 4xAF	2xEO 4xAF	1xEO 4xAF	0.5xEO 4xAF	0.25xEO 4xAF	0.125xEO 4xAF	4xEO	4xAF	Positive DMSO Control	Positive DMSO Control
16xEO 2xAF	8xEO 2xAF	4xEO 2xAF	2xEO 2xAF	1xEO 2xAF	0.5xEO 2xAF	0.25xEO 2xAF	0.125xEO 2xAF	2xEO	2xAF		
16xEO 1xAF	8xEO 1xAF	4xEO 1xAF	2xEO 1xAF	1xEO 1xAF	0.5xEO 1xAF	0.25xEO 1xAF	0.125xEO 1xAF	1xEO	1xAF		
16xEO 0.5xAF	8xEO 0.5xAF	4xEO 0.5xAF	2xEO 0.5xAF	1xEO 0.5xAF	0.5xEO 0.5xAF	0.25xEO 0.5xAF	0.125xEO 0.5xAF	0.5xEO	0.5xAF		
16xEO 0.25xAF	8xEO 0.25xAF	4xEO 0.25xAF	2xEO 0.25xAF	1xEO 0.25xAF	0.5xEO 0.25xAF	0.25xEO 0.25xAF	0.125xEO 0.25xAF	0.25xEO	0.25xAF		
16xEO 0.125xAF	8xEO 0.125xAF	4xEO 0.125xAF	2xEO 0.125xAF	1xEO 0.125xAF	0.5xEO 0.125xAF	0.25xEO 0.125xAF	0.125xEO 0.125xAF	0.125EO	0.125xAF		

Table 6: Interpretation of fractional inhibitory concentration index (FICI).

FICI= FIC of Oil* + FIC of Antifungal**

FICI ≤ 0.5	Synergistic
0.5 < FICI ≤ 1.0	Additive
1.0 < FICI ≤ 4.0	Indifferent
FICI > 4.0	Antagonistic

***FIC of Oil =** $\dfrac{\textbf{MIC of Oil in Combination}}{\textbf{MIC of Oil Alone}}$

****FIC of Antifungal =** $\dfrac{\textbf{MIC of Antifungal in Combination}}{\textbf{MIC of Antifungal Alone}}$

FICI= **FIC of Oil + FIC of Antifungal**

Results

Absorbance-Population Study

In all cases, the absorbance at 600 nm correlated linearly with the populations counted using the hemocytometer (Figures 5-16). The functions all appear as expected, with a slope between 1.0 – 8.0 x 10^8 (Table 7). Some variation in the correlation between OD600 and population size exists between the different species of yeasts. The most pronounced is *Saccharomyces cerevisiae,* which displays a substantially lower population at a given absorbance than the other yeasts. In all cases, the functions point towards a population in the range of 10^8 cells mL^{-1} at an OD600 of 1.0. This similarity is expected of related organisms of similar size.

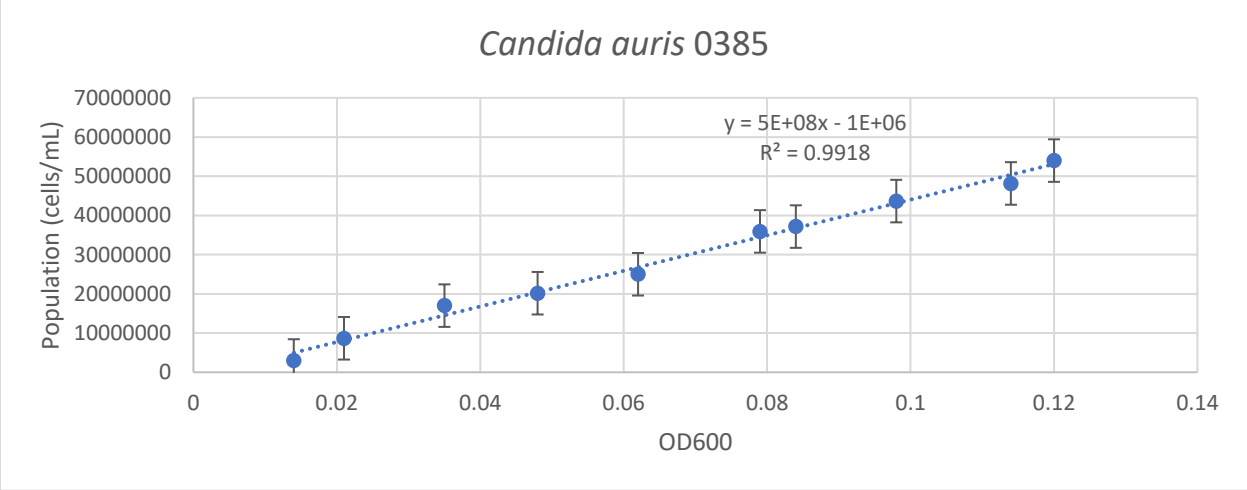

Figure 5. Absorbance at 600 nm to population standard curve for *Candida auris* AR0385.

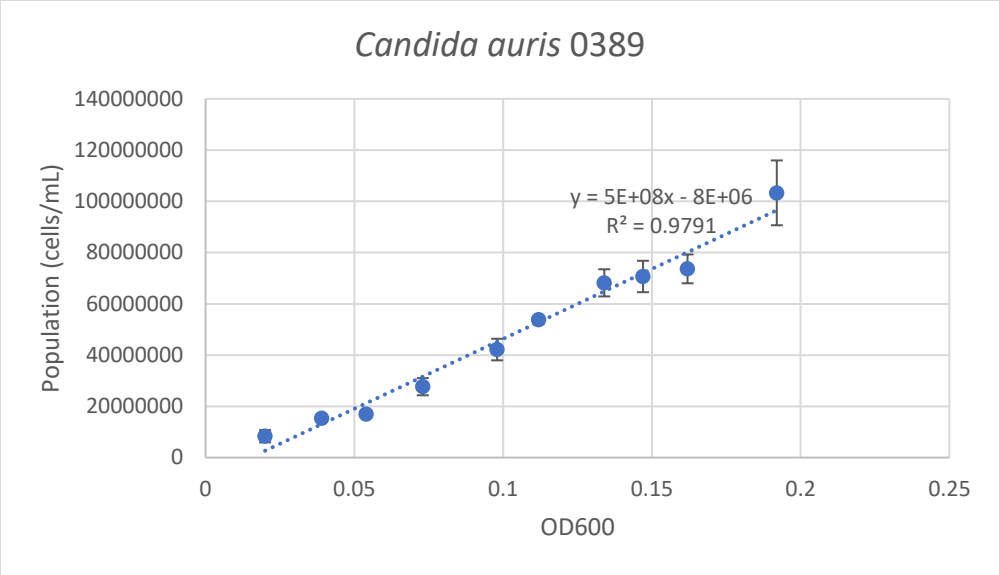

Figure 6: Absorbance at 600 nm to population standard curve for *Candida auris* AR0389.

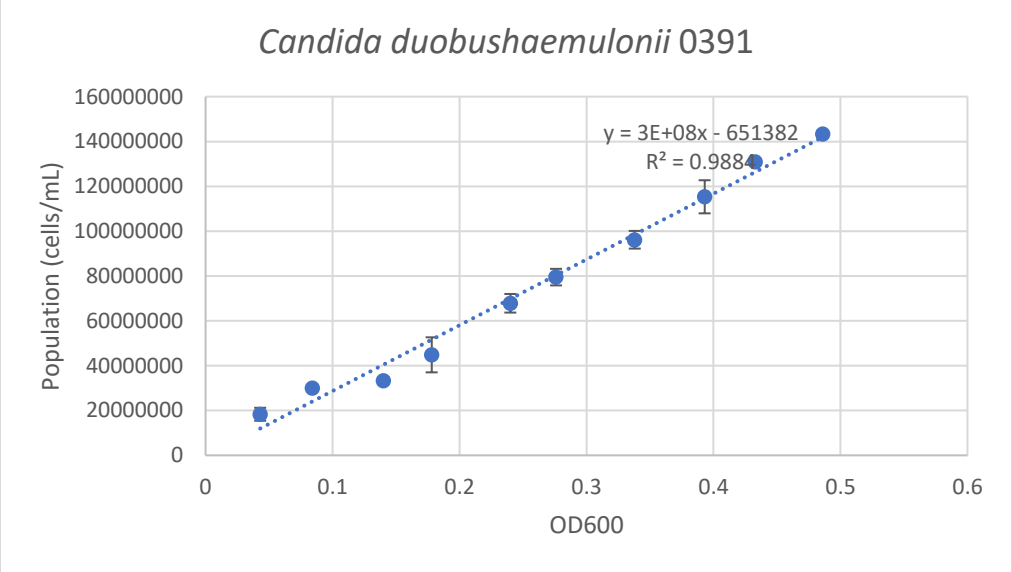

Figure 7: Absorbance at 600 nm to population standard curve for *Candida duobushaemulonii* AR0391.

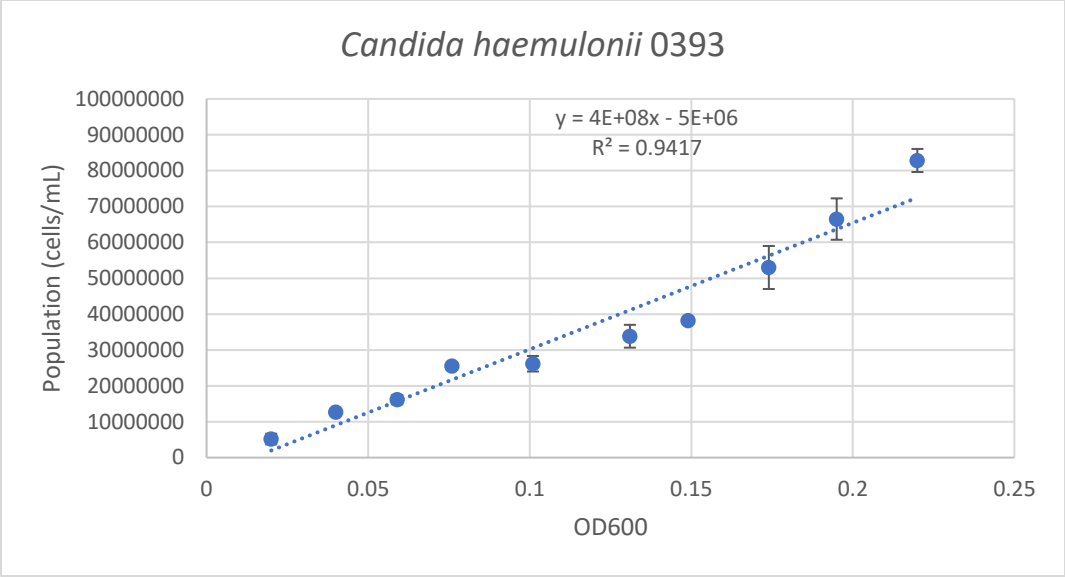

Figure 8: Absorbance at 600 nm to population standard curve for *Candida haemulonii* AR0393.

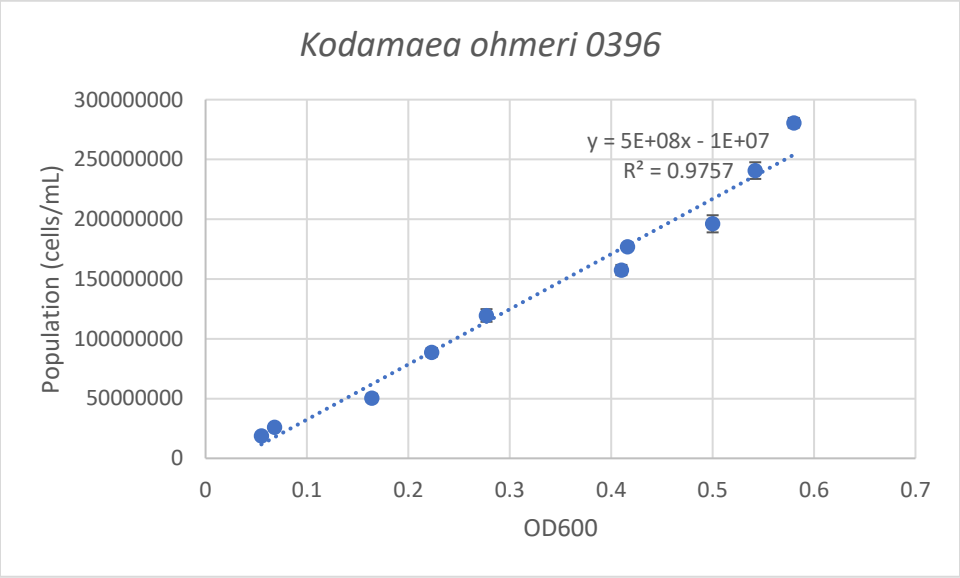

Figure 9. Absorbance at 600 nm to population standard curve for *Kodamaea ohmeri* AR0396.

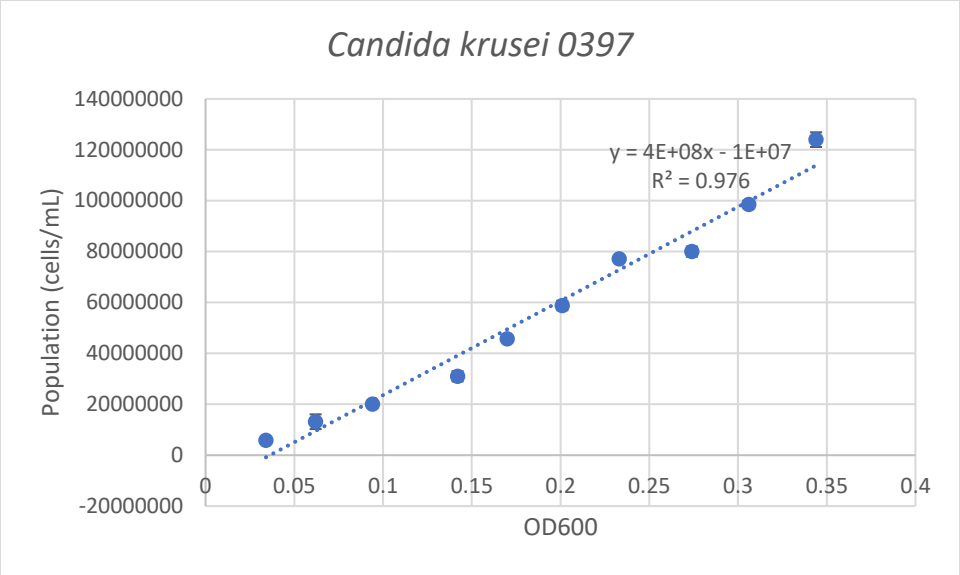

Figure 10: Absorbance at 600 nm to population standard curve for *Candida krusei* AR0389.

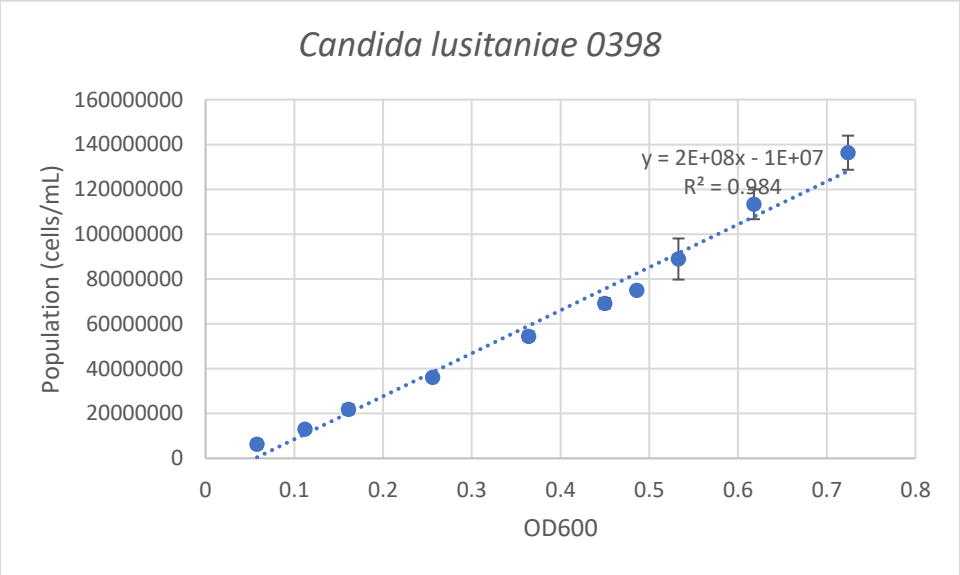

Figure 11: Absorbance at 600 nm to population standard curve for *Candida lusitaniae* AR0398.

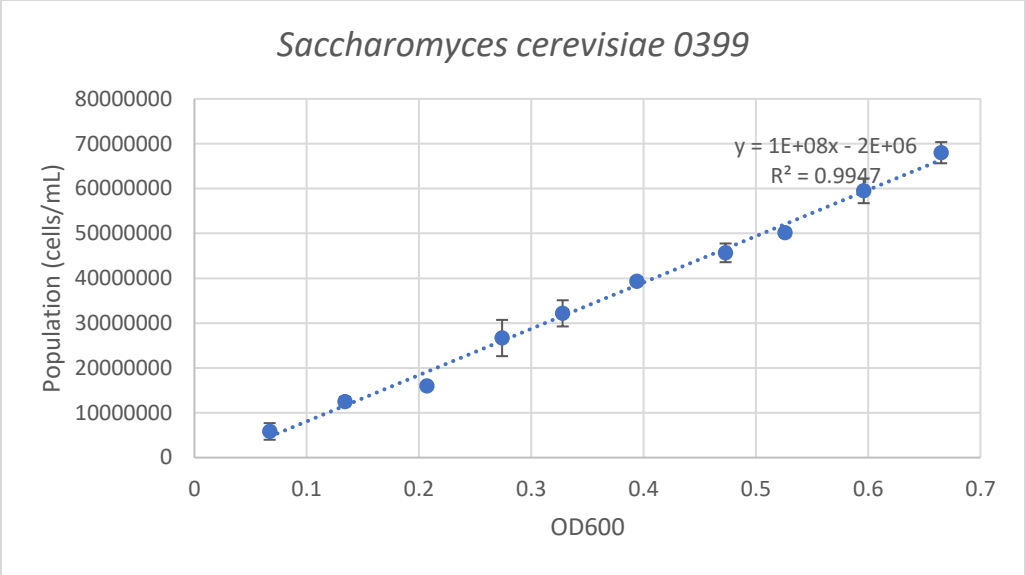

Figure 12: Absorbance at 600 nm to population standard curve for *Saccharomyces cerevisiae* AR0399.

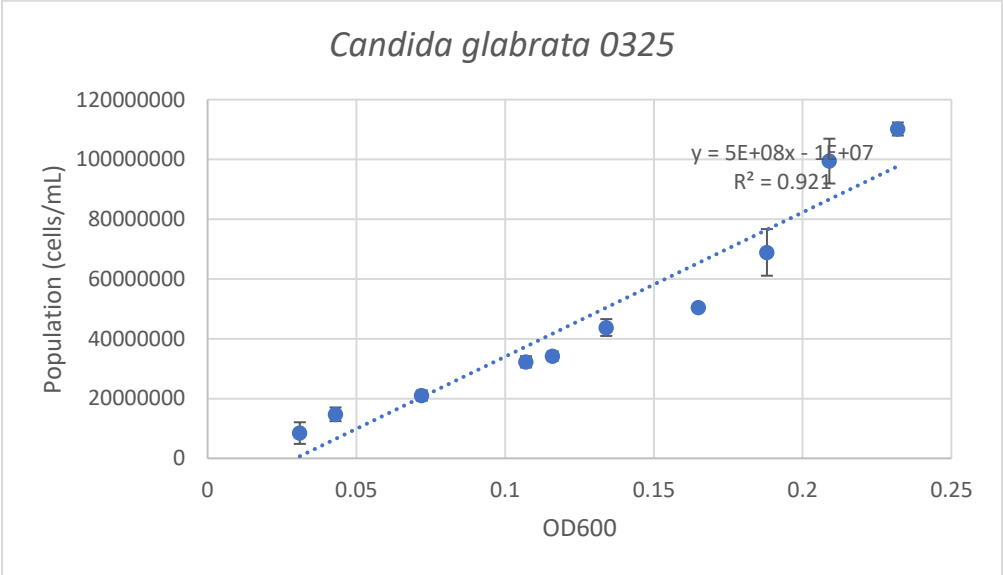

Figure 13: Absorbance at 600 nm to population standard curve for *Candida glabrata* AR0325.

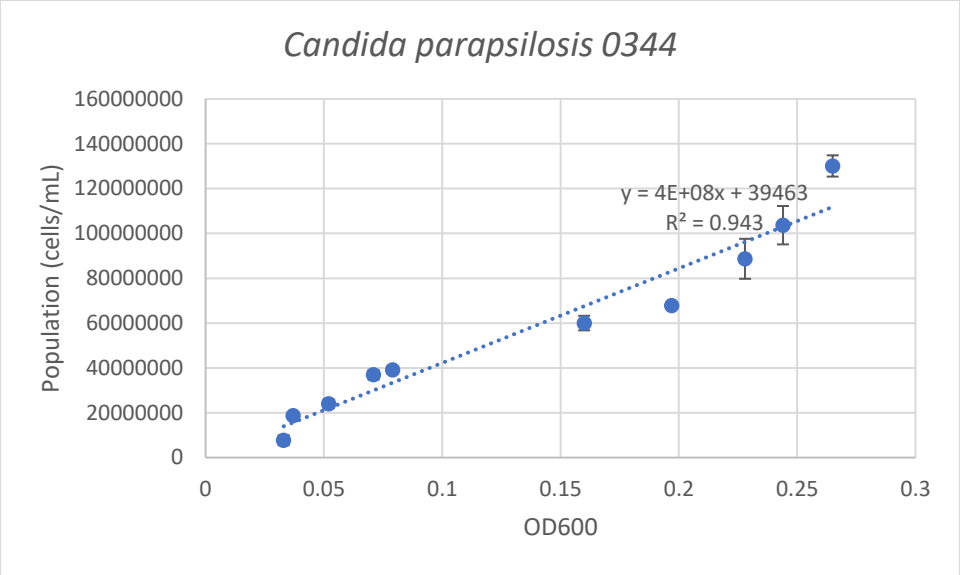

Figure 14: Absorbance at 600 nm to population standard curve for *Candida parapsilosis* AR0344.

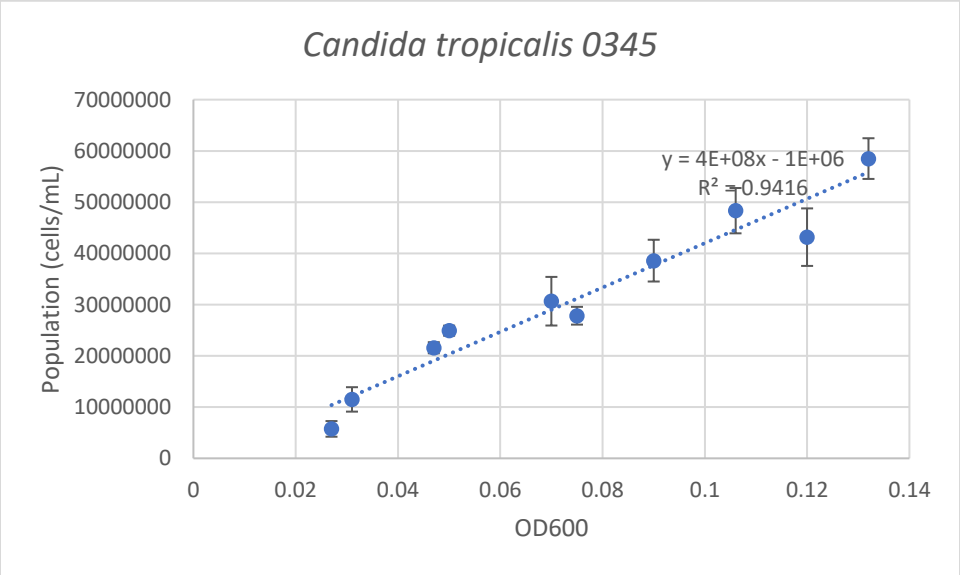

Figure 15: Absorbance at 600 nm to population standard curve for *Candida tropicalis* AR0345.

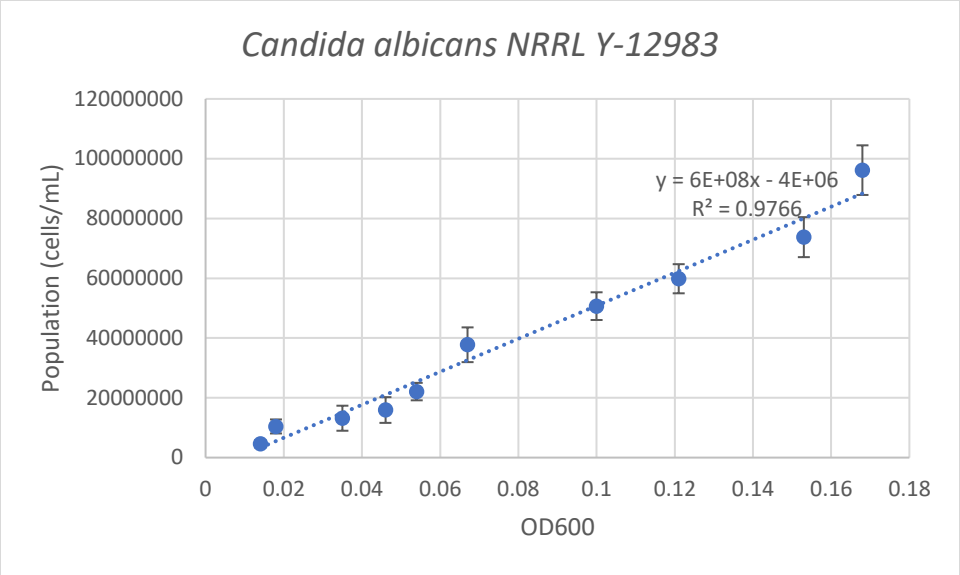

Figure 16. Absorbance at 600 nm to population standard curve for *Candida albicans* NRRL Y-12983.

Table 7: Absorbance to population functions.

Strain	**Function***	**R^2**
Candida auris 0385	$y = (5 \times 10^8)x - 1x10^6$	0.992
Candida auris 0389	$y = (5 \times 10^8)x - 8 x10^6$	0.979
Candida duobushaemulonii 0391	$y = (3 \times 10^8)x - 6 x10^5$	0.988
Candida haemulonii 0395	$y = (4 \times 10^8)x - 5 x10^6$	0.942
Kodamaea ohmeri 0396	$y = (5 \times 10^8)x - 1 x10^7$	0.976
Candida krusei 0397	$y = (4 \times 10^8)x - 1 x10^7$	0.976
Candida lusitaniae 0398	$y = (2 \times 10^8)x - 1 x10^7$	0.984
Saccharomyces cerevisiae 0399	$y = (1 \times 10^8)x - 2 x10^6$	0.995
Candida glabrata 0325	$y = (5 \times 10^8)x - 1 x10^7$	0.921
Candida parapsilosis 0344	$y = (4 \times 10^8x + 3 x10^4$	0.943
Candida tropicalis 0345	$y = (4 \times 10^8)x - 1 x10^6$	0.942
Candida albicans NRRL Y-12983	$y = (6 \times 10^8)x - 4 x10^6$	0.977

** y = population, x = absorbance*

Methylene Blue Validation

The boiled samples of both *C. auris* and *S. cerevisiae* displayed the typical blue staining in all cases, while the non-boiled samples remained hyaline (Figure 17). There was no significant difference in

viability (p=0.99) between the staining and plate count methods (Figure 18). No viability was detected from the boiled samples using either method.

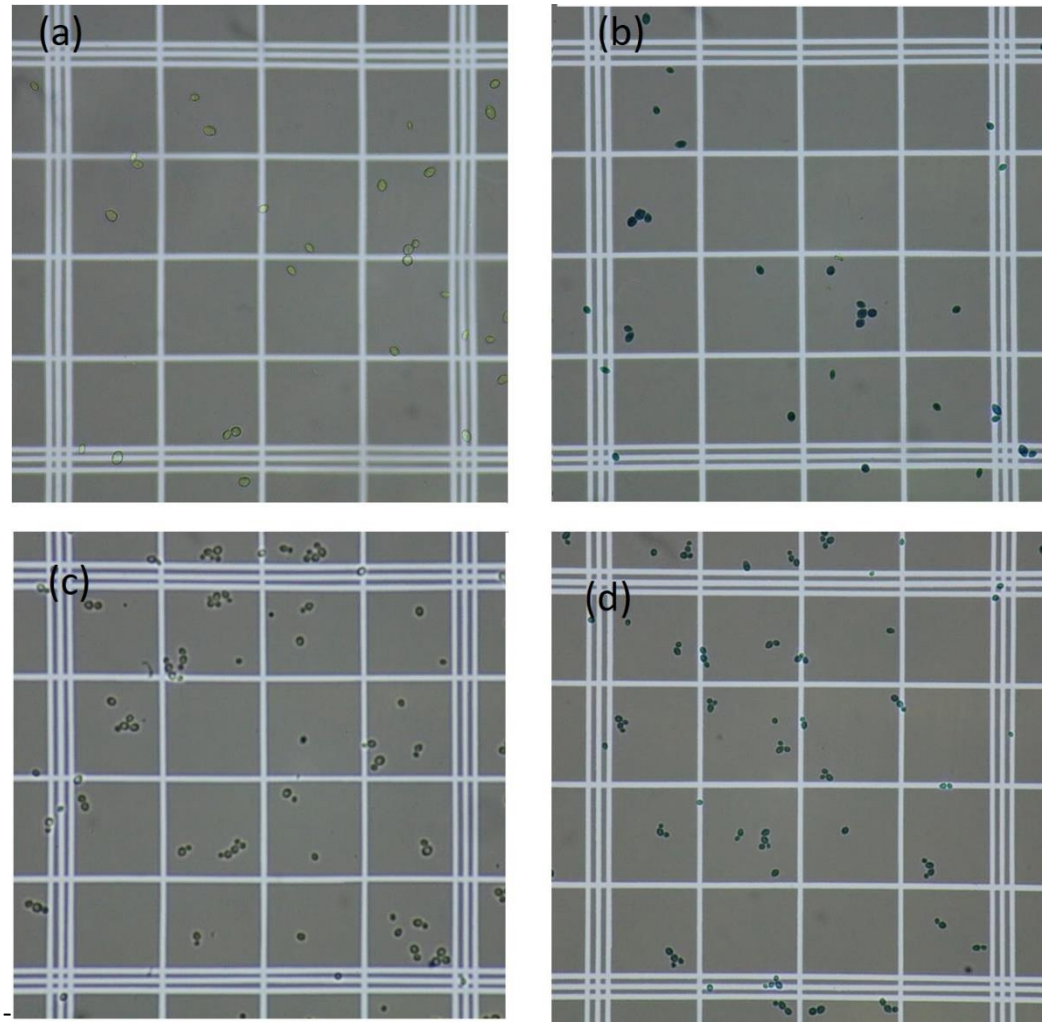

Figure 17: Hemocytometer grid at 400x magnification with boiled and non-boiled *Saccharomyces cerevisiae* and *Candida auris* after being stained with methylene blue. *S. cerevisiae* non-boiled (A), *S. cerevisiae* boiled (B), *C. auris* non-boiled (C), and *C. auris* boiled (D). Non-viable cells are stained blue. Smaller squares, outlined by single lines, are 0.0025 mm^2. Larger squares, outlined by 3 lines, are 0.04 mm^2.

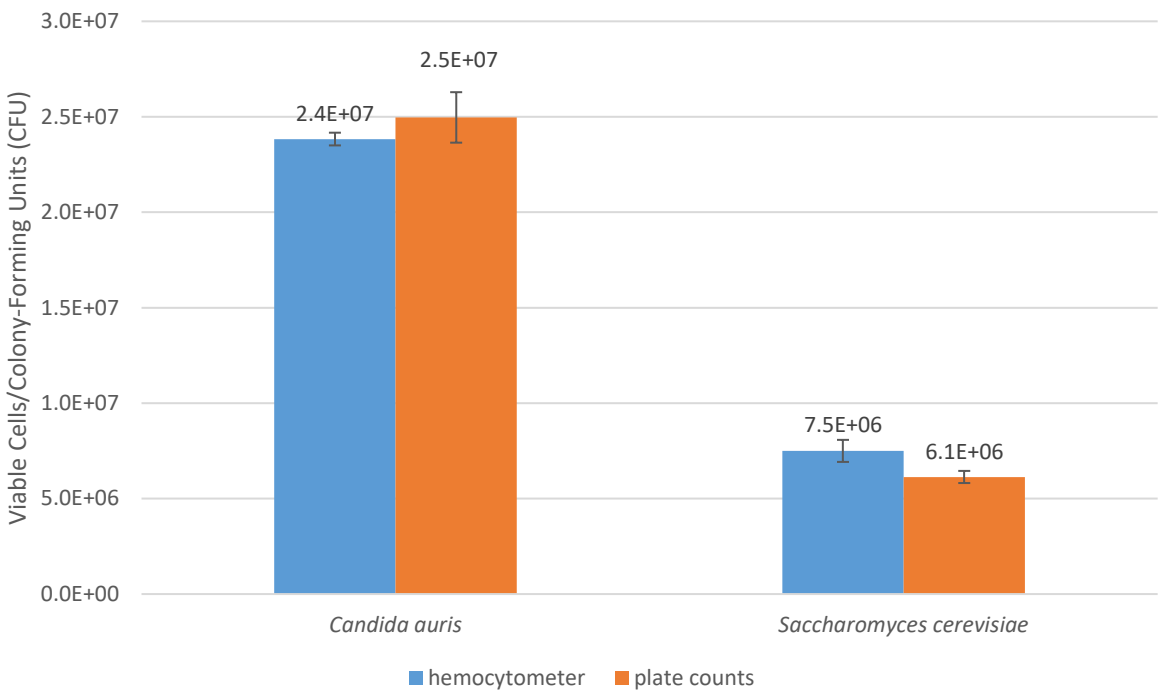

Figure 18: Viability of *Candida auris* and *Saccharomyces cerevisiae* using hemocytometer enumeration following methylene blue staining and traditional plate counts. Results are the average of triplicate counts for each, with the average being displayed above the corresponding bar. Units presented are viable cells for hemocytometer counts and colony-forming units for plate counts.

Substrate Utilization

Carbon Source Assimilation

Each species displayed a unique pattern of carbon assimilation. (Tables 7, 8 and 9). Negative results are indicated by red shading and are determined as an OD600 of less than 0.2, which corresponds to a lack of visible growth. Positive results are shaded green and determined as an OD600 of greater than 0.5, which corresponds with obvious visible growth. Those intermediate in this range are colored yellow and identified as weak growth. All isolates did not reach the positive range on several carbon sources by 24 hours. Some of these did not reach the needed OD600 until 72 hours. *C. auris* did not assimilate galactose or xylose, while all other species except *C. glabrata* can assimilate galactose, although *C. haemulonii* only does so weakly. Some substrates showed variable results between isolates at 72 hours for *C. auris*. These were lactose, L-arabinose, and dulcitol. The variability was not isolated by clade. *C. glabrata* only assimilated glucose. Lactose and L-arabinose display some variability among the other isolates as well. Raffinose is not assimilated well by *S. cerevisiae, C. parapsilosis,* or *C. tropicalis*,

and is only weakly utilized by *C. haemulonii.* Dulcitol, dodecane and xylose also display some variability between the two isolates of *S. cerevisiae.* The CHS controls remained negative as expected.

Table 7: Carbon usage profiles of *Candida auris* and other related species at 24 hours.

		D-glucose	D-galactose	D-maltose	sucrose	lactose	L-arabinose	D-raffinose	D-xylose	glycerol	mannitol	maltodextrin	dodecane	dulcitol
Species	**Isolate**													
Candida auris														
	0381	0.495667	0.006111	0.226556	0.390222	0.026111	0.219889	0.329111	0.009667	0.244111	0.187222	0.390333	0.322444	0.155667
	0382	0.510111	0.000222	0.209111	0.433444	0.105556	0.114778	0.409778	0.003889	0.163	0.203556	0.609667	0.265222	0.181
	0383	0.059111	0.005778	0.018778	0.037	0.017111	0.013333	0.042	0.002667	0.016667	0.025222	0.062667	0.152778	0.044889
	0384	0.008556	-0.01489	0.002	0.002778	0.004444	-0.00033	0.009778	0.000444	0.002889	0.015	0.024	0.049222	-0.01144
	0385	0.009111	0.006222	0.003778	0.008444	0.046444	0.028778	0.002444	0.000889	0.018333	0.003889	0.002333	0.072111	0.009889
	0386	0.426667	0.003111	0.223556	0.318889	0.092556	0.100556	0.347889	0.027556	0.084111	0.167222	0.346667	0.244444	0.045222
	0387	0.081778	-0.00878	-0.00778	0	0.001	-0.00833	0.003889	0.001	0.004556	0.003	0.013889	0.196111	0.002667
	0388	0.068556	-0.00622	0.007111	0.011556	0.006667	0.004111	0.017556	0.002111	0.000222	0.008222	0.051333	0.020889	-0.00244
	0389	0.056889	0.001889	0.009	0.010444	0.009111	0.007	0.014	0.002	0.008444	0.011444	0.031222	0.031889	-0.00744
	0390	0.166889	0.022667	0.014667	0.024889	0.013667	-0.00633	0.047111	0.001667	0.020778	0.015556	0.133222	0.178889	0.001778
Candida duobushaemulonii														
	0391	0.214778	0.259889	0.181111	0.164556	0.041333	0.018667	0.019889	0.003778	0.043	0.132333	0.285556	0.051889	0.041778
	0392	0.178889	0.017889	0.058444	0.117333	0.029333	0.022667	0.067111	0.002556	0.025889	0.052222	0.100556	0.142444	0.046556
	0394	0.130222	-0.00089	0.067111	0.088444	0.022222	0.009556	0.069222	0.003667	0.030889	0.066778	0.171222	0.124778	0.037889
Candida haemulonii														
	0393	0.248556	0.121	0.027889	0.075222	0.018111	0.018	0.052444	0.037556	0.011333	0.086889	0.182333	0.064556	0.020667
	0395	0.265222	0.025222	-0.01267	0.173889	0.056	0.016444	0.088778	0.037556	0.015222	0.130889	0.212667	0.195222	0.028889
Kodamaea ohmeri														
	0396	0.910111	0.069111	0.202556	0.594889	0.909444	0.102333	0.302222	0.024111	0.091	0.173111	0.324222	0.171111	0.204222
Candida krusei														
	0397	0.003778	0.016333	0.021667	-0.00833	-0.006	0.003333	0.097667	0.107333	0.036	0.077222	0.003	0.030889	-0.004
Candida Lusitaniae														
	0398	0.834778	0.171667	0.234111	0.198111	0.142556	0.140667	0.111222	0.026889	0.087667	0.187222	0.417556	0.115667	0.052111
Saccharomyces cerevisiae														
	0399	0.588111	0.025222	0.195	0.702444	0.076444	0.034333	0.184556	0.022778	0.030556	0.039	0.626	0.063444	0.032778
	0400	0.408222	0.003556	0.003111	0.041778	0.035111	0.021556	0.074111	0.002778	0.055	0.001667	0.103778	0.058556	0.002667
Candida glabrata														
	0325	1.447667	0.003222	0.015444	0.003444	0.004889	0.061556	0.060556	0.001556	0.002	0.003778	0.131333	0.018889	0.012889
	0333	1.043556	0.053667	0.015444	0.003588	0.008018	0.087307	0.092333	0.055222	0.002365	0.067444	0.087667	0.070778	0.073444
Candida parapsilosis														
	0344	0.242667	0.141333	0.104222	0.116778	0.071889	0.038556	0.013889	0.023778	0.072889	0.090444	0.191889	0.064222	0.084556
	0340	0.403619	0.272192	0.175826	0.229828	0.075485	0.062898	0.078889	0.044369	0.13451	0.133701	0.227409	0.119985	0.147193
Candida tropicalis														
	0345	0.488556	0.021333	0.070556	0.075556	0.093556	0.007222	0.101444	0.041333	0.019667	0.027333	0.316778	0.133889	0.130667
Candida albicans														
		0.707111	0.257111	0.067667	0.181667	0.016667	0.034444	0.776222	0.095111	0.049667	0.140111	0.274889	0.104222	0.029111

[a] Positive(Green):OD600>0.5; Negative(Red):OD600<0.2 Weak(Yellow):0.2≤OD600≤0.5; n = 3

Table 8: Carbon usage profiles of *Candida auris* and other related species at 48 hours.

		D-glucose	D-galactose	D-maltose	sucrose	lactose	L-arabinose	D-raffinose	D-xylose	glycerol	mannitol	maltodextrin	dodecane	dulcitol
Species	**Isolate**													
Candida auris														
	0381	1.231667	0.019556	0.672778	1.009556	0.055889	0.394778	0.795	0.011333	0.476111	0.610444	0.801	0.406444	0.245556
	0382	1.458667	-0.00611	1.092778	1.132222	0.381556	0.347778	0.835444	0.006556	0.460667	0.755556	1.265111	0.656556	0.390556
	0383	1.213473	0.025584	0.657497	0.862131	0.199122	0.223345	0.617092	0.012008	0.276023	0.443771	0.991868	0.305813	0.140592
	0384	1.170168	0.022885	0.611199	0.816776	0.189484	0.209212	0.587508	0.011694	0.262617	0.417297	0.950324	0.284467	0.129751
	0385	1.355111	0.034222	0.373556	0.634	0.117	0.177778	0.223	0.002	0.109	0.144333	0.780333	0.115889	0.088111
	0386	1.223222	0.052333	1.06	1.061222	0.294889	0.353111	0.932556	0.065444	0.418444	0.759556	1.150889	0.374556	0.261444
	0387	1.153789	0.027289	0.563211	0.746422	0.1764	0.192789	0.544078	0.010389	0.245944	0.400522	0.940244	0.27	0.128177
	0388	1.131333	0.004111	0.375	0.659444	0.073889	0.069556	0.379333	0.005333	0.102778	0.229222	1.132444	0.164333	0.059222
	0389	1.266889	-0.00333	0.860556	0.960444	0.403778	0.227778	0.684111	0.003556	0.261222	0.512444	1.025889	0.278222	0.017667
	0390	1.244111	0.062444	0.498667	0.860333	0.069333	0.034444	0.599556	0.003111	0.191111	0.253111	1.060667	0.276667	-0.00256
Candida duobushaemulonii														
	0391	1.152778	0.365111	0.735889	0.827333	0.308222	0.101222	0.354222	0.004	0.292556	0.595111	0.742444	0.309333	0.285444
	0392	1.178444	0.137222	0.430444	0.800667	0.179667	0.150667	0.439667	0.007778	0.214444	0.481778	0.705111	0.356222	0.210667
	0394	0.938037	0.174667	0.564259	0.803815	0.196222	0.12137	0.426259	0.007111	0.248593	0.53337	0.748593	0.346593	0.276111
Candida haemulonii														
	0393	1.177333	0.202556	0.527667	0.724778	0.172556	0.079556	0.201111	0.560444	0.059333	0.536667	0.665	0.082667	0.108111
	0395	1.204333	0.263	0.320222	0.610444	0.166778	0.074111	0.223778	0.341667	0.102444	0.544	0.485667	0.159444	0.253778
Kodamaea ohmeri														
	0396	1.428	0.930667	1.241	1.494	0.426333	0.10978	1.198333	0.13022	1.278333	1.228	0.673667	0.390667	0.416
Candida krusei														
	0397	1.204	0.063889	0.086444	-0.008	-0.00389	0.004556	0.263556	0.274222	0.113222	0.074556	0.01	0.167667	-0.00289
Candida Lusitaniae														
	0398	1.585889	0.591778	0.890444	0.895	0.276	0.577444	0.495222	0.636889	0.547111	0.718333	0.859444	0.349	0.401778
Saccharomyces cerevisiae														
	0399	1.175778	1.124778	1.034111	1.315778	0.241889	0.190333	0.432333	0.08111	0.08078	0.060667	0.488444	0.077222	0.109667
	0400	1.035333	1.062222	0.922889	1.149667	0.164	0.129111	0.08433	0.191222	0.06389	0.020111	0.671111	0.293667	0.019889
Candida glabrata														
	0325	1.442889	0.004111	0.012667	0.007333	0.029778	0.062222	0.104333	0.000222	0.001111	0.003333	0.070667	0.006	0.089889
	0333	0.779556	-0.15333	-0.17667	0.011388	0.03893	0.065144	0.097149	-0.15978	0.0012	-0.16389	0.095222	-0.15544	-0.12467
Candida parapsilosis														
	0344	1.033333	0.717778	0.617333	0.720778	0.638778	0.338889	0.05444	0.278333	0.406111	0.548	0.637667	0.325111	0.598222
	0340	1.146789	1.053011	1.189421	1.40783	0.465333	0.4538	0.022	0.378056	0.700469	0.901816	1.140702	0.334179	0.535667
Candida tropicalis														
	0345	1.149667	0.819	1.234333	1.016444	0.08644	0.050667	0.11411	0.521222	0.208667	0.568333	1.122111	0.231333	0.10733
Candida albicans														
	Y-12983	1.472111	1.013333	0.908222	0.661556	0.05344	0.236556	0.480333	0.612444	0.317222	0.647444	0.929778	0.334667	0.12833

[a] Positive(Green):OD600>0.5; Negative(Red):OD600<0.2 Weak(Yellow):0.2≤OD600≤0.5; n = 3

Table 9: Carbon usage profiles of *Candida auris* and other related species at 72 hours.

		D-glucose	D-galactose	D-maltose	sucrose	lactose	L-arabinose	D-raffinose	D-xylose	glycerol	mannitol	maltodextrin	dodecane	dulcitol
Species	**Isolate**													
Candida auris														
	0381	1.379444	0.035111	1.019889	1.210333	0.084889	0.542111	0.969667	0.019	0.688444	0.853222	1.036333	0.545667	0.621444
	0382	1.559778	-0.00144	1.572	1.369	0.690556	0.631	1.111333	0.034333	0.922111	1.080778	1.482222	0.670333	0.500667
	0383	1.341778	0.11	1.077333	1.142333	0.610444	0.588778	0.955778	0.014222	0.711778	0.960556	1.243889	0.689333	0.223778
	0384	1.363333	-0.00422	1.084333	1.175	0.593333	0.211	0.943778	0.006111	0.567333	0.760222	1.138	0.749111	0.091
	0385	1.419444	0.150222	0.948333	0.845222	0.385556	0.497222	0.786778	0.011778	0.355111	0.769667	1.033444	0.294556	0.249778
	0386	1.512778	0.086889	1.293222	1.184444	0.565667	0.571	1.055333	0.077556	0.816556	1.107556	1.399222	0.653778	0.639222
	0387	1.3972	0.070912	1.118967	1.128756	0.426589	0.4082	0.931356	0.021556	0.572044	0.877133	1.2417	0.555667	0.285078
	0388	1.456333	0.068667	1.310556	1.208111	0.223556	0.235556	0.884556	0.007889	0.231556	0.732333	1.315778	0.347222	0.170444
	0389	1.243	0.024556	1.203667	1.158556	0.804333	0.544222	0.863889	0.042222	0.630444	0.699444	1.029556	0.592667	0.103556
	0390	1.598	0.091111	1.231444	1.214333	0.118333	0.159889	0.971222	0.002889	0.564889	0.793333	1.349778	0.736111	0.030556
Candida duobushaemulonii														
	0391	1.213111	0.722	0.964444	1.024778	0.635222	0.285444	0.452778	0.030222	0.617222	0.837889	0.842667	0.517111	0.566
	0392	1.492444	0.781667	1.114444	1.438111	0.785	0.744556	1.100778	0.007111	0.748778	0.801333	0.701444	0.790778	0.75
	0394	1.118259	0.52637	0.980333	1.137593	0.545407	0.419222	0.723926	0.021741	0.644037	0.831444	0.814926	0.636148	0.635852
Candida haemulonii														
	0393	1.395111	0.447333	0.897667	1.043333	0.439556	0.293222	0.471556	0.837889	0.217444	0.803889	0.834	0.160556	0.326333
	0395	1.471556	0.450111	0.431667	0.750222	0.267	0.150444	0.390222	0.642333	0.298111	0.610556	0.553333	0.155222	0.351222
Kodamaea ohmeri														
	0396	1.647333	1.722222	1.761111	1.767778	0.806333	0.125	1.383444	0.14967	1.569667	1.704444	0.807667	0.522	1.196111
Candida krusei														
	0397	1.233	0.850111	0.797889	0.888111	0.752222	0.885444	0.912667	0.492556	1.197556	0.796	0.787222	0.722556	0.701667
Candida Lusitaniae														
	0398	1.517111	0.816444	1.313111	1.006778	0.365333	0.947222	0.581667	1.030889	0.743111	1.141556	0.980778	0.488889	0.549556
Saccharomyces cerevisiae														
	0399	1.239667	1.341778	1.190444	1.418333	0.591889	0.300778	0.311889	0.218556	0.13233	0.357778	0.624667	0.148111	0.377222
	0400	1.007	1.251111	1.098889	1.055556	0.382667	0.245667	0.099	0.164	0.10056	0.220556	0.958222	0.274	0.179333
Candida glabrata														
	0325	1.111444	0.005333	0.010333	0.042333	0.077333	0.058556	0.166556	0.001333	0.008222	0.021444	0.0567	0.016	0.080556
	0333	1.753024	0.007331	0.015306	0.044291	0.111372	0.105999	0.102042	0.00211	0.012081	0.030309	0.09333	0.02076	0.104456
Candida parapsilosis														
	0344	1.125778	0.978333	0.932444	0.970222	0.746444	0.743222	0.11944	0.722778	0.717	0.951111	0.901333	0.738333	0.776333
	0340	1.858853	1.089263	1.532753	1.601905	0.633111	1.384434	0.117	1.09245	1.358506	1.901996	1.363622	1.208192	0.675889
Candida tropicalis														
	0345	1.301667	1.229111	1.317667	1.203333	0.12356	0.279111	0.12778	1.011889	0.522	0.960222	1.209222	0.578111	0.12267
Candida albicans														
	Y-12983	1.474667	1.307222	1.317556	1.019	0.00367	0.456111	0.793	0.869667	0.533667	0.968889	1.151667	0.450111	0.07889

[a] Positive(Green):OD600>0.5; Negative(Red):OD600<0.2 Weak(Yellow):0.2≤OD600≤0.5; n = 3

Nitrogen Source Assimilation

The nitrogen assimilation patterns were similar for all species, except *C. glabrata* and *K. ohmeri*. (Tables 10, 11 and 12). All species were able to utilize ammonium acetate. All but *C. glabrata* were able to assimilate L-valine, L-proline, glycine, L-arginine and urea. Some variability was present for sodium nitrate and D-valine, although only weak or negative results were obtained. Curiously, *C. albicans* reached the positive threshold, an OD600 of greater than 0.5, when grown with sodium nitrate. *K. ohmeri* also demonstrated an ability to absorb many nitrogen sources the other species could not, reaching the positive threshold in all sources except sodium nitrite, sodium nitrate and thiamin. As was the case with the carbon source assimilation above, most organisms required at least 48 hours to reach the positive threshold for any nitrogen source. The CHS controls remained negative.

Table 10: Nitrogen usage profiles of *Candida auris* and other related species at 24 hours.

		ammonium sulfate	sodium nitrate	sodium nitrite	L-valine	glycine	L-proline	L-arginine	D-valine	urea	thiamin	pyridoxin	thymine	uracil
Species	**Isolate**													
Candida auris														
	0381	0.243	0.050556	0.005333	0.070667	0.059	0.060667	0.489333	0.013444	0.048333	0.020444	0.038667	0.040333	0.022
	0382	0.130667	-0.01311	0.003111	0.063778	0.156667	0.142111	0.356778	0.024	0.025222	0.041333	0.072	0.048889	0.028778
	0383	0.083222	0.102667	0.002556	0.022222	0.038333	0.046444	0.064	0.043	0.060333	0.042333	0.050111	0.068444	0.046556
	0384	0.003667	0.016667	0.001111	0.014556	0.007111	0.008333	0.015556	-0.004	0.012778	0.006333	0.010778	0.013778	0.007778
	0385	0.002333	0.116889	-0.00011	0.003778	0.001111	0.001667	0.001778	0.002333	0.005222	0.001222	0.003	0.002222	0.001222
	0386	0.332222	0.015111	0.001889	0.145778	0.175444	0.349333	0.428667	0.083111	0.146444	0.063667	0.077444	0.050333	0.045889
	0387	0.000778	0.004556	-0.00044	0.015556	0.004444	0.024	-0.00122	0.013889	0.000111	-0.00056	0.005444	0	0.013889
	0388	0.078111	0.061222	0.000778	0.031889	0.056667	0.067556	0.110333	0.051222	0.063667	0.044889	0.042444	0.054222	0.044222
	0389	0.021667	0.003889	0.000333	0.007333	0.039667	0.013	0.027444	0.012778	0.010222	0.006444	0.009333	0.012556	0.011667
	0390	0.253444	-0.00456	0.002333	0.073222	0.110222	0.086778	0.305778	-0.00344	0.231556	0.066111	0.059	0.059556	0.140444
Candida duobushaemulonii														
	0391	0.055889	0.017889	0.002556	0.017	0.021556	0.014222	0.213	0.023111	0.025111	0.007333	0.010333	0.011444	0.002
	0392	0.156	0.056333	0.001556	0.079111	0.105222	0.098222	0.166556	0.051222	0.139444	0.057111	0.039889	0.041	0.036333
	0394	0.197556	0.054778	0.016111	0.065889	0.095778	0.065778	0.147222	0.063667	0.121556	0.047667	0.025556	0.036	0.038667
Candida haemulonii														
	0393	0.036333	0.018778	0.001444	0.044333	0.027667	0.041667	0.037889	0.012778	0.037667	0.008222	0.008889	0.017111	0.022333
	0395	0.065556	0.040778	0.001778	0.040444	0.186222	0.216889	0.082333	0.049444	0.171889	0.030111	0.028	0.032111	0.032778
Kodamaea ohmeri														
	0396	0.567333	0.266556	0.008111	0.123556	0.157889	0.229778	0.398333	0.057889	0.381333	0.095111	0.096222	0.095889	0.175111
Candida krusei														
	0397	0.138556	0.179667	-0.006	0.064222	0.071667	-0.00533	0.049667	0.050111	0.031556	0.008444	0.085444	0.002444	0.021778
Candida Lusitaniae														
	0398	0.649	0.057556	0.002778	0.050111	0.060333	0.220667	0.867556	0.018222	0.312889	0.106889	0.097222	0.043222	0.023111
Saccharomyces cerevisiae														
	0399	0.562	0.091	0.013111	0.138556	0.030333	0.156778	0.517	0.080556	0.402444	0.055667	0.073333	0.075667	0.093556
	0400	0.363	0.019333	0.003111	0.060889	0.011444	0.037778	0.275222	0.017556	0.064333	0.018889	0.016333	0.016444	0.011333
Candida glabrata														
	0325	1.273111	-0.07522	-0.09578	-0.08044	-0.08967	-0.09622	-0.02911	-0.09678	-0.07444	-0.095	0.019333	-0.08278	-0.09056
	0333	1.136222	0.044889	-0.004	-0.14326	-0.08998	-0.14104	-0.05602	0.042	-0.11115	0.047333	0.039222	0.041222	0.048889
Candida parapsilosis														
	0344	0.472333	0.039889	0.001444	0.033111	0.045889	0.020444	0.600444	0.017444	0.014667	0.009111	0.011556	0.008889	0.014111
	0340	0.653461	0.073334	0.002237	0.057315	0.025062	0.192111	0.650327	0.018629	0.016778	0.01234	0.021278	0.016176	0.02165
Candida tropicalis														
	0345	0.150444	0.029111	0.004222	0.035222	0.052333	0.061222	0.255889	0.041222	0.119	0.024556	0.009667	0.03	0.064778
Candida albicans														
		0.782111	0.039222	0.002333	0.239111	0.258444	0.364556	0.921111	0.079	0.818889	0.071	0.060889	0.078444	0.068444

[a] Positive(Green):OD600>0.5; Negative(Red):OD600<0.2 Weak(Yellow):0.2≤OD600≤0.5; n = 3

Table 11: Nitrogen usage profiles of *Candida auris* and other related species at 48 hours.

		ammonium sulfate	sodium nitrate	sodium nitrite	L-valine	glycine	L-proline	L-arginine	D-valine	urea	thiamin	pyridoxin	thymine	uracil
Species	**Isolate**													
Candida auris														
	0381	0.864	0.101556	0.024889	0.497556	0.666667	0.637	1.174444	0.075444	0.537778	0.063111	0.092556	0.083556	0.077
	0382	0.660444	0.063889	0.003667	0.499778	0.806333	0.963222	0.864111	0.099778	0.641111	0.074889	0.110889	0.254667	0.078556
	0383	0.768303	0.11652	0.004766	0.57273	0.728752	0.762413	0.966386	0.13169	0.811813	0.105412	0.104252	0.131138	0.133612
	0384	0.726831	0.120523	0.004939	0.529038	0.704007	0.705568	0.92024	0.129708	0.763517	0.10193	0.103401	0.128388	0.125486
	0385	0.371556	0.18333	-0.00022	0.047556	0.072222	0.113333	0.376333	0.054778	0.669	0.033444	0.031222	0.079222	0.160222
	0386	1.131222	0.043778	0.001111	0.937667	1.013222	0.960111	1.189333	0.185667	0.989889	0.162222	0.152222	0.149	0.144778
	0387	0.747867	0.114344	0.004389	0.504378	0.646844	0.649789	0.918844	0.1283	0.714389	0.104633	0.099122	0.1221	0.1203
	0388	1.037	0.131	0.006222	0.735556	1.028222	0.922333	1.240778	0.186222	1.062111	0.192778	0.188222	0.202444	0.170889
	0389	0.812333	0.122667	0.000333	0.748556	0.894667	0.925556	1.161667	0.255111	0.791222	0.102111	0.083667	0.101333	0.099111
	0390	0.735333	0.112	0.002667	0.775111	0.951889	0.914	1.088	0.031333	1.128667	0.112	0.067778	0.074778	0.179889
Candida. duobushaemulonii														
	0391	0.865222	0.109556	0.005333	0.617333	0.876889	0.627111	1.056444	0.333667	0.986111	0.055667	0.042222	0.069111	0.293
	0392	1.013444	0.202778	0.002333	0.686222	0.939667	0.766222	1.153222	0.138556	1.089	0.162333	0.090333	0.200444	0.099333
	0394	1.010148	0.169408	0.015556	0.685333	1.006815	0.533778	1.230259	0.201333	1.081222	0.122185	0.06963	0.132555	0.186481
Candida haemulonii														
	0393	0.797444	0.111889	0.031444	0.370889	0.699111	0.778222	1.143222	0.055778	0.994778	0.056889	0.052667	0.083	0.167778
	0395	0.735778	0.124778	0.001	0.407556	1.032556	0.504889	0.850111	0.129556	0.791333	0.073	0.044222	0.086778	0.074556
Kodameaea ohmeri														
	0396	1.039667	0.224667	0.09	1.041667	1.163333	1.112	1.186667	0.369	1.169667	0.382	0.593	0.827333	0.791333
Candida krusei														
	0397	0.183222	0.260333	-0.00567	0.175	0.194889	-0.00367	0.152556	0.143778	0.095111	0.017778	0.014111	0.003778	0.020778
Candida Lusitaniae														
	0398	0.907778	0.128	0.047667	0.998444	1.147111	1.337222	1.276111	0.071444	1.141444	0.197	0.182889	0.066667	0.028889
Saccharomyces cerevisiae														
	0399	0.924889	0.074	0.026667	0.841	0.076444	0.877556	1.056111	0.056556	1.060111	0.059778	0.056444	0.067222	0.056556
	0400	0.719444	0.060556	0.059111	0.629556	0.108444	0.572	0.795222	0.052	0.842556	0.055556	0.050222	0.052667	0.032333
Candida glabrata														
	0325	1.251	-0.09111	-0.10867	-0.08967	-0.07678	-0.10767	-0.01344	-0.10889	-0.08589	-0.10756	-0.00133	-0.09567	-0.10244
	0333	0.827667	-0.22244	-0.23144	-0.14025	-0.1285	-0.15159	-0.0147	-0.21567	-0.16772	-0.21756	-0.22444	-0.22156	-0.21167
Candida parapsilosis														
	0344	0.961	0.089333	0.013444	0.284333	0.174444	0.242444	1.080444	0.044667	0.086444	0.024222	0.041111	0.026667	0.042444
	0340	1.040784	0.148874	0.015598	0.429674	0.10467	0.394333	1.889417	0.05479	0.106566	0.033038	0.072562	0.046999	0.065554
Candida tropicalis														
	0345	0.875222	0.204667	0.016222	0.766111	0.913556	0.776667	0.892222	0.079667	0.942889	0.100222	0.104	0.133111	0.112111
Candida albicans														
	Y-12983	0.853111	0.382889	0.003667	0.918778	0.983667	1.001778	0.986111	0.174889	1.041333	0.121667	0.113778	0.118222	0.112889

[a] Positive(Green):OD600>0.5; Negative(Red):OD600<0.2 Weak(Yellow):0.2≤OD600≤0.5; n = 3

Table 12: Nitrogen usage profiles of *Candida auris* and other related species at 72 hours.

		ammonium sulfate	sodium nitrate	sodium nitrite	L-valine	glycine	L-proline	L-arginine	D-valine	urea	thiamin	pyridoxin	thymine	uracil
Species	**Isolate**													
Candida auris														
	0381	1.297667	0.118	-0.023	1.072556	1.233889	1.260778	1.420778	0.093	1.134889	0.066111	0.107778	0.082778	0.088889
	0382	0.594333	0.150444	0.003444	0.895111	1.156889	1.233778	1.029556	0.288667	0.909667	0.167	0.299556	0.420222	0.130222
	0383	0.774222	0.161444	0.007667	0.847333	1.003	1.142333	1.158889	0.272	1.330444	0.158667	0.166111	0.126889	0.278333
	0384	0.831	0.295889	0.003778	0.622778	1.356444	0.856667	1.276778	0.178778	1.103222	0.113333	0.150333	0.175667	0.194333
	0385	0.738333	0.390222	-0.001	0.929333	1.264778	0.920778	0.917778	0.150667	1.345	0.143667	0.126778	0.162111	0.361444
	0386	1.356889	0.068222	0.038556	1.253889	1.330778	1.267222	1.336111	0.227444	1.274333	0.200556	0.196111	0.194556	0.192444
	0387	0.983422	0.209155	0.0042	1.017	1.217156	1.076722	1.212789	0.212144	1.207189	0.158067	0.159367	0.188222	0.1914
	0388	1.111111	0.238333	0.004111	1.206	1.269333	1.272556	1.325556	0.246778	1.228333	0.239778	0.241778	0.254556	0.217889
	0389	1.107667	0.21111	0.006556	1.175556	1.352889	1.339222	1.340667	0.379222	1.168	0.141556	0.110111	0.130444	0.128111
	0390	0.666333	0.23111	0.003222	0.913667	1.009778	1.017778	0.992889	0.056	1.239556	0.126667	0.078889	0.153889	0.182
Candida duobushaemulonii														
	0391	1.113111	0.145111	0.026667	1.114444	1.256444	1.105444	1.261556	0.438667	1.255222	0.102778	0.074444	0.106889	0.502
	0392	1.270556	0.488	0.002111	1.402222	1.527556	1.426222	1.537111	0.285222	1.560889	0.469222	0.117444	0.403111	0.217444
	0394	1.112259	0.319222	0.022444	1.174741	1.311667	0.955074	1.317185	0.328556	1.319889	0.230778	0.086963	0.245926	0.299
Candida haemulonii														
	0393	1.074333	0.135556	0.080333	0.845778	1.100556	1.073	1.250111	0.101222	1.271222	0.096222	0.092333	0.112778	0.215222
	0395	0.786889	0.176222	0.004667	0.851444	1.120889	0.492667	0.879667	0.263444	0.917111	0.099222	0.059333	0.172444	0.113222
Kodamaea ohmeri														
	0396	1.330889	0.445556	0.287889	1.33	1.571444	1.435778	1.429111	0.532111	1.330444	0.5	0.770778	1.131222	1.034333
Candida krusei														
	0397	1.062444	0.116889	0.089	0.999444	0.560778	0.979778	1.396	0.333778	1.168444	1.274556	0.172444	0.151222	0.122333
Candida Lusitaniae														
	0398	1.156889	0.144333	0.069111	1.164778	1.260889	1.399556	1.377778	0.042222	1.229889	0.190222	0.167444	0.054111	0.042556
Saccharomyces cerevisiae														
	0399	1.001222	0.082556	0.046111	0.92	0.370222	1.004667	1.086778	0.065778	1.041556	0.056556	0.064889	0.061222	0.060444
	0400	0.858333	0.071	0.111	0.723444	0.514111	0.776444	0.914667	0.055333	0.949111	0.064889	0.058889	0.071556	0.040333
Candida glabrata														
	0325	1.134667	-0.098	-0.11044	-0.04833	-0.00856	-0.10644	0.031556	-0.12067	-0.09411	-0.11867	0.001333	-0.095	-0.11489
	0333	1.260416	-0.14862	-0.21	-0.08848	-0.01469	-0.19292	0.060757	-0.13	-0.09523	-0.12747	0.002564	-0.18253	-0.11713
Candida parapsilosis														
	0344	1.084667	0.125889	0.062556	1.102778	0.499778	1.075	1.262778	0.122	0.880222	0.06	0.536	0.259111	0.225778
	0340	1.259833	0.132271	0.095859	1.521669	0.822361	0.609889	2.059369	0.213444	0.885558	0.076875	0.682587	0.489966	0.338873
Candida tropicalis														
	0345	1.005889	0.255444	0.031	0.902556	1.020667	0.925889	0.946111	0.075111	0.940889	0.125556	0.112333	0.168556	0.203
Candida albicans														
	Y-12983	0.914889	0.684889	0.003	0.930556	0.996333	1.084222	1.029111	0.214222	1.039222	0.128556	0.129778	0.130889	0.109889

[a] Positive(Green):OD600>0.5; Negative(Red):OD600<0.2 Weak(Yellow):0.2≤OD600≤0.5; n = 3

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Biolog Testing

The different isolates displayed a similar pattern of fermentation and assimilation (Tables 13 and 14). The Microlog system classifies results as positive, partial, or negative. These are colorized as green, yellow and red, respectively. In the case of isolates 0385, 0398 and 0399, which were tested in triplicate, three results may be listed. This is only the case when one of the replicates disagreed with the others. If only one result is presented, all three replicates were in accord. There are, however, several differences between isolates. The type strain, isolate number 0381, displays some interesting discrepancies with the other isolates. It is the only isolate that did ferment or assimilate n-acetyl-D-glucosamine. It was also the only isolate of *C. auris* that presented a negative result for assimilation of 2-keto gluconic acid. The other isolates presented at least partial growth here. Isolate 0381 also displayed more positive results in the assimilation of the combinations of D-xylose and another carbon source. Isolates 0384, 0385, and 0386 also assimilated most of these. With one exception, however, all of these instances of growth were only in the partial range. Without the *C. auris* in the database, it was misidentified in every case (Table 15). In fact, the only species the system correctly identified was *C. haemulonii* (AR0393), even though *C. lusitaniae* (AR0398) and *S. cerevisiae* (AR0399) were tested and both species are listed in the database. In all cases, the most likely identity given by the software for *C. auris* was either *C. haemulonii* or *Rhodotorula acheniorum*. These identifications did appear to be aligned with the clades, although the probability values of correct identification were far lower for some than others (Table 15).

Table 13: Carbon source fermentation of *C. auris and related species* using the Biolog YT plate.

Carbon Source	0381	0382	0383	0384	0385*	0386	0387	0388	0389	0390	0391+	0393+	0398*+	0399*+
acetic acid	w	n	n	w	n	n	n	n	n	n	n	w	n	n
formic acid	w	n	n	w	n	n	n	n	n	n	n	n	n	n
propionic acid	w	n	n	w	n	n	n	n	w	w	n	n	n	n
succinic acid	n	n	w	w	w	n	w	n	n	n	n	n	N	n
succinic acid mono-methyl ester	n	n	n	n	w	n	n	n	n	n	n	n	wwn	n
L-aspartic acid	w	n	n	w	n	n	n	n	n	n	n	n	W	n

L-glutamic acid	w	w	n	w	n	n	w	n	w	n	w	n	wwn	n
L-proline	w	w	w	w	p	w	w	w	w	w	n	n	w	n
D-gluconic acid	p	w	p	w	p	w	p	w	w	w	w	p	p	n
dextrin	p	w	w	w	w	w	p	w	w	w	n	w	w	n
inulin	p	w	p	p	p	p	w	w	p	w	w	p	W	w
D-cellobiose	w	n	n	w	n	n	n	n	n	n	n	n	P	n
gentibiose	w	n	n	w	n	n	n	n	n	n	n	n	wwn	wwn
maltose	p	p	p	p	ppw	p	p	p	p	p	p	p	p	P
Maltotriose	p	p	p	p	ppw	p	p	p	p	p	p	p	p	wwn
D-melezitose	p	p	p	p	p	p	p	p	p	p	w	n	p	wwn
D-melibiose	n	n	n	w	n	n	n	n	n	n	n	w	w	N
palatinose	p	p	p	p	p	p	p	p	p	p	p	p	p	p
D-raffinose	p	p	p	w	wwn	w	p	p	w	w	w	w	w	p
stachyose	p	w	w	w	P	w	p	w	w	w	n	n	n	n
sucrose	p	p	p	p	P	p	p	p	p	p	p	p	p	p
D-trehalose	p	w	p	p	ppw	p	p	p	w	p	p	p	p	w
turanose	p	p	p	p	P	p	p	p	p	p	w	p	p	ppw
n-acetyl glucosamine	n	p	p	p	p	p	p	p	p	p	p	p	wwn	w
α-D-Glucose	p	p	p	p	p	p	p	p	p	p	p	p	p	p
D-galactose	n	n	n	n	n	n	n	n	n	n	n	w	w	p
D-psicose	p	w	w	n	w	n	p	w	w	w	n	p	w	n
L-sorbose	n	n	n	n	n	n	n	n	n	n	n	n	w	n
salicin	n	n	n	n	n	n	n	n	n	n	n	n	p	n
D-mannitol	p	p	p	p	p	p	p	p	p	p	w	p	p	n
D-sorbitol	p	p	p	w	ppw	w	p	p	p	w	p	p	p	n
D-arabitol	w	n	n	n	w	n	w	n	n	n	n	p	wwn	n
xylitol	w	n	w	n	p	n	w	w	w	n	n	n	p	n
glycerol	w	n	w	n	p	n	w	w	n	n	n	n	p	wwn
tween 80	p	w	w	w	w	w	w	n	w	w	n	w	n	n

**n=3, otherwise n=1, + 0391: Candida duobushaemulonii, 0393: Candida haemulonii 0398: Candida lusitaniae, 0399: Saccharomyces cerevisiae;* p(green): positive, w(yellow): weak n(red): negative

Table 14: Carbon source assimilation of *C. auris and related species* using the Biolog YT plate.

Carbon Source	0381	0382	0383	0384	0385*	0386	0387	0388	0389	0390	03911+	03923+	0398*+	0399*+
fumaric acid	n	n	n	n	w	n	n	n	n	n	n	n	w	n
L-malic acid	w	n	n	n	w	n	w	n	n	n	n	n	wwn	n
succinic acid mono-methyl ester	n	w	n	n	n	n	n	n	n	w	n	n	w	n
bromo-succinic acid	n	n	n	n	n	n	n	n	n	n	n	n	n	n
L-glutamic acid	w	n	n	n	n	n	n	n	n	w	w	n	w	n
γ-amino-Butyric acid	w	n	n	n	n	n	n	w	n	n	n	w	n	n
α-Keto-glutaric acid	n	n	n	n	n	n	w	n	n	w	w	n	w	n
2-keto-gluconic acid	n	p	w	w	w	w	p	w	w	w	w	w	w	N
D-gluconic acid	w	p	w	w	p	w	p	w	w	w	w	w	p	ppw
dextrin	p	w	w	w	w	w	w	w	w	w	n	w	n	n
inulin	p	p	p	p	w	p	w	p	w	p	p	p	w	w
D-cellobiose	n	n	n	n	n	n	n	n	n	n	n	n	ppw	n
gentibiose	w	n	n	w	n	n	n	n	n	n	n	n	w	n
maltose	p	p	p	p	p	p	p	p	p	p	p	p	w	p
Maltotriose	p	p	p	p	p	p	p	p	p	p	p	p	w	n
d-melezitose	p	p	p	p	p	p	p	p	p	p	w	n	p	n
D-melibiose	n	n	n	n	n	n	n	n	n	n	n	n	w	n
palatinose	p	p	p	p	p	p	p	p	p	p	p	p	w	p
D-raffinose	p	w	p	w	w	w	w	p	w	w	p	w	n	p
stachyose	p	w	w	w	w	w	p	p	w	w	w	n	n	n

sucrose	p	p	p	p	p	p	p	p	p	p	p	p	p	p
D-trehalose	p	p	p	p	p	p	p	p	w	p	p	p	wwn	p
turanose	p	p	p	p	p	p	p	p	p	p	p	p	w	p
n-acetyl-d-glucosamine	n	p	p	p	p	p	p	p	p	p	p	p	wwn	n
D-glucosamine	n	w	w	w	w	w	n	w	n	w	p	w	n	n
α-D-Glucose	p	p	p	p	p	p	p	p	p	p	p	p	p	p
D-galactose	n	n	n	n	n	n	n	n	n	n	p	p	wwn	p
D-psicose	w	w	w	n	n	n	n	w	n	w	w	p	w	n
L-rhamnose	n	n	n	n	n	n	n	n	n	n	n	n	w	n
L-sorbose	n	n	n	n	n	n	n	n	n	n	w	n	w	n
α-Methyl-d-glucoside	w	n	n	n	n	n	n	n	n	n	n	n	n	n
β- Methyl-d-glucoside	w	n	n	n	n	n	n	n	n	n	n	n	w	n
amygdalin	n	n	n	n	n	n	n	n	n	n	w	n	n	w
arbutin	w	n	n	n	n	n	n	n	n	w	n	n	n	n
salicin	w	n	n	n	n	n	n	n	n	n	n	n	w	n
maltitol	p	w	n	w	p	w	n	w	n	w	n	n	w	n
D-mannitol	p	p	p	p	p	p	p	w	p	p	p	p	w	n
D-sorbitol	w	w	p	w	p	w	w	p	p	p	p	p	w	n
adonitol	n	n	n	n	n	n	w	n	n	w	w	w	wwn	n
D-arabitol	w	n	n	n	n	n	n	n	n	n	n	w	w	n
xylitol	p	w	n	w	w	w	n	w	n	w	w	n	w	n
I-erythritol	n	n	n	n	n	n	n	n	n	n	n	n	n	n
glycerol	w	n	w	w	w	w	w	w	n	w	w	n	n	n
tween 80	p	w	w	w	w	w	n	n	w	w	n	n	n	n
L-arabinose	w	n	n	n	w	n	w	n	n	n	n	n	n	n
D-arabinose	n	n	n	n	n	n	n	n	n	n	n	n	n	n
D-ribose	w	n	n	n	n	n	n	n	n	n	n	n	n	n
D-xylose	w	n	n	n	n	n	n	n	n	n	n	w	w	n

succinic acid mono-methyl ester plus D-xylose	w	w	n	n	w	n	n	n	n	n	n	n	n	n
n-acetyl-D-glutamic acid plus D-xylose	w	w	n	n	n	n	n	n	n	w	w	w	w	n
quinic acid plus D-xylose	w	w	n	n	w	n	n	n	n	n	n	n	n	n
D-glucuronic acid plus D-xylose	w	n	n	n	n	n	w	n	n	n	w	w	n	n
dextrin plus D-xylose	p	n	w	w	N	w	n	w	n	n	w	w	n	n
α-D-Lactose plus D-xylose	p	n	n	w	w	w	n	n	n	n	n	n	n	n
D-melibiose plus D-xylose	p	n	n	w	w	w	n	n	n	n	w	w	w	n
D-galactose plus D-xylose	w	n	n	n	wwn	n	n	n	n	n	w	w	w	p
m-inositol plus D-xylose	p	n	n	w	w	w	n	n	n	n	w	n	n	n
1,2-propanediol plus D-xylose	p	n	n	w	p	w	n	n	n	n	w	n	w	n
acetoin plus D-xylose	p	n	n	w	w	w	n	n	n	n	n	n	n	n

**n=3, otherwise n=1, + 0391: Candida duobushaemulonii, 0393: Candida haemulonii 0398: Candida lusitaniae, 0399: Saccharomyces cerevisiae;* p(green): positive, w(yellow): weak n(red): negative

Table 15: Microlog Identification of Panel Isolates, colorized by clade.

Isolate	Species	Identification	Probability
0381	*Candida auris*	*Rhodotorula acheniorum*	0.99
0382	*Candida auris*	*Candida haemulonii*	0.844
0383	*Candida auris*	*Rhodotorula acheniorum*	0.916
0384	*Candida auris*	*Rhodotorula acheniorum*	0.994
0385	*Candida auris*	*Candida haemulonii*	0.988
0386	*Candida auris*	*Candida haemulonii*	0.959
0387	*Candida auris*	*Candida haemulonii*	0.964
0388	*Candida auris*	*Candida haemulonii*	0.913
0389	*Candida auris*	*Candida haemulonii*	0.714
0390	*Candida auris*	*Candida haemulonii*	0.896
0391	*Candida duobushaemulonii*	*Candida haemulonii*	0.997
0393	*Candida haemulonii*	*Candida haemulonii*	0.993
0398	*Candida lusitaniae*	*Candida haemulonii*	0.93
0399	*Saccharomyces cerevisiae*	*Zygosaccharomyces fermentati*	0.804

Essential Oils Testing

Antifungal Activity

Higher concentrations of many essential oils negatively impacted the growth of all species. All essential oils except eucalyptus, lemon, grapefruit and bitter orange displayed inhibitory activity at the concentrations tested (Tables 16 and 17). Bergamot, cinnamon bark, cinnamon leaf, clove bud, geranium, lemongrass, lime peel, peppermint, spearmint, and tea tree oils all showed inhibitory activity at concentrations considered safe for dermal use (Appendix II). Basil and lavender oils had MIC values above the recommended safe concentrations and the remainder had no maximum concentration listed (Appendix II). Of the essential oils that displayed lethal activity against *C. auris*, only bergamot, lavender and basil oils were above the safe concentrations. Interestingly, *C. auris* (AR0391 and AR0395) displayed mostly lower MIC and MFC values than either *C. lusitaniae* (AR0398) or *S. cerevisiae* (AR0399). Both strains of *C. auris* also displayed MIC and MFC values within one microdilution of each other, except for in the case of the manuka and basil oil MIC values. Cinnamon leaf, cinnamon bark, clove bud and lemongrass oils displayed the most potent antifungal activity, with cinnamon bark displaying the lowest MIC and MFC values against all organisms. In some cases, the MIC was below the tested concentrations and required further dilution during the checkerboard assays.

Table 16: MIC by percentage (v/v) of select essential oils against *C. auris* (0381, 385), *Candida lusitaniae* (0398) and *Saccharomyces cerevisiae* (0399). Tests were run in triplicate.

Essential Oil	0381	0385	0398	0399
Tea Tree	0.25%	0.13%	0.50%	0.50%
Geranium	0.13%	0.06%	0.50%	0.25%
Lime Peel	0.25%	0.13%	1.0%	>1.0%
Eucalyptus	>1.0%	>1.0%	>1.0%	>1.0%
Peppermint	0.25%	0.25%	1.0%	1.0%
Manuka	0.25%	1%	>1.0%	1.0%
Clove Bud	0.01%	0.02%	0.06%	0.25%
Myrrh	0.13%	0.13%	1.0%	1.0%
Spearmint	0.13%	0.06%	0.50%	>1.0%
Cinnamon Leaf	<0.01%	<0.01%	0.13%	0.25%
Cinnamon Bark	<0.01%	<0.01%	<0.01%	<0.01%
Bergamot	0.25%	0.13%	>1.0%	>1.0%
Lemon	>1.0%	>1.0%	>1.0%	>1.0%
Frankincense	1.0%	1.0%	>1.0%	>1.0%
Coriander	0.50%	0.50%	1.0%	1.0%
Bitter Orange	>1.0%	>1.0%	>1.0%	>1.0%
Grapefruit	>1.0%	>1.0%	>1.0%	>1.0%
Lavender	1.0%	1.0%	1.0%	>1.0%
Ginger	1.0%	>1.0%	>1.0%	>1.0%
Basil	0.13%	0.50%	>1.0%	>1.0%
Lemongrass	0.02%	0.03%	0.13%	0.25%

Table 17: MFC of select essential oils against *C. auris* (0381, 385), *Candida lusitaniae* (0398) and *Saccharomyces cerevisiae* (0399).

Essential Oil	0381	0385	0398	0399
Tea Tree	0.50%	1.0%	ND	1.0%
Geranium	0.50%	0.25%	1.0%	0.50%
Peppermint	1.0%	1.0%	ND	ND
Clove Bud	0.06%	0.13%	ND	ND
Cinnamon Leaf	0.50%	0.25%	0.50%	0.50%
Cinnamon Bark	0.02%	0.02%	0.01%	0.02%
Bergamot	0.50%	0.25%	ND	ND
Coriander	1.0%	1.0%	1.0%	ND
Lavender	1.0%	ND	1.0%	ND
Basil	1.0%	1.0%	ND	ND
Lemongrass	0.13%	0.06%	1.0%	0.50%

Vapor-Phase Testing

Only lemongrass, clove bud and cinnamon bark oils displayed any inhibition in vapor phase (Table 18). These oils were also the most effective in direct contact. All three oils were lethal at 100 µL. At 10 µL, cinnamon bark again displayed lethality, clove bud oil showed complete inhibition, and lemongrass oil elicited no effect.

Table 18: Antifungal activity of essential oils in vapor-phase on *C. auris* **(0385).**

Essential Oil	**Result**	**Essential Oil**	**Result**
Tea Tree	No inhibition	Cinnamon Bark (1.0µL)	No inhibition
Geranium	No inhibition	Bergamot	No inhibition
Lime Peel	No inhibition	Lemon	No inhibition
Eucalyptus	No inhibition	Frankinsence	No inhibition
Peppermint	No inhibition	Coriander	No inhibition
Manuka	No inhibition	Bitter Orange	No inhibition
Clove Bud (100 µL)	Lethal	Grapefruit	No inhibition
Clove Bud (10 µL)	Inhibition	Lavender	No inhibition
Myrrh	No inhibition	Ginger	No inhibition
Spearmint	No inhibition	Basil	No inhibition
Cinnamon Leaf	No inhibition	Lemongrass (100 µL)	Lethal
Cinnamon Bark (100 µL)	Lethal	Lemongrass (10 µL)	No inhibition
Cinnamon Bark (10 µL)	Lethal		
n=3, volumes of essential oils are 100 µL unless stated otherwise			

Synergism Testing

A range of interactions were observed between the essential oils and the various antifungal drugs (Tables 19, 20, 21 and 22). FICI values ranged from 0.0625 to 5.0. This range includes at least one instance of each interaction. Antagonism was only present with the combination amphotericin B and clove bud oil. However, clove bud oil also displayed synergistic activity with fluconazole for all three organisms and an additive to synergistic association with flucytosine. Lemongrass oil was generally additive with all of the drugs. The only exception was an indifferent association with amphotericin B when used on *C. lusitaniae* and a synergistic one when used with micafungin on *S. cerevisiae.* Cinnamon oil was generally indifferent in most cases. When looked at from the perspective of the drugs, the results were more varied. All drugs except amphotericin B had instances of synergism, while

amphotericin B had the only instances of antagonism. All four drugs displayed instances of indifference and additivity.

Table 19: Interactions between select antifungal drugs and essential oils on *C. auris.*

Antifungal	Essential Oil	FIC(AF)	FIC(EO)	FICI	Interpretation
Micafungin	Cinnamon Bark	1	1	2	Indifferent
Micafungin	Clove Bud	1	1	2	Indifferent
Micafungin	Lemongrass	0.5	0.125	0.625	Additive
Flucytosine	Cinnamon Bark	0.5	0.25	0.75	Additive
Flucytosine	Clove Bud	0.0625	0.125	0.1875	Synergistic
Flucytosine	Lemongrass	0.125	0.5	0.625	Additive
Amphotericin B	Cinnamon Bark	1	0.5	1.5	Indifferent
Amphotericin B	Clove Bud	4	0.5	4.5	Antagonistic
Amphotericin B	Lemongrass	0.25	0.5	0.75	Additive
Fluconazole	Cinnamon Bark	1	0.5	1.5	Indifferent
Fluconazole	Clove Bud	0.03125	0.25	0.28125	Synergistic
Fluconazole	Lemongrass	0.125	0.5	0.625	Additive

Table 20: Interactions between select antifungal drugs and essential oils on *C. lusitaniae.*

Antifungal	Essential Oil	FIC(AF)	FIC(EO)	FICI	Interpretation
Micafungin	Cinnamon Bark	1	1	2	Indifferent
Micafungin	Clove Bud	0.5	1	1.5	Indifferent
Micafungin	Lemongrass	0.125	0.5	0.625	Additive
Flucytosine	Cinnamon Bark	1	0.5	1.5	Indifferent
Flucytosine	Clove Bud	0.25	0.5	0.75	Additive
Flucytosine	Lemongrass	0.5	0.5	1	Additive
Amphotericin B	Cinnamon Bark	1	0.5	1.5	Indifferent
Amphotericin B	Clove Bud	4	1	5	Antagonistic
Amphotericin B	Lemongrass	1	0.25	1.25	Indifferent
Fluconazole	Cinnamon Bark	2	0.5	2.5	Indifferent
Fluconazole	Clove Bud	0.03125	0.03125	0.0625	Synergistic
Fluconazole	Lemongrass	0.25	0.5	0.75	Additive

Table 21: Interactions between select antifungal drugs and essential oils on *S. cerevisiae.*

Antifungal	Essential Oil	FIC(AF)	FIC(EO)	FICI	Interpretation
Micafungin	Cinnamon Bark	1	1	2	Indifferent
Micafungin	Clove Bud	0.5	0.5	1	Additive
Micafungin	Lemongrass	0.25	0.125	0.375	Synergistic
Flucytosine	Cinnamon Bark	1	0.75	1.75	Indifferent
Flucytosine	Clove Bud	0.25	0.5	0.75	Additive
Flucytosine	Lemongrass	0.5	0.5	1	Additive
Amphotericin B	Cinnamon Bark	1	0.5	1.5	Indifferent
Amphotericin B	Clove Bud	4	1	5	Antagonistic
Amphotericin B	Lemongrass	0.25	0.5	0.75	Additive
Fluconazole	Cinnamon Bark	1	0.5	1.5	Indifferent
Fluconazole	Clove Bud	0.25	0.25	0.5	Synergistic
Fluconazole	Lemongrass	0.125	0.5	0.625	Additive

Table 22: Summary of Essential Oil-Drug Interactions.

	Cinnamon Bark	Clove Bud	Lemongrass
Micafungin	Indifferent	Indifferent-Additive	Additive-Synergistic
Flucytosine	Indifferent-Additive	Additive-Synergistic	Additive
Amphotericin B	Indifferent	Antagonistic	Indifferent-Additive
Fluconazole	Indifferent	Synergistic	Additive

Discussion

Absorbance-Population Conversion

A rapid means to estimate and standardize microbial populations is essential to performing high throughput methods, such as microbial identification and antimicrobial testing. While the use of OD600 as a means to estimate population is not a new concept, it was used here on several novel organisms, namely *Candida auris*. At any point when approximating cell density is required, reading the absorbance of a sample is much faster than counting using a hemocytometer or traditional plates. The purpose here was to increase the speed of preparation and precision of starting populations. Because absorbance is a linear function of cell density, with single and uniform celled organisms, a small but representative range of population densities and their respective absorbance values allow the creation of a standard curve that can extrapolate population values outside the range initially used to construct the curve. This

relation will be slightly different for different species due to a variety of factors. The factor with the greatest impact is likely the cell size. Larger cells absorb more light because there is simply more biomass. It was observed that *S. cerevisiae* is qualitatively much larger than *C. auris* (Figure 17). This contributes to the reason why the slope of the *S. cerevisiae* curve was about a quarter of the slope of the *C. auris* curve. It was also noticed that some of the curves fluctuated more than others. The most pronounced of these were the curves drafted for *C. glabrata, C. parapsilosis* and *C. tropicalis*. In each of these instances, the organisms were noticed to aggregate more heavily than the other species. This aggregation of cells would cause more variability in the curve. CHS contains a small amount of Tween 80 to act as a detergent and prevent this. These three organisms still maintained some aggregation despite this. Standard curves such as these could also be used as a replacement for other standards that estimate population based on turbidity, such as MacFarland standards. To replace the turbidity standards, however, either the starting population predicted by the turbidity standard or its absorbance value need to be known. Standard curves are more precise than visually comparing culture to the turbidity standards because the sensor of a spectrophotometer is more sensitive than the human eye.

Methylene-Blue Staining

Methylene blue was successful in differentially staining *C. auris* to determine viability. Staining is simple, inexpensive, and time-efficient when compared to traditional plate counts and could easily be applied to many antimicrobial testing methods where assessing viability is desired. Plate counts are currently used for this assessment and require two to three days of incubation to obtain results. Using methylene blue and a hemocytometer, equivalent results were obtained in less than an hour. Because this stain has worked on two different yeast species, it is also reasonable to believe that this method could be extended to other *Candida* species and possibly even other pathogenic yeasts. One limitation of this method is that hemocytometers have a lower limit of detection of 10^4 cells per mL. For testing requiring a starting inoculum lower than this, the method could still be used, but an extra dilution step would be required and could be a source of error.

New multi-drug-resistant *C. auris* isolates continue to be identified. Coupled with the fact that many other fungal species are developing resistance to a variety of drugs, this underscores the need for production of novel antifungal drugs and higher throughput testing methods. With drug development being undermined by misdiagnosis and a lack of funding, cheap and time-saving tools are invaluable for combatting this emerging pathogen. Using methylene blue, a low-cost viability estimate can be obtained

in less than an hour. These qualities can enhance the rapid production of antimicrobials to prevent and treat infection by *C. auris* and thus save lives.

Carbon and Nitrogen Sources Assimilation Patterns

The presence of distinctive patterns of carbon and nitrogen source assimilation for *Candida auris* could provide a step towards developing a cheap and effective diagnostic test or enrichment medium. Because all species tested utilize galactose except *C. auris* and *C. glabrata,* this could provide a major differentiating component of diagnostic tests. Tests requiring a negative result would be not ideal, and it would thus be wise to include other tests for verification. Using a quad plate, a simple test media containing quadrants with either D-galactose, D-xylose, D-raffinose and dodecane as a sole carbon source would be worth examining. *C. auris* is unable to assimilate either D-galactose or D-xylose, so the two substrates could be used to rule out *C. auris* when examining a suspected isolate. *C. auris* will assimilate D-raffinose and dodecane, so these carbon sources could help differentiate *C. auris* from the related species that cannot utilize them.

Lactose, L-arabinose and dulcitol presented variable results for the different isolates of *C. auris*. These discrepancies were not aligned with clade. These were each retested repeatedly, and the results were consistent. In addition, the standard error of the mean calculated from each triplicate could not account for the variation between isolates (Table 23). It is, however, quite common for this type of variability to appear between isolates of the same yeast, especially when they are geographically separated [68, 69]. According to one study, the metabolic capabilities of several isolates of *Saccharomyces paradoxus* from a geographic region the size of a state were examined. The results show that the ability to utilize various substrates of a core group of carbon sources, such as glucose and sucrose, that are more common and nutritive is conserved among isolates. The remainder, which the study dubs the auxiliary group, includes less common and nutritive substrates like lactose and L-arabinose. The capability to utilize these is more variable and depends on the organism's environmental history [69]. This makes sense, as it is inefficient to maintain genes and enzymes to process less nutritive or absent substrates when more valuable ones are readily available. This type of variability can also pose a problem for utilization-based diagnostic test. Substrates from the core group are not appropriate because the ability to utilize them will likely be conserved between related species. On the other hand, many of the auxiliary sources are not good choices because they are not conserved between isolates of the same species. The former case could yield false positive identification, while the latter could yield

false negative results. Either type of misidentification would lead to mistreatment, thus complicating the clinical outcome.

The nitrogen sources yielded little in differential potential. All isolates tested showed very similar patterns of assimilation. The differences were primarily in the OD600 range that was classified as weak growth and the variability was not isolated to any one species. “Weak” growth could be the result of carryover of media or nutrients within the cell. This would allow the cells to continue to grow and reach a low density before running out of nutrients. In total, more studies are needed to generate a diagnostic media. More carbon and nitrogen sources can be tested to find additional combinations that can differentiate between *C. auris* and related species. Similarly, additional isolates of the species besides *C. auris* should be included. Many species had only one isolate tested, and several species that turn up as misidentifications were not tested at all (Table 1). Many species had only one isolate tested, and this would help to validate the results for species examined here. By examining additional species that were not tested here, it would help to ensure that the assimilation patterns of *C. auris* are not shared by these other species and to avoid future misidentification. Further studies could also explore the situations where organisms could not assimilate some sources. Sodium nitrite is a well-known food preservative. Not only were nearly all isolates unable to assimilate it, it is possible that they were inhibited by it as well. This could be true of the other negative results as well. In any case, the metabolic capabilities are a necessary component to understanding any organism. These fundamental properties often contribute to the pathogenic potential and the vulnerabilities in the organism’s defense and warrant exhaustive exploration.

Table 23: Selected standard error of the mean for carbon source utilization testing.

Strain	Lactose	Arabinose	Dulcitol
0381	0.0050	0.0721	0.0543
0382	0.0729	0.0257	0.0298
0383	0.0348	0.0497	0.2013
0384	0.0900	0.0217	0.0162
0385	0.0443	0.0135	0.0329
0386	0.1570	0.1185	0.0300
0387	0.0194	0.1057	0.0984
0388	0.0401	0.0561	0.0032
0389	0.0511	0.0878	0.0314
0390	0.0308	0.0426	0.0053

Identification and Substrate Utilization using the Biolog YT Plate

The utilization data gathered using the Biolog YT plate adds significantly to the data produced in the carbon source testing described above. Despite the fact that the Biolog system did not accurately identify the *Candida lusitaniae* or *Saccharomyces cerevisiae* isolates used in this study, the information provided by the system is valuable. The Biolog YT plate contains 35 carbon source fermentation assay, 48 sole carbon source assimilation assays and 11 assimilation assays for paired carbon sources. Some of the carbon sources were the same as those previously tested. These were D-galactose, D-glucose, maltose, sucrose, D-raffinose, D-mannitol, L-arabinose, glycerol and D-xylose. The results for all overlapping sources aside from L-arabinose, glycerol and D-xylose agree with the previous results for *C. auris*. However, each of these sources resulted in partial growth on the Biolog plate. For L-arabinose and glycerol, this doesn't align with the 72-hour results. However, the Biolog plates were only incubated for 48-hours. When looking at the 48-hour utilization results, L-arabinose and glycerol show only weak growth. They do not reach the positive threshold until 72 hours, thus aligning with the Biolog results. Isolate 0381 shows partial growth in the D-xylose well of the Biolog plate. It also utilized several of the D-xylose combinations. Since this isolate was not tested in triplicate, it becomes difficult to rule out common sources of error such as contamination or nutrient transfer. This could be the cause of the differences between this isolate and the others. However, discussion with a colleague (Dr. Joe Sexton, mycotic diseases branch, CDC) working with this panel have reported similar metabolic oddities in this strain. More replication will be needed to confirm these anomalies.

Other prevalent issues with comparing these results come from the proprietary nature of the Biolog system and thus many specific parameters are not released to the end-user. The wavelength used by the turbidimeter and thus the starting population are not known, although the starting populations for the Biolog plates were visibly more turbid. The concentrations of the carbon sources are also not known in the YT plates. This is relevant because the chemicals may be nutritive at some levels, but toxic at higher concentrations, either directly or by affecting properties like pH or osmotic pressure of the cells [70]. Finally, the thresholds for the partial and positive results on the Biolog plates are unknown. This could obviously complicate the comparison if the thresholds for one are significantly different from the other. The preparation is also different between the two methods. The Biolog preparation had cultures suspended in water, while the other method used CHS. The CHS serves to separate the yeast cell aggregates and ensure a more homogenous solution. Thus, the Biolog starting inoculums would likely be more variable if the organism tended to aggregate. Even though the comparison must be taken

with some skepticism, the differences are still worth examining. It is very interesting that, despite the variance, the species *C. auris* was identified as was consisted within each clade. Obviously, more testing is needed to validate the trends identified here. Preferably, the methods should be developed by the researcher so all parameters are known, and testing conditions can be replicated with the use of the Biolog YT plate.

Antifungal Activity of Essential Oils

The antifungal activity elicited by the tested essential oils at levels considered safe for dermal use further strengthens their potential application in microbial control, and the applications of the findings are numerous. First, the antifungal activity is present against multiple species. Essential oils have been tested on a range of yeasts and molds [58], and this activity may be expanded to many more. The essential oils might be effective in disinfecting formulations for surfaces [71]. Because this activity occurred at concentrations considered safe for dermal use, it is reasonable to predict that the essential oils could be used in topical remedies for fungal infections. Future work could also examine if this antifungal activity is maintained against bloodstream infections. Another line of inquiry that could be pursued is to continue this testing with an expanded pool of essential oils. Since only a small number of essential oils were tested here, there could be many more with undiscovered potency already widely available. As a foil to the broad-spectrum testing, the most effective oils could be analyzed in depth. Gas-Chromatography and Mass-Spectrophotometry could be used to identify the most prevalent compounds in these essential oils. These prevalent components could then be subjected to the same testing as the essential oils themselves. If activity is retained, the components themselves could be used in formulations for disinfection or for the treatment of infection. This may be beneficial because individual chemicals are much easier to standardize than the essential oils themselves.

Vapor-Phase Activity of Essential Oils

Cinnamon Bark, lemongrass and clove bud essential oils all retained their antimicrobial activity in vapor-phase. Despite these being the only three that elicited inhibition of growth, the other oils may exhibit the same inhibitory activity at higher doses, especially considering that they required greater doses in direct contact. In suboptimal conditions outside of growth media, such as on a healthcare surface where the organism does not have access to a nutrient-dense substrate, these effects may become relevant. The easily diffused vapor-phase oils could have applications in the home or expansive

areas where surface disinfection is needed. More testing is needed under more controlled conditions to ensure the gaseous concentration, even dispersal and contact time of the gaseous oils to truly elucidate the vapor-phase activity of the oils, but these results are promising. In addition, much of the additional testing mentioned for direct contact above is applicable in vapor phase as well. Additional essential oils or the major constituents of the essential oils could be test as they may also display vapor-phase antimicrobial activity [60]. The essential oils could also be tested against a widened range of organisms.

Interaction of Essential Oils and Antifungal Drugs

Several additive or synergistic combinations of essential oils and antifungal drugs where discovered. While additive combinations indicate that the two components are substitutable and thus the overall effective dose is not reduced, two points make these valuable discoveries, nonetheless. First, even though the overall effective dose is the same as each component alone, the component doses of the essential oil and antifungal are reduced and likely the side effects of each [61]. Second, synergism may actually be present, despite the checkerboard method indicating otherwise. While the checkerboard assay is one of the better options for large scale screening of combinations, other methods exist that are more sensitive [61]. Even as the best option for large-scale screening of combinations, checkerboard assays are roughly ten to twenty times more labor-intensive than traditional microdilution MIC testing when considering the quantity of wells needed to test one combination. This has led to less attention and standardization of the method [66]. Because the method is still not fully standardized, several things must be considered when evaluating the results. First, because microdilutions are utilized, a fairly large range of concentrations is represented over a small number of wells, and thus a lower sensitivity. A difference of one microdilution can have significant impact on the FICI. To validate the results produced by the checkerboard assay, smaller dilutions can be performed. Second, the methods of analysis for this type of synergism assay are still not well defined [50]. FICI cutoffs for different interaction categories are different for different publications, and some even combine multiple categories [66]. The selection of the well that determines the MIC values in combination can significantly impact the results. Thus, a more standardized method of analysis and better-defined categories of interaction is needed for the practice of combination therapy to prosper.

The results here show a range of interactions. Cinnamon bark oil appears to be the most neutral, displaying mostly indifferent interactions with all antifungals tested. Current thought is that the essential oil acts by disrupting membrane integrity [72]. The lack of synergism is unexpected in this case,

as increased membrane permeability should give other drugs greater access to drug targets, as is seen with amphotericin b in combination. Further examination could explore the molecular basis for cinnamon oil's mode of action. This may elucidate why there is an apparent lack of interaction with the antifungal drugs.

Clove bud oil displayed a range of interactions. In all cases but amphotericin b, these interactions were generally positive. The primary component of clove oil, eugenol is thought to impact yeast cell wall and membrane integrity [73]. In the case of clove oil, the essential oil likely allows enhanced access of flucytosine, fluconazole and micafungin to their respective drug targets as with combinations of amphotericin B and other antifungals [61]; however, antagonism was observed between clove oil and amphotericin. This is worth examining with future work because the conditions causing this antagonism could be mimicked by other chemicals *in vivo* and complicate treatment with amphotericin B. Because both appear to act on the cellular envelope, there may be interference between the two chemicals. Eugenol has been demonstrated to bind to steroid receptors [74, 75]. Sterols and steroids have been demonstrated to inhibit the activity of amphotericin B [76, 77]. It is possible that the eugenol behaves similarly to a sterol or steroid and thus impedes the function of amphotericin B. It is also possible that clove bud oil and amphotericin B chemically interact and interfere with each other's access to targets.

Lemongrass oil displayed positive interactions with all of the tested drugs. While there have been attempts to elucidate the mode of action of lemongrass oil, the mechanisms appear to be various and dose dependent [78]. In the cited study, cytoplasmic leakage appeared to be low, and the majority of the effects of lemongrass oil seem to be targeted on intercellular components. One thought was that the lemongrass oil causes the cell to swell [78]. The increased surface area could lead to an increase in permeability. However, the primary components of lemongrass oil are citral isomers (terpenoids) [79]. Citral has been demonstrated to decrease membrane fluidity [80]. Decreased membrane fluidity has been linked to increased susceptibility of drug-resistant bacteria and *Candida* [81, 82].

In total, several potentially therapeutic combinations of essential oils and antifungal drugs were uncovered. More data is needed to validate the results *in vivo* and examine if toxicity towards human cells is also increased, but the results thus far are promising. Many other future lines of inquiry are possible. First, additional combinations could be screened for synergism. Since synergism screening is labor-intensive, these results could be used to carefully select new combinations for screening by considering essential oils similar to those tested here and testing them in appropriately matched pairs.

The major chemicals components of the oils could also be examined in place of the oils. The pool of isolates and species of organisms could also be expanded, to see if the interactions extend beyond the small group of organisms tested here. Other, more sensitive methods of synergism screening could be pursued with the additive combinations to detect any borderline synergism. Finally, the molecular modes of actions of the various combinations could be examined. Understanding these could help researchers replicate the effects in future drug development.

Integrative Nature of the Work

While much of this work was built on classical microbiology, the integrative nature is in the application and ramifications of the findings. The work has generated data and techniques that can be applied to a wealth of situations. Disease is an interdisciplinary issue and future study would require techniques beyond microbiology to answer those questions. To expand the vapor-phase testing to better controlled settings and eventually healthcare facilities would require the use of physics to determine the variables that yield a treatment appropriate to the setting. Variables such as humidity, air current and temperature all impact the dispersal of a vaporous substance, including essential oils. Chemistry techniques are required to elucidate the chemical constituents of the essential oils and, coupled with molecular biology, to understand why they function alone or in combination the way that they do. Genetics is broadly applicable as well. Traits such as resistance to antifungal drugs and essential oils are coded into the genetic material of the organisms. The same can be said of the ability of various strains to assimilate the different carbon and nitrogen sources and why their capabilities are different between strains, clades and species. Looking on the macroscopic scale, ecology will play a fundamental role in the application of this work. How do any of the treatments emerging from this data impact the microflora of the healthcare environment or people within? Will the use of vaporous essential oils to sterilize a hospital room create conditions ideal for hardier and dangerous species? Will the application of combination therapy put patients at risk of candidemia or pave the way for other resistant pathogens by eliminating the person's skin and gut flora, much as the overuse of antifungals probably led to the rise of *Candida auris* [26]? Ecological and microbiological techniques will be needed to answer these questions. These results could even reach beyond the traditional sciences. Economic analysis is needed to assess the cost of any of the treatments arising from this data and their viability as a treatment option. Even psychology has its place. For instance, hospitals are a stressful environment for patients. One study even shows a higher incidence of depression in those under hospital care than the general population [65]. Therefore, some patients are dealing with both medical and psychological disorders. A

link has also been found between impaired immune function and depression [84]. It has been demonstrated that olfactory stimulation can impact mood and that aromatherapy with essential oils may help treat depression [85, 86]. It is possible that essential oil aromatherapy could both help control the microbial burden of the hospital environment and ease the psychological burden of the patients. It would be worth evaluating the psychological changes and clinical outcomes of patients in rooms treated with traditional cleaners, such as bleach, as compared to those using natural and pleasant-smelling essential oils.

General Discussion of Research Outcomes

Despite the need for further study in many areas to fully realize the potential applicability of these results, this research has added to the groundwork for future work. The absorbance-population curves allow for a quick population estimates of 12 different species using a spectrophotometer, while the methylene blue staining allows for the estimation of viability in *C. auris* and possibly other species of pathogenic yeasts. The combination of the carbon and nitrogen assimilation assays and the Biolog trials provides some idea into the basic metabolic capabilities of several species, including two novel organisms, *C. duobushaemulonii* and *C. auris.* The latter portion of the work discovered the MIC values of 21 essential oils on three different species of yeast, as well as MFC values of several of the essential oils. This research also lays the foundation for future research examining the gaseous activity of these oils against *Candida auris* and possibly other species. Finally, additive or synergistic associations between three essential oils and three antifungal drugs were discovered. *Candida auris* is an emerging fungal pathogen that is commonly misidentified and nearly always resistant to at least one antifungal drug. Here, we added to the basic understanding of the organism needed to better our diagnostic procedures and found several potential treatment options to help control it. Drug-resistant pathogens are becoming an increasing problem; this research should aid in controlling this emerging global threat.

Appendix I: Identity and Minimum Inhibitory Concentrations of Isolates Used

AR Bank Num	Organism Name	Amphotericin B	Anidulafungin	Caspofungin	Fluconazole	Flucytosine	Isavuconazole	Itraconazole	Micafungin	Posaconazole	Voriconazole
0381	*Candida auris*	0.38	0.25	0.125	4	2	#N/A	0.125	0.125	0.06	0.03
0382	*Candida auris*	0.38	0.25	0.5	16	0.125	#N/A	1	0.25	0.5	0.5
0383	*Candida auris*	0.38	1	0.25	128	0.5	#N/A	0.5	1	0.5	4
0384	*Candida auris*	0.5	2	16	128	0.5	#N/A	1	2	0.5	1
0385	*Candida auris*	0.5	1	0.5	>256	0.5	#N/A	1	0.5	1	16
0386	*Candida auris*	0.5	1	0.5	>256	0.5	#N/A	0.5	0.25	0.5	16
0387	*Candida auris*	0.75	0.5	0.25	8	8	#N/A	0.5	0.5	0.25	0.06
0388	*Candida auris*	1.5	0.5	1	>256	0.125	#N/A	0.5	0.125	0.25	2
0389	*Candida auris*	4	1	0.5	256	128	#N/A	0.25	0.25	0.125	4
0390	*Candida auris*	4	1	0.5	>256	128	#N/A	1	0.25	0.5	8
0391	*Candida duobushaemulonii*	#N/A	0.06	0.03	8	<0.125	#N/A	0.5	0.125	0.25	0.25
0392	*Candida duobushaemulonii*	#N/A	0.06	0.03	8	<0.125	#N/A	0.5	0.125	0.25	0.06
0393	*Candida haemulonii*	#N/A	0.25	0.125	0.5	<0.125	#N/A	0.125	0.25	0.125	0.008
0394	*Candida duobushaemulonii*	#N/A	0.03	0.008	4	<0.125	#N/A	0.06	0.06	0.016	0.125
0395	*Candida haemulonii*	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
0396	*Kodamaea ohmeri*	#N/A	1	0.25	2	0.5	#N/A	0.125	0.5	0.06	0.03
0397	*Candida krusei*	#N/A	0.03	0.125	64	2	#N/A	1	0.125	1	1
0398	*Candida lusitaniae*	0.38	0.125	0.125	1	<0.125	#N/A	0.125	0.125	0.5	0.016
0399	*Saccharomyces cerevisiae*	#N/A	0.125	0.06	2	<0.125	#N/A	0.06	0.25	0.5	0.03
0400	*Saccharomyces cerevisiae*	0.032	0.5	0.5	1	<0.125	#N/A	0.5	0.5	0.5	0.06
0314*	*Candida glabrata*	0.38	1	0.5	64	<0.12	#N/A	>16	1	16	4
0315*	*Candida glabrata*	0.38	2	16	4	>256	#N/A	1	4	1	0.25
0316*	*Candida glabrata*	0.05	1	1	4	<0.12	#N/A	0.25	0.25	0.25	0.06
0317*	*Candida glabrata*	0.19	0.5	1	32	<0.12	#N/A	1	0.25	1	0.5
0318*	*Candida glabrata*	0.19	4	16	32	<0.12	#N/A	1	4	1	1
0319*	*Candida glabrata*	0.125	0.5	1	4	<0.12	#N/A	0.5	2	0.25	0.12

0320*	*Candida glabrata*	0.19	0.5	1	4	<0.12	#N/A	1	0.25	1	0.12
0321*	*Candida glabrata*	0.09	2	4	64	<0.12	#N/A	1	1	2	2
0322*	*Candida glabrata*	0.19	2	2	8	<0.12	#N/A	0.5	0.25	0.5	0.12
0323*	*Candida glabrata*	0.19	4	16	4	<0.12	#N/A	0.25	4	0.25	0.06
0324*	*Candida glabrata*	0.25	4	16	8	0.12	#N/A	0.5	2	0.5	0.25
0325	*Candida glabrata*	0.38	4	>16	128	<0.12	#N/A	16	4	8	16
0326*	*Candida glabrata*	0.016	0.06	0.06	4	<0.12	#N/A	1	0.015	1	0.125
0327*	*Candida glabrata*	0.25	0.125	0.125	16	0.25	#N/A	1	0.015	1	0.25
0328*	*Candida glabrata*	0.25	0.03	0.03	8	<0.12	#N/A	0.5	0.015	0.5	0.25
0329*	*Candida glabrata*	0.19	0.06	0.06	8	<0.12	#N/A	1	0.03	1	0.25
0330*	*Candida glabrata*	0.19	0.03	0.06	8	<0.12	#N/A	1	0.015	1	0.25
0331*	*Candida glabrata*	0.25	0.03	0.06	64	<0.12	#N/A	2	0.015	2	1
0332*	*Candida glabrata*	0.125	0.06	0.06	128	<0.12	#N/A	4	0.015	2	4
0333	*Candida glabrata*	0.125	0.06	0.06	64	<0.12	#N/A	1	0.03	2	1
0334*	*Candida glabrata*	0.125	0.06	0.06	128	<0.12	#N/A	>16	0.03	>16	4
0335	*Candida parapsilosis*	0.19	4	0.5	16	0.125	#N/A	0.5	1	0.25	1
0336*	*Candida parapsilosis*	0.047	1	0.25	32	<0.12	#N/A	0.125	1	0.125	1
0337*	*Candida parapsilosis*	0.094	1	0.25	64	<0.12	#N/A	0.125	0.5	0.125	1
0338*	*Candida parapsilosis*	0.125	1	1	16	<0.12	#N/A	0.5	1	0.25	0.25
0339*	*Candida parapsilosis*	0.047	1	0.25	32	0.25	#N/A	0.25	1	0.25	0.5
0340*	*Candida parapsilosis*	0.03	2	0.25	0.05	0.125	#N/A	0.125	0.5	0.125	0.015
0341*	*Candida parapsilosis*	0.023	1	0.5	0.5	0.125	#N/A	0.06	1	0.06	0.015
0342*	*Candida parapsilosis*	0.023	2	0.5	0.5	0.125	#N/A	0.06	2	0.06	0.015
0343*	*Candida parapsilosis*	0.06	0.5	0.25	1	0.125	#N/A	0.125	1	0.125	0.06
0344	*Candida parapsilosis*	0.06	2	0.25	0.25	<0.12	#N/A	0.03	1	0.06	0.015
0345	*Candida tropicalis*	0.38	0.06	0.06	>256	<0.12	#N/A	>16	0.06	>16	16
NRRL-Y12983	*Candida albicans*	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A

Isolate #0381-0400 from Candida auris panel, Isolate # 0323-0345 from Drug Resistant Candida Species panel, adapted from [28]

**Not used in data presented, but included for completeness*

Appendix II: Layout of the Biolog YT Plate

water	acetic acid	formic acid	propionic acid	succinic acid	succinic acid mono-methyl ester	L-aspartic acid	L-glutamic acid	Ll-proline	D-gluconic acid	dextrin	inulin
D-cellobiose	gentibiose	maltose	maltotriose	D-melezitose	D-melibiose	palatinose	D-raffinose	stachyose	sucrose	D-trehalose	turanose
n-acetyl-D-glucosamine	α-D-Glucose	D-galactose	D-psicose	L-sorbose	salicin	D-mannitol	D-sorbitol	D-arabitol	xylitol	glycerol	tween 80
water	fumaric acid	L-malic acid	succinic acid mono-methyl ester	bromo-succinic acid	L-glutamic acid	γ-amino-Butyric acid	α-Keto-glutaric acid	2-keto-D-gluconic acid	D-gluconic acid	dextrin	inulin
D-cellobiose	gentibiose	maltose	maltotriose	D-melezitose	D-melibiose	palatinose	D-raffinose	stachyose	sucrose	D-trehalose	turanose
n-acetyl-D-glucosamine	D-glucosamine	α-D-Glucose	D-galactose	D-psicose	L-rhamnose	L-sorbose	α-Methyl-D-glucoside	β- Methyl-D-glucoside	amygdalin	arbutin	salicin
maltitol	D-mannitol	D-sorbitol	adonitol	D-arabitol	xylitol	I-erythritol	glycerol	tween 80	L-arabinose	D-arabinose	D-ribose
D-xylose	succinic acid mono-methyl ester plus D-xylose	n-acetyl-D-glutamic acid plus D-xylose	quinic acid plus D-xylose	D-glucuronic acid plus D-xylose	dextrin plus D-xylose	α-D-Lactose plus D-xylose	D-melibiose plus D-xylose	D-galactose plus D-xylose	M-inositol plus D-xylose	1,2-propanediol plus D-xylose	acetoin plus D-xylose

Cells outlined in red represent fermentation assays, the remainder are assimilation assays

Appendix III: Essential Oils Examined

Essential Oil	**Species**	**MSDC(%)***
Basil	*Osimum basilicum*	0.10
Bergamot	*Citrus bergamium*	0.40
Bitter Orange	*Citrus aurantium*	1.25
Cinnamon Bark	*Cinnamomum zeylancium*	0.07
Cinnamon Leaf	*Cinnamomum zeylancium*	0.60
Clove Bud	*Syzygium aromaticum*	0.50
Coriander	*Coriandrum sativum*	NA
Eucalyptus	*Eucalyptus globulus*	20.00
Frankincense	*Boswellia carteri*	NA
Geranium	*Pelargonium graveolens*	17.50
Ginger	*Zingiber officinale*	NA
Grapefruit	*Citrus paradisi*	4.00
Lavender	*Lavandula angustifolia*	0.10
Lemon	*Citrus limon*	2.00
Lemongrass	*Cymbopogon flexuosus*	0.70
Lime Peel	*Citrus aurantifolia*	0.70
Manuka	*Leptospermum scoparium*	NA
Myrrh	*Commiphora myrrha*	NA
Peppermint	*Mentha piperita*	5.40
Spearmint	*Mentha spicata*	1.70
Tea Tree	*Melaleuca alterenifolia*	15.00
**MSDC: Maximum Safe Dermal Concentration, taken from* [59]		

Appendix IV: Supply and Equipment List

Item Name	Manufacturer	Item Number
Glycerol	Sigma Aldrich	G5516
Yeast Nitrogen Base w/o Amino Acids	Sigma Aldrich	Y0626
D-glucose	Sigma Aldrich	G8270
D-galactose	Sigma Aldrich	G0750
L-arabinose	Sigma Aldrich	A3256
D-xylose	Sigma Aldrich	X1500
Maltose	Sigma Aldrich	M5885
Lactose	Sigma Aldrich	L254
Sucrose	Sigma Aldrich	S0389
D-raffinose	Sigma Aldrich	83400
Maltodextrin	Sigma Aldrich	419680
Dulcitol	Sigma Aldrich	D0256
Mannitol	Sigma Aldrich	M4125
Dodecane	Sigma Aldrich	297879
96-well Plates	Celltreat	229596
Yeast Carbon Base	Sigma Aldrich	Y3627
Ammonium Acetate	Sigma Aldrich	A1542
Sodium Nitrate	Sigma Aldrich	S5506
Sodium Nitrite	Sigma Aldrich	237213
Urea	Sigma Aldrich	U0631
L-arginine	Sigma Aldrich	A5006
L-valine	Sigma Aldrich	V0500
Glycine	Sigma Aldrich	410225
L-proline	Sigma Aldrich	P0380
D-valine	Sigma Aldrich	855987
Uracile	Sigma Aldrich	U0750
Thymine	Sigma Aldrich	T0376
Thiamin Hydrochloride	Sigma Aldrich	T4625
Pyridoxin Hydrochloride	Sigma Aldrich	P9755
Biolog Universal Yeast Agar	Biolog	70005
Turbidimeter	Biolog	3587
Biolog YT Standard	Biolog	3415
YT Microplate	Biolog	1005
Reservoirs	MedSupplyPartners	62-1012-6
Flucytosine b	ApexBio	A8433
Micafungin	ApexBio	A3606
Fluconazole	Sigma Aldrich	F8929
Amphotericin b	Sigma Aldrich	A9528
Dimethyl Sulfoxide	Sigma Aldrich	D4540

Coriander Seed Essential Oil	Mountain Rose Herbs	eo_co1/2
Ginger Essential Oil	Mountain Rose Herbs	eo_gi1/2
Eucalyptus Essential Oil	Mountain Rose Herbs	eo_eu1/2
Geranium Essential Oil	Mountain Rose Herbs	eo_ge1/2
Tea Tree Essential Oil	Mountain Rose Herbs	eo_tto1/2
Peppermint Essential Oil	Mountain Rose Herbs	eo_pep1/2
Lemongrass Essential Oil	Mountain Rose Herbs	eo_lg1/2
Cinnamon Bark Essential Oil	Mountain Rose Herbs	eo_cb1/2
Orange, Bitter Essential Oil	Mountain Rose Herbs	eo_or_b1/2
Grapefruit Essential Oil	Mountain Rose Herbs	eo_gr1/2
Clove Bud Essential Oil	Mountain Rose Herbs	eo_clo1/2
Spearmint Essential Oil	Mountain Rose Herbs	eo_sp1/2
Basil Essential Oil	Mountain Rose Herbs	eo_ba1/2
Bergamot Essential Oil	Mountain Rose Herbs	eo_be1/2
Cinnamon Leaf Essential Oil	Mountain Rose Herbs	eo_cl1/2
Frankincense Essential Oil	Mountain Rose Herbs	eo_fr1/2
Lavender Essential Oil	Mountain Rose Herbs	eo_la_o1/2
Lemon Essential Oil	Mountain Rose Herbs	eo_le1/2
Lime Peel Essential Oil	Mountain Rose Herbs	eo_lp1/2
Manuka Essential Oil	Mountain Rose Herbs	eo_manu1/2
Myrrh Essential Oil	Mountain Rose Herbs	eo_mro1/2
Breathable Sealing Film	Diversified Biotech	BEM-1
Methylene blue	EK industries	EK-7161
Malt extract	Acumedia	7341A
Agar	EMD Millipore	EM-140500
Hemocytometer	Brightline	400180
Hemocytometer	LW Scientific	MKT-7.5.3L120
Spectrophotometer	VWR	UV6300PC
Microscope	Motic	BA410E
Biolog Microstation	Biolog	65361
Microlog 3 Software	Biolog	V 5.2.1.35
Plate Reader	Biotek	SynergyH1
Gen5 Software	Biotek	V2.01
Excel Software	Microsoft	V1905

Appendix V: Media and Solution Recipes

Malt Extract Broth (MEB): 30 grams Malt Extract per 1.0 L deionized water

Malt Extract Agar (MEA): 30 grams Malt Extract & 20 grams Agar per 1.0 L deionized water

Conidia Harvesting Solution (CHS): 0.05% Tween 20 & 0.9% sodium chloride in deionized water.

Yeast Nitrogen Base without amino acids (YNB): 6.8 grams dehydrated YNB powder per 1.0 L deionized water. Supplement with 2.0% of selected carbon source.

YNB plus Glucose (YNBG): 6.8 grams dehydrated YNB powder per 1.0 L deionized water. Supplement with 20 grams of Glucose.

Yeast Carbon Base (YCB): 11.7 grams dehydrated YCB powder per 1.0L deionized water. Supplement with 2.0% selected nitrogen source.

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