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Survival of Candida auris in the environment and the development of resistance to disinfection

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Survival of *Candida auris* in the environment and the development of resistance to disinfection

Orefemetse Dire
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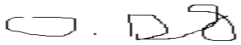
Degree of Master of Science in Medicine by research only

Dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science in Medicine

Johannesburg 2022

DECLARATION

I, Orefemetse Dire hereby declare that this dissertation is my own work. It is being submitted for the degree of Master of Science in Medicine to the University of Witwatersrand, Johannesburg. It has not submitted before for any degree or examination at this or any other University



…… (Signature of candidate)

……26th……..day of …August……2022

DEDICATION

To my loved ones, those that are with me and those that passed…

ACKNOWLEDGEMENTS

I would like to thank my supervisor **Professor Mrudula Patel** (Department of Clinical Microbiology and Infectious Diseases, University of the Witwatersrand) for welcoming me when I came knocking on her door, providing me with such an interesting research topic and guiding me throughout the project, I truly am grateful; my co-supervisor **Dr. Aijaz Ahmad** (Department of Clinical Microbiology and Infectious Diseases, University of the Witwatersrand) for his guidance and expertise; **Dr. Musa Marimani** (Department of Molecular Medicine and Haemotology, University of the Witwatersrand) for his guidance and assistance when I needed it the most; The whole CMID postgraduate room for moral support and assistance in the laboratory.

I would also like to thank **Dr. Morne Du Plessis** (National Institute for Communicable Diseases) and **Miss Thina Duze (**Department of Clinical Microbiology and Infectious Diseases, University of the Witwatersrand**)** for assistance with gene sequencing and genetic analysis; and also **Dr. Zvifadzo Matsena Zingoni** for assistance with statistical analysis.

To each and every one that has been cheering me on through the years, I am because you are, thank you.

Abstract

Introduction:

The increasing number of cases of *Candida auris* across continents since its emergence in 2009, continue to raise an alarm and the significance of *C. auris* as a pathogen. The fungus has been found mainly in critical care patients causing nosocomial infections, hospital outbreaks and high rate of mortality. This pathogen has indicated the ability to survive for long periods outside of their host which facilitate the transmission from healthcare workers to patients. In the hospital environment many different types of surface materials are used which can become contaminated. Many disinfectants with different efficacies are used to decontaminate these surfaces. Therefore, it is important to study the survival of *C. auris* on these surfaces in different conditions and the effect of disinfectants.

Aim:

The aim of this study was to investigate the survival of *C. auris* on various environmental surfaces under different conditions and a possible development of resistance to repeated use of disinfectants.

Methods:

Known size of glass, fabric, plastic, wood and steel blocks were fabricated and artificially contaminated with planktonic 3 strains of *C. auris* and one strain of *C. albicans*. One set was kept wet and one dry for 3 weeks, and at intervals, *C. auris* counts were performed using a dilution and culture technique. Similarly, biofilms were grown, and counts were performed. The resulted counts were compared to the initial counts using a student t-test. In the disinfectant study, 4 commonly used disinfectants such as sodium dichloroisocyanurate (NADCC), benzalkonium chloride (BC), 70% ethyl alcohol (Eth) and hydrogen peroxide (HP) were selected and minimum inhibitory concentrations (MIC) were determined using a broth microdilution technique. Time-kill assays were performed using MIC, ½ and ¼ MIC dilutions. *C. auris* was pulsed daily with 10 minute exposure for 15 days and MIC was repeated. In addition, ergosterol quantities and *ERG11* gene expression were measured for the exposed and unexposed *C. auris* cells. Finally, whole-genome sequencing was performed on the exposed and unexposed cells of *C. auris* and mutations were identified.

Results:

The survival results showed that *C. auris* survived on all the surfaces for more than 3 weeks with either increase or reduction in the viable cells. Planktonic cells survived the most on wood and steel with 390 and 148% increase, and least on glass with 93% reduction in the counts. Survival was significantly better in the wet condition (p <0.001) compared to the dry condition. In the biofilm form *C. auris* flourished on all the surfaces with an increase of up to 575% regardless of wet and dry conditions. *C. albicans* behaved similarly on all the surfaces and conditions.

In the time-kill study, at recommended concentration NADCC and BC killed 100% of *C. auris* within 30 seconds whereas Eth required one minute and HP was found to be very weak requiring 5 minutes to kill 100% of *C. auris*. When *C. auris* was pulsed for 10 minutes daily with disinfectants for 15 days, it was found that for NADCC and BC the MIC increased in the exposed cells compared to the unexposed cells. NADCC and HP exposed cells had increased level of ergosterol and both induced a significant level of *ERG11* gene expression. Genome sequencing results showed that BC treated Cau4888 was found to have more genetic variations than NaDCC treated *C. auris*. Most of the changes/variations were made upstream of the genes, possibly the promoter regions. Even though some of the mutated genes were identified, most are unannotated genes.

Conclusions:

In wet and dry conditions, planktonic and biofilm form of *C. auris* can survive on many different types of surfaces for more than 3 weeks. It is important to use an effective disinfectant at a recommended concentration. Repeated exposure to sublethal concentrations may develop resistance to the disinfectant in this organism. This highlights the importance of immediate cleaning and disinfection of surfaces after exposure to *C. auris* to minimize transmission.

Table of Contents

Chapter 1 Introduction and literature review

Introduction……19

1. Literature review

1.1.Candida auris…….....19

1.1.1. General information……...19

1.1.2. Morphology and Cultural characteristics of *C. auris*…….....20

1.1.3. Identification of *C. auris*……21

1.2. *C. auris*: Epidemiology and Infections…….22

1.2.1. Epidemiology……...22

1.2.2. Infections…….....24

1.3. Pathogenicity of *C. auris*…….25

1.3.1. Adherence…….25

1.3.2. Hydrolytic enzymes……..25

1.3.3. Aggregation……..26

1.3.4. *C. auris* vs *C. haemulonii* pathogenicity..……27

1.3.5. Immune response in *C. auri*s infections……..….....27

1.3.6. Hyphae formation…….28

1.4. Environmental survival of *C. auris*…….29

1.4.1. Abiotic surfaces……29

1.4.2. Biofilm formation……30

1.5. Disinfection and *C. auris*……....31

1.5.1. Importance of infection control in hospitals……31

1.5.2. Disinfectants…….....33

1.5.3. Resistance to disinfectants and antifungal agents…...…….34

1.6. Ergosterol…….....…….35

1.7. Rationale for the study…….....36

1.8. Aim……...……...36

1.9. Objectives……....……36

Chapter 2 Methods and materials

2.1. *C. auris* strains……...38

2.2. Disinfectants…….....38

2.3. Survival of planktonic *C. auris* on wet and dry surfaces….....38

2.3.1. Surface material samples and test conditions…….....38

2.3.2. Preparation of Inoculum……...39

2.3.3. Test procedure……..39

2.4. Survival of biofilm form of *C. auris* on wet and dry surfaces……....40

2.4.1. Surface material samples and test conditions……...40

2.4.2. Preparation of Inoculum……...40

2.4.3. Biofilm formation……40

2.4.4. Test procedure……..42

2.5. Disinfection study……...42

2.5.1. Minimum Inhibitory Concentration (MIC)……..42

2.5.2. Time Kill Study……42

2.5.3. Disinfection Pulsing and second MIC……..43

2.5.4. ERG11 gene expression……...43

2.5.4.1. RNA Extraction and cDNA Construction……..43

2.5.4.2. Standard and real-time PCR……43

2.5.4.3.Ergosterol extraction and quantification……..44

2.5.5. Genomic DNA Extraction and Sequencing……..45

2.6. Statistical analysis……...45

Chapter 3 Results

3.1.Survival of planktonic form of *C. auris* on the wet and dry surfaces……46

3.1.1. *C. auris* counts on various surfaces……46

3.1.1.1. Glass surfaces……..46

3.1.1.2. Fabric surfaces……48

3.1.1.3. Plastic surfaces……50

3.1.1.4. Wood surfaces……52

3.1.1.5. Steel surfaces……...54

3.1.2. Comparison of surfaces wet and dry combined with planktonic cells……..……...56

3.1.3. Comparison of wet and dry condition combining all the surfaces with planktonic cells……...57

3.2. Survival of biofilm form of cells of *C. auris*…….58

3.2.1. Counts of biofilm of *C. auris* on various surfaces…….58

3.2.1.1. Glass surfaces……..58

3.2.1.2. Fabric surfaces…….60

3.2.1.3. Plastic surfaces……62

3.2.1.4. Wood surfaces…….64

3.2.1.5. Steel surfaces……...66

3.2.2. Comparison of surfaces wet and dry combined with biofilm……68

3.2.3. Comparison of wet and dry condition combining all the surfaces with biofilms……...69

3.3. Comparison of planktonic and biofilm forms of *C. auris*……70

3.3.1. Comparison of planktonic cells to biofilm cells combining all the surfaces…….70

3.3.2. Comparison of planktonic cells to biofilm cells in wet and dry condition combining all the surfaces……...71

3.4. Comparison of planktonic and biofilm forms of *C. albicans* strain Cal5339……...72

3.5. Disinfectant study……..74

3.5.1. Minimum inhibitory concentrations of disinfectants (MIC)……..74

3.5.2. Time-kill study of disinfectants…….75

3.5.3. Pulsing of *C. auris* with disinfectant …….77

3.5.4 Ergosterol quantification of disinfectant exposed *C. auris*…….69

3.5.5 Effect of disinfectant on the *ERG11* gene expression……...82

3.5.5.1. Standard PCR …….82

3.5.5.2. Real time PCR results…….84

3.5.5.3. Whole genome sequencing of disinfectant exposed *C. auris*……..87

Chapter 4 Discussion

4.1. Survival of planktonic and biofilm *C. auris* and the effects of wet and dry surfaces…...90

4.1.1. Glass Vs steel surfaces……...91

4.1.2. Wood surfaces……91

4.1.3. Fabric surfaces……..……..93

4.1.4. Wet Vs Dry surfaces……...93

4.1.5. Survival of *C. auris* under 1 week……..94

4.1.6. Survival of *C. albicans*……...94

4.1.7. Increase of *C. auris* on certain surfaces…….95

4.2. Disinfection study (Effect of suboptimum concentrations of disinfectants)…….96

4.2.1. Minimum Inhibitory Concentration (MIC)……96

4.2.2. Time kill…….96

4.2.3. Ergosterol quantification and *ERG11* gene expression in disinfectant exposed *C. auris*

……..…97

4.2.4. Effects of disinfectant on the erg 11 gene expression……99

4.2.4.1. Whole gene Sequencing ……..……..87

4.2.4.2. *C. auris* adaptation, survival and spread**……...**101

Chapter 5 Conclusions, limitations, and future research

5. Conclusions, limitations, and future research…….....103

5.1. Conclusions…….103

5.2. Limitations……..104

5.3. Future research……104

Chapter 6 references

6. References……..……105

Chapter 7 Appendices

7.1 Appendix 1 Ethics clearance certificate……..……...……...113

7.2 Appendix 2 Composition and preparation of media and reagents……...114

7.3 Appendix 3 Statistics……116

7.4 Appendix 4 Turn-it-in report for the similarity index and plagiarism…….146

List of Figures

Figure 1.1 Colonies of *C. auris* grown on Sabouraud dextrose agar (SDA) for 48 hours…..21

Figure 1.2 List of Species that are often misidentified with *C. auris* depending on the method used……..……22

Figure 1.3 The distribution of *C. auris* clades across the globe as of February 2019 (b) RaxML phylogeny indicating the relationship among the *C. auris* clades…….…..23

Figure 1.4 Human neutrophils engage with *C. albicans* and not *C. auris* 28

Figure 1.5 Flow diagram showing the study layout that was used to achieve these objectives…….….37

Figure 2.1 A visual representation of the test procedure carried out to determine the survival of *C. auris* cells on wet and dry environmental surfaces. …..…….41

Figure 3.1 Survival of planktonic cells of C. *auris* on wet and dry glass surface. …….46

Figure 3.2 Survival of planktonic cells of *C. auris* on wet and dry fabric surface. …….48

Figure 3.3 Survival of planktonic cells of *C. auris* on wet and dry plastic surface. ……..……...50

Figure 3.4 Survival of planktonic cells of *C. auris* on wet and dry wood surface. ……52

Figure 3.5 Survival of planktonic cells of *C. auris* on wet and dry steel surface ……54

Figure 3.6 Comparison of planktonic *C. auris* counts on various surfaces including wet and dry conditions ……..56

Figure 3.7 Comparison of planktonic *C. auris* counts in wet and dry conditions on various surfaces…….....57

Figure 3.8 Survival of Biofilms of *C. auris* on wet and dry glass surface…….....58

Figure 3.9 Survival of Biofilms of *C. auris* on wet and dry fabric surface. ….……60

Figure 3.10 Survival of Biofilms of *C. auris* on wet and dry plastic surface.....62

Figure 3.11 Survival of biofilms of *C. auris* on wet and dry wood surface……..64

Figure 3.12 Survival of Biofilms of *C. auris* on wet and dry steel surface……66

Figure 3.13 Comparison of biofilm form of *C. auris* counts on various surfaces including wet and dry conditions……68

Figure 3.14 Comparison of biofilm form of *C. auris* counts in wet and dry conditions on various surfaces……69

Figure 3.15: Comparison of planktonic *C. auris* counts to biofilm counts over 3 weeks combining all the surfaces under wet and dry conditions……..…….70

Figure 3.16 Comparison of planktonic cells to biofilm cells in wet and dry condition combining all the surfaces……..71

Figure 3.17 Comparison of planktonic and biofilm counts of the *C. albicans* strain Cal5339 combining all the surfaces, and wet and dry conditions…….....73

Figure 3.18 Ultra-Violet spectrophotometric sterol profile representing the quantification of ergosterol in the *C. auris* and *C. albicans* strains after 15 days of disinfection pulsing with 5 different disinfectants separately……81

Figure 3.19 A 2% agarose gel profile following amplification of cDNA derived from *C. auris* and *C. albicans*...…….83

Figure 3.20 A 2% agarose gel validating amplification of *Candida* cDNA derived from *C. auris* and *C. albicans*...…….83

Figure 3.21: Fold change in gene expression relative to control (untreated) samples graphed for the different strains for the tested disinfectants……..…85

List of Tables

Table 1.1 Antiseptics tested for effectiveness against *Candida auris* (Ku et al., 2018)…….32

Table 1.2 Surface disinfectants tested for effectiveness against *Candida auris* (Ku et al., 2018)…….33

Table 3.1 Survival of planktonic cells of *C. auris* on wet and dry glass surface……47

Table 3.2 Survival of planktonic cells of *C. auris* on wet and dry fabric surface……..…...49

Table 3.3 Survival of planktonic cells of *C. auris* on wet and dry plastic surface……..51

Table 3.4 Survival of planktonic cells of *C. auris* on wet and dry wood surface……53

Table 3.5 Survival of planktonic cells of *C. auris* on wet and dry steel surface…….55

Table 3.6 Comparison of planktonic *C. auris* counts on various surfaces including wet and dry conditions……...56

Table 3.7 Comparison of planktonic *C. auris* counts in wet and dry conditions combining various surfaces……57

Table 3.8 Survival of Biofilms of *C. auris* on wet and dry glass surface……59

Table 3.9 Survival of Biofilms of *C. auris* on wet and dry fabric surface……..61

Table 3.10 Survival of Biofilms of *C. auris* on wet and dry plastic surface……...63

Table 3.11 Survival of biofilms of *C. auris* on wet and dry wood surface…….....65

Table 3.12 Survival of Biofilms of *C. auris* on wet and dry steel surface……..67

Table 3.13 Comparison of biofilm form of *C. auris* counts on various surfaces including wet and dry conditions……68

Table 3.14 Comparison of biofilms of *C. auris* counts in wet and dry conditions combining various surfaces……....69

Table 3.15 Comparison of planktonic counts of *C. auris* to biofilm combining all the surfaces, and wet and dry conditions……70

Table 3.16 Comparison of *C. auris* planktonic cells to biofilm cells in wet and dry condition combining all the surfaces……71

Table 3.17 Survival of Planktonic *C. albicans* strain Cal5339 on wet and dry glass, fabric, plastic, wood and steel surfaces……..……..72

Table 3.18 Survival of Biofilm *C. albicans* strain Cal5339 on wet and dry glass, fabric, plastic, wood and steel surfaces……..……...……..73

Table 3.19 Minimum inhibitory concentrations of disinfectants (MIC) of disinfectants against *C. auris*……74

Table 3.20 Mean percentage kill of *C. auris* cells obtained for different concentrations of ethanol……..76

Table 3.21 Mean percentage kill of *C. auris* cells obtained for different concentrations of sodium dichloroisocyanurate (NaDCC)……76

Table 3.22 Mean percentage kill of *C. auris* cells obtained for different concentrations of benzalkonium chloride…….77

Table 3.23 Mean percentage kill of *C. auris* cells obtained for different concentrations of hydrogen peroxide……77

Table 3.24 MIC results of *C. auris* and *C. albicans* against disinfectants before and after the 15 day limited exposure to sub-inhibitory concentration…….78

Table 3.25 MIC results of *C. auris* Cau4888 against disinfectants before and after the 15 day limited exposure……...79

Table 3.26 Quantities of ergosterol in disinfectant treated *C. auris* and *C. albicans* ……..82

Table 3.27 *ERG11 expression quantity (ΔCp) results for C. auris and C. albicans as observed by real-time PCR* ……..……85

Table 3.28 Results of SNPS identified in BC treated Cau4888 mapped against untreated CAU4888 *C. auris*……87

Table 3.29 Results of SNPS identified in NaDCC treated Cau4888 mapped against untreated CAU4888 *C. auris*……88

Table 4.1 Summary results: MIC assay, ergosterol quantification and *ERG11* gene expression…….99

List of Abbreviations and accronyms

BC	Benzalkonium chloride
CDC	Center for Disease Control and Prevention
cfu	Colony forming unit
cm	Centimetre
Cal	*Candida albicans*
Cau	*Candida auris*
DNA	Deoxyribonucleic acid
^{o}C	Degree Celsius
Eth	Ethanol
g	Gram
HREC	Human Research Ethics Committee
HP	Hydrogen peroxide
IL	Interleukin
ml	Millilitre
mg	Milligram
µl	Microliters
NaOCl	Sodium hypochlorite
NADCC	Sodium dichloroisocyanurate
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
ppm	Parts per million
%	Percentage
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
SDA	Sabouraud Dextrose Agar
SD	Sabouraud Dextrose

TNF	Tumor necrosis factor
UV	Ultra violet
VBNC	Viable but nonculturable

CHAPTER 1 INTRODUCTION AND LITERATURE REVIEW

Introduction

Candia auris was first reported in Japan in 2009 following isolation from the ear canal of a patient, and has since been linked to outbreaks in all six continents**.** The fungus has been found mainly in critical care patients, causing nosocomial infections. Compared to the common and well known species of *C. albicans*, the newly emerged *C. auris* produces fewer virulence factors, suggesting weaker pathogenicity (Jeffery-Smith et al., 2018). However, it has shown high mortality rates that reach up to 72% (Lone & Ahmad 2019), and has the ability to cause outbreaks in hospital settings, particularly among critically ill patients, and widespread multidrug resistance to commonly used antifungal agents (Jeffery-Smith et al., 2018). It has been found to be resistant to fluconazole, which is commonly used as a prophylaxis, and antifungal agents that are used for the treatment of systemic infections (Kindo et al., 2015). The ability to cause outbreaks could be related to the survival and persistence of this organism in the environment (Jeffery-Smith et al., 2018). Studies have shown that it can form biofilm on environmental surfaces and survive for up to 14 days, tolerating disinfection (Welsh et al., 2017). It can survive on plastic surfaces and even on different types of gloves. As such, the purpose of this study was to do further research into the persistence of *C. auris* in the environment on different types of surfaces and under different conditions (i.e. wet and dry conditions) as well as to study the development of resistance due to repeated exposure to disinfectants at sub-lethal levels.

1. Literature review

1.1. Candida auris

1.1.1. General Information

Candida auris was so named because it was first described as an isolate from a patient's ear canal (Larkin et al., 2017). This yeast, however, has now been isolated from several other parts of the body in patients around the world (Jeffery-Smith et al., 2018). As at this time, several studies have been done on it, aimed at characterizing the phenotypic and genotypic features of the yeast and comparing different isolates from different regions. *Candida. Auris* has a haploid genome which is about 12.5 Mb and has a guanine-cytosine content of around 45%. Analysis of the *C. auris* genome has suggested that there are about 6500-8500 sequences that code for proteins. These analyses have indicated that there has been a very striking divergence of *C. auris* from other *Candida* species, while it remains closely related to *C. lusitaniae* and *C.*

haemulonii. In addition, there is a huge variation between geographical clades consisting of thousands of single nucleotide polymorphism (SNP) differences. Currently *C. auris* is separated into four geographic clades, which are South African, South Asian, East Asian and South American clades (Sharma et al., 2016). In each clade there are additional minimum genetic differences. This is thus in agreement with the proposed hypothesis that claims that *C. auris* emerged simultaneously and independently on the different continents (Govender et al., 2019).

A yeast genus consists of a heterogeneous group of organisms, and as a result, *Candida* species are often classified using clades. *C. auris* then belongs to the Metschnikowia/Clavispora clade. More than 90% of all the invasive infections caused by *Candida* species in humans have been due to the following: *Pichia kudriavzevii* (formerly known as *Candida krusei*), *Candida tropicalis*, *Candida dubliniensis, Candida parapsilosis, Candida glabrata* and *Candida albicans*. Recently however, *C. auris* has posed a much more serious threat to global health, and this is owed to the fact that this particular yeast is multi-drug-resistant and causes invasive infections. Among the species mentioned above *C. albicans* have been the most prevalent in infections. The recently emerged *C. auris* has now become the biggest concern for researchers and health workers. In addition, it is associated with a high mortality rate and is very difficult to diagnose (de Cássia Orlandi Sardi et al., 2018).

1.1.2. Morphology and Cultural characteristics of *C. auris*

When cultured on SDA, *C. auris* forms white to cream colonies that have smooth edges (Figure 1.1) and they grow well at 37°C. In comparison, when grown on chromogenic agar *C. auris* forms pink colonies which grow well at around 42°C, but has variable growth at higher temperatures. The colonies are formed of elongated or oval cells, which occur as single cells, pairs or as groups of cells; however, they do not form hyphae or pseudohyphae (Satoh et al., 2009). Studies have shown that different *C. auris* strains behave differently, with some having the ability to form aggregates while others do not aggregate. In addition, it was found that isolates that do not form aggregates show greater pathogenicity than isolates that form aggregates. The ability to form biofilms has been recently demonstrated with the non-aggregating strains of *C. auris,* while the aggregate-forming isolates have been shown to form biofilms at a lesser degree (Jeffery-Smith et al., 2018).



Figure 1.1: Colonies of *C. auris* grown on Sabouraud dextrose agar (SDA) for 48 hours.

1.1.3. Identification of *C. auris*

In laboratory settings, *C. auris* is often misidentified as other *Candida* species, particularly in conventional diagnostic laboratories that use biochemical typing. In most cases, these isolates have been mistaken for *C. haemulonii* which rarely causes infections in humans. In addition, *C. auris* has been misidentified as *Rhodotorula mucilaginosa, Rhodotorula glutinis, Candida Sake, Candida famata* and *Saccharomyces* species. Also, in very rare cases, *C. auris* has been mistaken for *Candida parapsilosis, Candida guilliermondii, Candida lusitaniae* or *Candida catenulate*. *C. auris* is closely related phylogenetically to the *C. haemulonii* species, which are detected at a lesser frequency as compared to *C. auris*. A number of molecular techniques have, however, been developed for the accurate identification of this pathogen; for example, the use of PCR assays that are specific for *C. auris,* using cultured colonies, have proven to be effective in terms of fast and accurate identification of *C. auris,* and this has proven to be very useful in outbreak situations (Kathuria et al., 2015).

Candida species identification methods have thus been under continuous improvement over past years, ranging from biochemical methods to nucleic acid-based methods. Conventional identification methods such as commercial systems like API20, BD Phoenix, and Vitek-2 are unable accurately to identify *C. auris* isolates. Isolating and identifying *C. auris* using traditional methods can be very challenging. Some phenotypic methods (API 20C, VITEK-2 YST), MicroScan and BD Phoenix yeast identification system, have often confused *C. auris* with other microorganisms (de Cässia Orlandi Sardi et al., 2018). Figure 1.2 shows the

identification methods and the species that these methods tend to confuse with *C. auris*. The most reliable method of identification of *C. auris* has been through sequencing targets in the nuclear ribosome RNA gene (D1/D2 region or the internal transcribed spacer domain) of the yeast, or by using matrix-assisted laser desorption/ionization – time of flight mass spectrometry (MALDI-TOF MS).

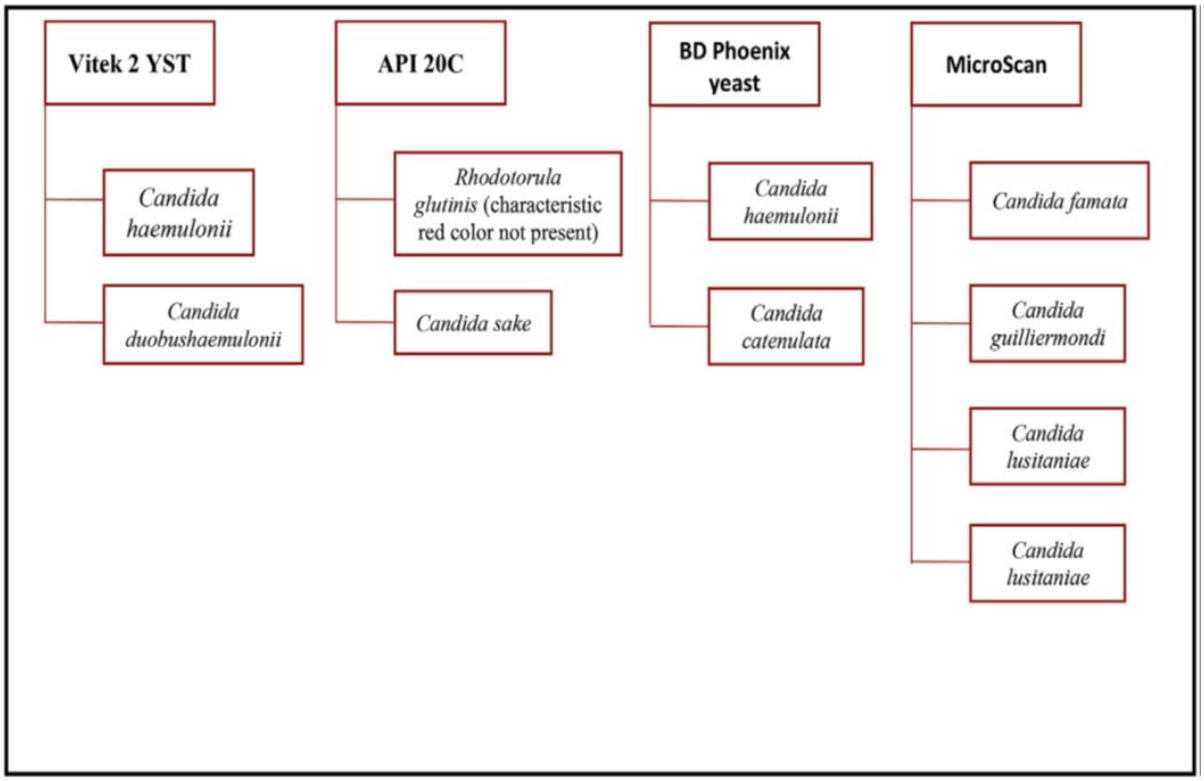


Figure 1.2: List of Species that are often misidentified as *C. auris,* depending on the method used (de Cássia Orlandi Sardi et al., 2018).

1.2. *C. auris*: Epidemiology and Infections

1.2.1. Epidemiology

C. auris was first described in 2009 at Tokyo Metropolitan Geriatric Hospital in Japan, after being isolated on the external ear canal of a 70 year old female patient (Lockhart et al., 2017). Later that same year, 15 isolates of the yeast were discovered from 15 patients from 5 different hospitals in South Korea. About two years after this occurrence, 3 blood stream infections (BSI) were reported in South Korea; indicating that *C. auris* is able to cause both superficial and invasive infections. The Fungi persisted in all 3 patients for about 10 to 31 days (Chowdhary et al., 2017). Not long after, outbreaks of this pathogen were reported in different parts of the globe, including several hospitals in 6 different continents. After affecting South Korea, a *C. auris* outbreak was reported in India in the year 2013, followed by South Africa in

2014 and then different parts of the globe in subsequent years. Figure 1.3 shows the distribution of *C. auris* clades across the globe, and the phylogenic relationships between the clades (Rhodes & Fisher, 2019).

Although *C. auris* cases were first reported in the year 2009, there has been detection of earlier cases in cultural depositories that date as far back as 1996. In the year 2018, all the six continents that are inhabited had reported cases of *C. auris*. This pathogen causes hospital-related outbreaks, and in the year 2019 *C. auris* had been detected in almost a 100 hospitals in South Africa, resulting in large outbreaks in some of the facilities. This pathogen now accounts for 10% (1 in 10) of candidaemia cases. Hospital settings have been found to be the main reservoirs for *C. auris* infections, and the reasons for its dramatic spread in these settings are not very clear. This may, however, be owing to some of the intrinsic properties of this pathogen, such as the ability to form biofilms, the ability to adhere to surfaces, the ability to survive outside of the host for notable periods of time, and resistance to chemical disinfectants used in these facilities (Govender et al., 2019).

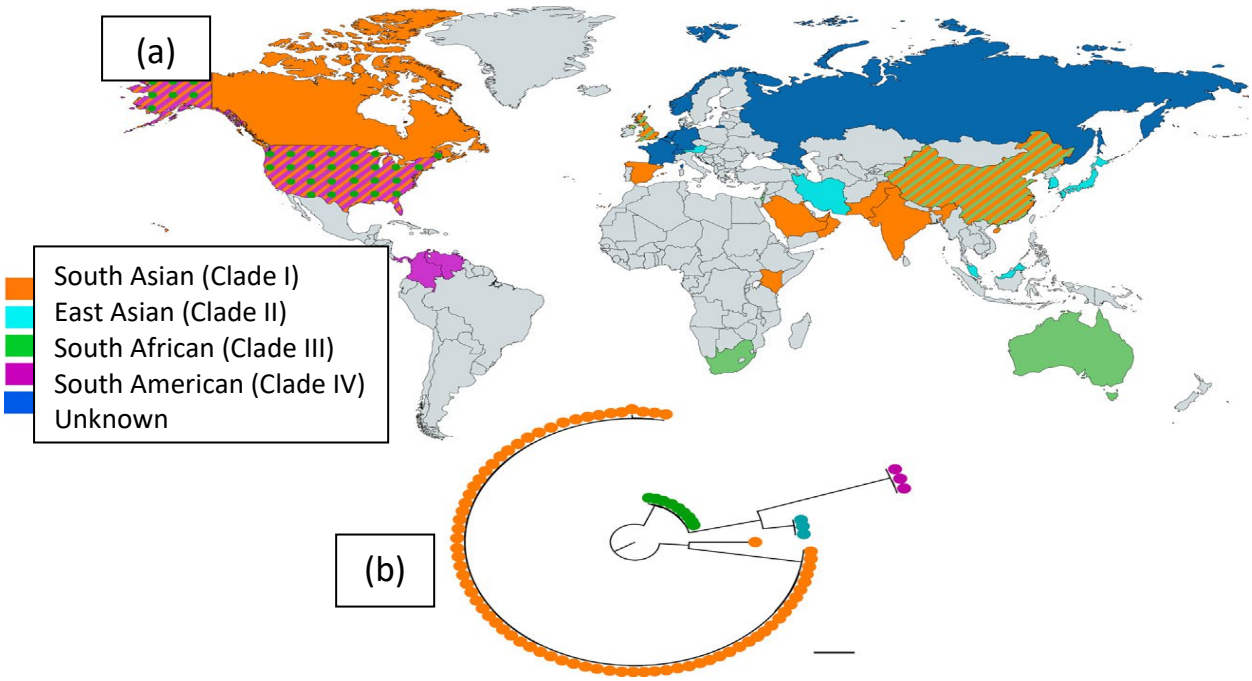


Figure 1.3: (a) The distribution of *C. auris* clades across the globe as of February 2019 (b) RaxML phylogeny indicating the relationship among the *C. auris* clades (Rhodes & Fisher, 2019).

1.2.2. Infections

Candida auris colonization has been detected at several body sites, which include the rectum, axilla and groin, and it has been detected 3 months after initial detection, even with use of certain antifungal agents. Risk of infection increases when one comes into contact with patients who harbour *C. auris* or their environment, and the contact time required for one to contract *C. auris* is only 4 hours, while invasive infections have been found to occur within only 48 hours of intensive care unit (ICU) admission. Invasive *C. auris* cases have been highly associated with candidemia, and they include cases associated with respiratory and urinary tract infections, among others. The majority of the *C. auris* invasive infection cases, however, occur in critically ill patients, in particular those patients that have been admitted to intensive care facilities or undergone invasive procedures. These patients often have serious underlying medical conditions which may often result in immunosuppression (Sarma et al., 2013).

As may be expected, the majority of patients diagnosed with invasive *C. auris* infections have often received broad spectrum antimicrobial agents or antifungal agents prior to invasive candidiasis development (Lee et al., 2011). However, owing to its multidrug resistance, the pathogen survives. These infections have also been associated with certain medical devices, such as urethral catheters and central venous catheters (CVCs). In addition, *C. auris*-associated deaths have been shown to differ with respect to geographical regions: The 30 day mortality rates have been found to be over 50% for those with invasive *C auris* infections in the United States, Asia and the Far East, while in Colombia it was found to be 35.2% (Jeffery-Smith et al., 2018). Ways have been implemented to try and curb the spread and infection rates of *C. auris* in hospital settings, and these recommendations include isolating patients, cleaning equipment and environments that have come into contact with infected persons and also observing contact precautions. Since not much is known about *C. auris,* these recommendations are all empirical, as they were deduced based on studies of other well-known resistant fungi (Jeffery-Smith et al., 2018).

Candida auris persistently colonises different materials and equipment in hospital environments along with multiple body-sites of patients, thus increasing the transmission of this pathogen and so leading to the occurrence of outbreaks (de Jong & Hagen, 2019). For example, there was an occurrence of a very large outbreak in a London Cardio-thoracic Centre between April 2015 and July 2016, consisting of 50 *C. auris* cases. It was discovered that these outbreaks were due to the persistent presence of *C. auris* around the bed space areas in the

centre. One of the healthcare workers from the London Cardio-thoracic Centre who carted for a *C. auris* infected patient also tested positive for the pathogen. Similarly, an earlier report of 7 cases that had happened in the United States of America between May 2013 and August 2016 indicated that *C. auris* has the ability to colonise and remain viable on the skin and other body sites of a patient after weeks or months of initial infection, which in turn can lead to further contamination of the hospital environment; thus creating a chain of infections (Chowdhary et, al*.,* 2017).

1.3. Pathogenicity of *C. auris*

1.3.1. Adherence

The emergence of *C. auris* has posed a great challenge in clinical settings in terms of controlling nocosomal infections. This pathogen is transmittable from one patient to the other, and it is resistant to a notable number of antifungal agents and disinfectants. The high prevalence of *C. auris* infections and the high mortality rates caused by this pathogen are associated with its virulence factors. These virulence factors include multi-resistance to the available antifungal agents and disinfectants, biofilm formation, phospholipase and proteinase production, as well as the ability to adhere to biotic and abiotic surfaces. Even though *C. auris* produces less virulence factors in comparison with *C. albicans,* it has been found to be highly virulent and much more virulent than *C. albicans* (de Cässia Orlandi Sardi et al., 2018).

Virulence factors that are associated with *C. auris* infections are not understood. *C. albicans,* for example, makes use of surface adhesion proteins like hyphal wall protein 1 (Hwp1), agglutin-like sequence proteins and GPI-anchored protein, among others, to attach to the host cell. Genome comparison has however indicated that *C. auris* is able to adapt to a number of environments and has many pathogenic mechanisms that are present in *C. albicans* and other *Candida* species. The identified *C. auris* attributes that aid with adhesion include pathways that are required for cell wall modelling, production of hydrolytic enzymes and lytic enzymes, and several orthologs such as orthologs of agglutinin that mediate attachment to host cells (Ahmad & Alfouzan., 2021).

1.3.2. Hydrolytic enzymes

The *C. auris* complete genome was only recently investigated and thus the roles of different genes in the virulence and pathogenicity of this pathogen are not yet fully understood. This is because the *C. auris* genome expresses a number of proteins that are uncharacterized; thus it is

not very clear whether these proteins promote the pathogenicity of this pathogen or not. When the *C. auris* genome is compared with that of *C. albicans,* which has been well studied and characterised, it may be suggested that the *C. auris* genome contains genes that have been well annotated as virulence factors in other *Candida*. These include genes for the formation of biofilms, adhesins, lipases, proteinases, secreted aspartyl proteases, phopholipases and transporters that belong to the ABC and major facilitator superfamily (Multidrug-resistance transcription factors) which are involved in antifungal drug resistance (Rossato & Colombo, 2018). The yeast was discovered to produce a number of enzymes that assist it in invading a human host, which include transferases, oxidoreductases and hydrolases.

In order to understand the pathogenicity and virulence of this pathogen, a genomic study was carried out by de Cässia Orlandi Sardi et al., (2018) using *C. albicans* as a reference. This study found 8 oligopeptide transporters which were related to the versatility of this pathogen in the acquisition of nutrients, thus allowing *C. auris* the ability to adapt to a number of different environments. This showed that the presence of these transporters is key when it comes to establishing an infection. Enzymes that are involved in the invasion of host cells were also uncovered, and these include lipases, mannosyl transferases and aspartyl-secreted proteases. These enzymes allow the invasiveness of *C. auris* and so are key in the establishment of an infection and thus the development of fungaemia. In addition, the study revealed 686 proteins that are involved in the formation of biofilms, along with orthologous transporters that function as efflux pumps and thus may be involved in the intrinsic resistance of *C. auris* to antifungal agents. Another study that analyzed *C. auris* isolates from a vaginal sample led to the demonstration of proteinase, phospholipase and hemolysin activity, thus putting the emphasis in the ability of *C. auris* to adhere to and invade host cells.

1.3.3. Aggregation

When *in vitro* growth of *C. auris* was observed, it was found that at times *C. auris* does not release its daughter cells after budding; as a result forming a large aggregation of cells that have a high physical resistance. This then gives *C. auris* resistance, both on environmental surfaces and on body tissue. It was uncovered however that the non-aggregated cells of the pathogen show greater pathogenicity as compared to the aggregated version, and these non-aggregated cells are able to form much stronger biofilms as compared to their aggregated counter parts (de Cässia Orlandi Sardi et al., 2018). In addition, it was discovered that the "aggregate" phenotypic character of the isolates was maintained even in *vivo.* This suggested

that the aggregate form of the pathogens might be the mode used to invade the immune defense system or for persistence in tissue; however this is yet to be confirmed through further studies (Rossato & Colombo, 2018).

1.3.4. *C. auris* vs *C. haemulonii* pathogenesis

A study was carried out on a mouse model and this study was aimed at comparing the virulence of *C. auris* to that of *C. haemulonii* (Rossato & Colombo, 2018). The mice were immunosuppressed with cyclophosphamide (150 mg/kg intraperitoneally) and then inoculated with the pathogens and observed for 12 days. After 12 days, 100% of the mice that were inoculated with *C. haemulonii* had survived and showed no signs of being ill, which meant that the *C. haemulonii* isolates were non-virulent. On the other hand, the mice that were infected with *C. auris* died rapidly, with only 20% of them surviving after 5 days of inoculation. This shows that although these two species are similar, *C. auris* is much more virulent and pathogenic than *C. haemulonii* (Rossato & Colombo, 2018).

1.3.5. Immune response in *C. auris* infections

To test the immune response to *C. auris,* an *in vitro* study was carried out where the efficacy of the phagocytic response of neutrophils as a first line of defense was tested against a strain of *C. auris* that was collected from India. Neutrophils are the key leukocytes that act against invasive candidiasis. These leukocytes clear away the invading fungi by phagocytosis and by delivering antimicrobial contents during neutrophil extracellular trap (NET) formation. During this study, human neutrophils were found to have no effect against *C. auris*. The leukocytes did not engage with the fungus, nor phagocytose it, not release NET's. In comparison, the human neutrophils were able to engage with and kill *C. albicans,* and this can be observed in figure 1.4. An *in vivo* analysis of neutrophil response for both *C. auris* and *C. albicans* was also carried out in a zebra fish and the same scenario was observed, where the neutrophils killed *C. albicans* but not *C. auris*. Very little is known with regard to the mechanisms however it may explain why the *C. auris*-associated mortality rates remain high, including those in patients that receive antifungal treatment (Nett, 2019).

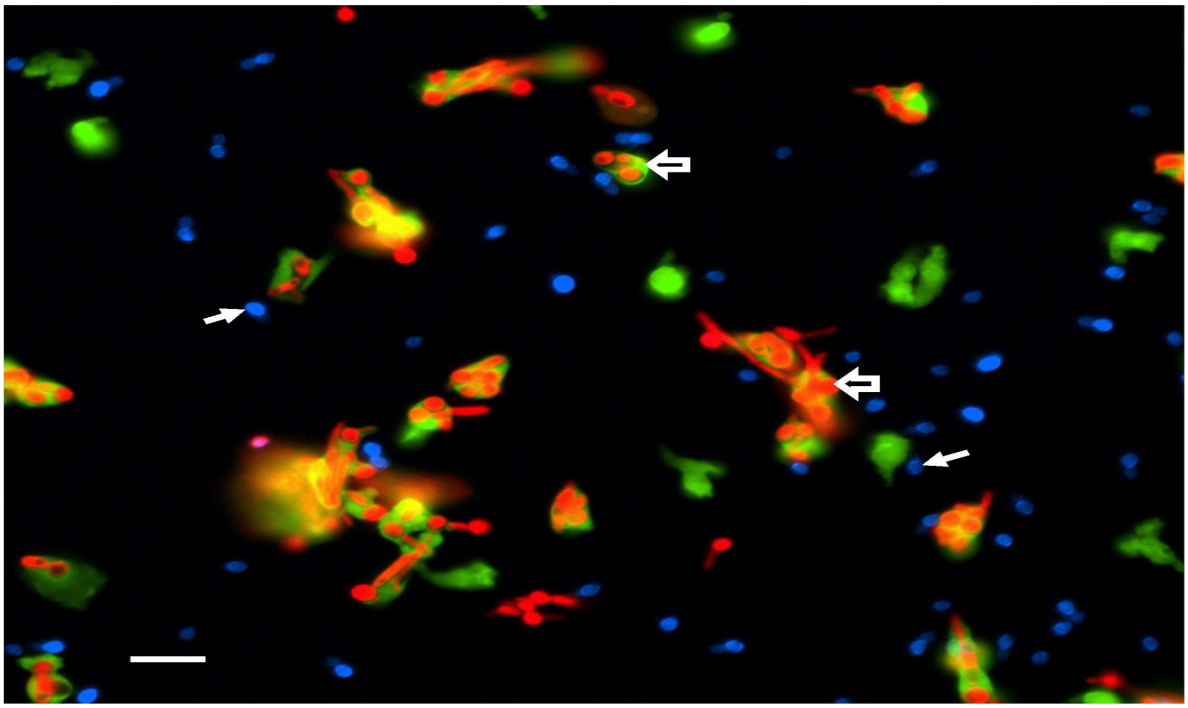


Figure 1.4: Human neutrophils engage with *C. albicans* and not *C. auris*. Calcein AM-labelled neutrophils (green) were co-cultured with calcofluor white-stained *C. auris* (blue) and red fluorescent protein-tagged *C. albicans* (red) for 30 minutes. The neutrophils preferentially engaged *C. albicans* while ignoring *C. auris*. Closed arrows show *C. auris* cells while open arrows point to the neutrophils phagocytosing *C. albicans*. Only a few neutrophils engage *C. auris* (Nett, 2019).

A study that was carried out by Kean et al (2020) showed that viable *C. auris,* tested against other *Candida* species, was unable to induce a significant inflammatory response in human peripheral blood mononuclear cells while the other species (*C. tropicalis, C. krusei* & *C. guilliermondii*) were able to induce a greater release of IL-6, IL1-β and TNF-α. In addition, it was discovered that human monocyte-derived macrophages do interact with and phagocytose *C. auris,* however not as efficiently as they do with other *Candida* species; thus showing that the host is able to recognize the pathogen but is not able to establish an effective immune response against it. This shows that *C. auris* makes use of sophisticated mechanisms in order to invade the host immune response and thus escape expulsion.

1.3.6. Hyphae formation

Previous studies have shown that *C. auris* do not form hyphae, pseudohyphae or chlamydospores, but rather they form pseudohyphae-like phenotypic structures under high salt stress and in biofilm communities. These forms of growth are characterized by rudimentary growth, whereby the *C. auris* cells have an elongated shape and undergo an incomplete cell

division (Rossato & Colombo, 2018). Recent studies however has shown that *C. auris* isolates are able to form true hyphae under certain conditions. For example, a study has shown that *C. auris* grown on yeast extract peptone dextrose (YPD) medium containing 10% NaCl, formed elongated/hyphae like cells (Ahmad & Alfouzan, 2021).

Some of the yeast isolates grow into clumps, whereby they do bud off, but the daughter cells are not released, thus forming cell aggregates. The ability of *C. auris* to form aggregates was shown to be an inducible trait that is stimulated by exposure to echinocandins or triazoles. The aggregated forms of the yeast have been found to have a higher capability of forming biofilms and are able to persist more in tissues as compared to non-aggregated phenotypes (Ahmad & Alfouzan., 2021).

1.4. Environmental survival of *C. auris*

1.4.1. Abiotic Surfaces

Studies of *C. auris* have indicated that environmental surfaces in the hospital facilities are actually the source of transmission of this fungus. This is because during multiple tests, the yeast was discovered on the environmental surfaces and was often found to be still viable. The yeast has been found on several different surfaces, including plastic, hospital beds and covers, gloves and even hospital equipment, and it has been found on both moist and dry surfaces. In a study done to compare the survival rate of 8 strains of *C. auris* with that of 3 strains of each of the following; *C. parapsilosis, C. glabrata*, and *C. albicans,* over a period of 7 days, it was discovered that all 8 of the *C. auris* strains survived the 7 days on both the dry and moist environments. In addition, *C. auris* showed a greater ability to survive on both the dry and wet surfaces, as compared to *C. albicans;* however the other *Candida* species (*C. parapsilosis* and *C. glabrata*) showed a greater propensity to survive than *C. auris*. These results thus prove that the environmental surfaces are potential reservoirs for the spread of *C. auris* and other *Candida* species (Piedrahita et al., 2017).

Another study was conducted by Welsh et al (2017) to investigate the ability of *C. auris* to persist and survive on plastic surfaces that are common in health care facilities; under controlled conditions, mimicking those found in health care facilities. The survival of *C. auris* was compared to that of *C. parapsilosis.* They inoculated 1cm^2 plastic coupons with *C. auris* and *C. parapsilosis* and at 1, 2, 4, 7, 14, 27 & 28 day intervals cultured the coupons. Through colony counts they found that *C. auris* survived for up to 14 days while *C. parapsilosis* remained viable for up to 28 days. This study thus shown that *C. auris* is able to survive and

persist outside of the host for a long time period. This has made it necessary for other environmental materials to be investigated as well, since this shows that plastic surfaces and possibly other surface materials in hospital facilities are the potential sources of *C, auris* transmission (Piedrahita et al., 2017).

1.4.2. Biofilm Formation

Candida auris also exhibits a resilient biofilm characteristic, which increases its chance of survival on the environmental surfaces. In addition, these biofilm-forming strains can be resistant to disinfection (Short et al., 2019). This pathogen has been reported to be able to tolerate a number of stresses including high temperature and salt concentration, and so this tolerance or ability to persist through stressors further allows the survival of *C. auris* both on the environmental surface and in the host. *C. auris* has also been found to be resistant to some chemical disinfectants used to disinfect environmental surfaces; while other factors like time of exposure to the disinfectant and type of environmental surface also play a key role in the environmental survival of *C. auris* and resistance to environmental disinfectants (Kean et al., 2018). Further research is needed in this area in order to determine the type (s) of environmental surfaces (s) on which *C. auris* is more persistent with respect to the form of *C. auris* i.e. whether planktonic or biofilm, and the type (s) of disinfectant needed to disinfect these surfaces.

A study conducted by Horton et al (2020) compared biofilm formation between *C. auris* and *C. albicans* on the skin niche conditions, and this was done by inoculating an artificial human axillary sweat medium with 10^6 cells/ml of *C. auris* and *C. albican* cells for comparison. These were incubated for 24 hours at 37 ^{o}C and the biofilm burdens were measured by absorbance at 600nm. They found that *C. auris* produced 10-fold more biofilm burden than *C. albicans*. They then continued to compare the biofilm formation between the two on porcine skin using pig skin. This was done because pig skin shares a number of similarities with human skin, which include thickness of layers, mechanisms of skin repair and the types and distribution of skin cells. The skin was placed in semi-solid medium to supply the dermis with nutrients. The skin was then inoculated with 10^7 cells/ml of the pathogens and incubated for 24 hours at 37 ^{o}C. This study uncovered that *C. auris* forms biofilms of about 15-fold more burden than *C. albicans* does on porcine skin. This study also found that *C. auris* biofilms can grow much more on "dry" skin than *C. albicans* can; such that after placing the skin for 24 hours in a laminar flow for desiccation, *C. auris* biofilms grew 30-fold more than *C. albicans* biofilms 7

days post-desiccation, and *C. auris* biofilms remained viable after 14 days post-desiccation, while *C. albicans* cells did not (Horton et al., 2020).

1.5. Disinfection and *C. auris*

1.5.1. Importance of infection control in hospital

According to reports from several outbreaks studied, *C. auris* is transmitted through hospital environments. Disinfection of these environments is thus of key importance since prevention is better than cure, and so curbing transmission begins in hospital environments. Several recommendations have been made in order to curb transmission, which include isolation of *C. auris*-infected patients, cleaning equipment and environments that have been in contact with infected patients, and contact precautions. In one unit, certain precautions were put in place in order to reduce *C. auris* transmission. These included decolonizing patients by making them use chlorhexidine mouthwashes, chlorhexidine gluconate body washes and hand washes, along with chlorhexidine impregnated pads for CVC exit sites (Vallabhaneni et al., 2016). *C. auris* as a pathogen has been discovered to be resistant to a number of antifungal agents and disinfectants. This makes it very difficult effectively to disinfect *C. auris*-contaminated environments and surfaces, or colonised hosts (Cadnum et al., 2017).

The earliest case of *C. auris* infection in South Africa was reported in 2009. This pathogen however was misidentified for *C. haemulonii,* and was only retrospectively confirmed in 2014. Since then, *C. auris* cases have been detected in many South African hospitals, resulting in large outbreaks at some facilities. From October 2012 to November 2016, 1692 confirmed or probable cases of *C. auris* were reported in both private and public hospitals and 92% of these were reported from Gauteng. Only 647 of these cases were confirmed to be of *C. haemulonii* (not of *C. auris*), while the other 1045 cases were confirmed to be those of *C. auris*. Of the 1045 *C. auris* cases, only 114 of them were reported from public sectors, while the remaining 931 cases were from private hospitals (Govender et al., 2018).

While only 1692 probable *C. auris* cases were reported from 2012-2016 (4 years), a study conducted by van-Schalkwyk et al (2019) of the cases reported between 01 January 2016- 31 December 2017 (2 years) showed that 6669 probable or confirmed cases of *C. auris* were reported in both private and public South African health care facilities. Of these, 2529 cases were reported in the public sector, while 4140 were reported in the private sector. Of the 6669 reported cases, only 794 were confirmed to be due to *C. auris,* while the other 5876 cases were due to other *Candida* species. These reports occurred mostly (86%) in hospitals in Gauteng

province, and this is most probably due to the fact that this province is the most densely populated in the country and so there is an ongoing clustering of health-care facilities. In addition, most of the cases were reported in private sector facilities (88%) as compared to public sector facilities (van-Schalkwyk et al., 2019). This thus makes it necessary for disinfection of these facilities, particularly in Gauteng province, to be intensified, in order to reduce *C. auris* infections while continuing to ensure that infections in the public sector do not increase.

Knowing which disinfectants are effective against *C. auris* allows for the decontamination of contaminated environments, thus curbing *C. auris* infections. As a matter of fact, the environments of patients who are colonized by *C. auris* must be decontaminated after every bodily discharge. It has been found that decontaminating these environments with high concentration chlorine solutions combined with hydrogen peroxide vapour or UV light, is effective in eliminating *C. auris* from them. It has also been highlighted that every patient contact item must be thoroughly decontaminated before and after every use. Examples of these include temperature probes, and pulse oximeter probes, and it has been advised that in communal hospital settings patients that are known to be colonized with *C. auris* must not share facilities with those that are immunosuppressed (Vallabhaneni et al., 2016). While it is of the utmost importance to disinfect the environment and surfaces that may harbour *C. auris,* it is also equally important to decolonize patient bodies along with those of health practitioners to reduce the risk of infection and transmission of the pathogen. Table 1.1 shows a number of antiseptics that have been tested against *C. auris* for use in decolonising patients, health practitioners and anyone who may be exposed to and be colonized by *C. auris* (Ku et al., 2018).

Table 1.1: Antiseptics tested for effectiveness against *Candida auris* (Ku *et al.,* 2018).

Disinfectant	**Concentrations tested (contact time in minutes used)**	**Effective**	**Level of Evidence**
Chlorhexidine gluconate	<0.02% (1440), 0.5% (0.5), 2% (2), 4% (3, 180, 1800)	Yes	Good
Chlorhexidine gluconate in isopropyl alcohol	2%/70% (2)	Yes	Low
Povidone-iodine	10% (2, 3, 180, 1800)	Yes	Moderate
Alcohol	70%	Yes	Low

1.5.2. Disinfectants

The efficacy of different germicides on *C. auris* has been studied over time and it has been found that this yeast is resistant to a number of disinfectants and that its biofilm form is much more resistant as compared to its planktonic form (Short et al., 2019, Ledwoch and Maillard, 2018). Ku et al. (2018) tested for the ability of a number of disinfectants to inhibit the survival of *Candida* planktonic cell species (Table 1.2). This study showed that quaternary ammonium detergent and phenol-based disinfectants are effective at inhibiting the growth of these cells and they are effective on all *Candida* species. This study was, however, limited since it was only conducted with planktonic cells and not on biofilms. On the other hand, Chlorine-based disinfectants such as sodium dichloroisocyanurate and sodium hypochlorite (NaOCl) were found to be quite effective against *C. auris*. Other disinfectants that were found to be effective in inhibiting *C. auris* growth include hydrogen peroxide vapour, hydrogen peroxide with silver nitrate, Ethyl alcohol, glutaraldehyde, peracetic acid, and peracetic acid with hydrogen peroxide and acetic acid. Of all the above-mentioned disinfectants, chlorine based disinfectants have been found to be the most effective against *C. auris* (Ku et al., 2018). Moore et al., (2017) has also suggested that the use of chlorine-based and iodine-based disinfectants are more effective in reducing environmental and skin contamination.

Table 1.2: Surface disinfectants tested for effectiveness against *Candida auris* (Ku et al., 2018)

Disinfectant	**Concentrations tested (contact time in minutes)**	**Effective**	**Level of evidence**
Chlorine	0.39% (1), 0.65% (1), 0.825% (1), 1% (10), 2% (10), 1000 ppm (3, 5, 180, 1800), 10000 ppm (3, 180, 1800	Yes	Good
Hydrogen peroxide	8 g/m3 (?), 1.4% (1)	Yes	Moderate
Hydrogen peroxide + silver nitrate	11% (60)	Yes	Low
Phenolics	5% (60)	Yes	Low
Glutaraldehyde	2% (20)	Yes	Low
Alcohols	29.4% (0.5)	Yes	Low
Peracetic acid	2000 ppm (5, 10)	Yes	Low
Peracetic acid + hydrogen peroxide + acetic acid	1200 ppm/<1% (3)	Yes	Low
Quaternary Ammonium compounds	2% didecyldimethyl ammonium chloride (60), alkyl dimethyl ammonium chlorides (10), didecyldimethyl ammonium	No	Low

	chloride/dimethylbenzyl ammonium chloride (10		
Acetic acid	>5% (3)	No	Low

While quaternary ammonium compound has been found to be ineffective against *C. auris*, those disinfectants that are effective in inhibiting *C. auris* growth are dependent on concentration and contact time to effectively inhibit the yeast (Ku et al., 2018). For example, 5% phenol and 2% glutaraldehyde were found to be effective against *C. auris* only when the contact period was 60 and 20 minutes, respectively. This means that at a lesser contact time or concentration the yeast could still be viable and maintain a degree of resistance to the disinfectant. Other disinfectants like Ethyl alcohol, for example, have been found to be able to inhibit *C. auris* growth to a lesser degree as compared to other disinfectants such as chlorine-based disinfectants. This shows that in a population of yeast, some of the cells may have traits that render them resistant to Ethyl alcohol. On the other hand, quaternary ammonium compounds as well as acetic acid were found to be completely ineffective against *C. auris;* thus showing that the yeast is resistant to these disinfectants. Similarly, it has been shown that *C. auris* selectively tolerates clinically relevant concentrations of disinfectants on environmental surfaces (Kean et al., 2018).

1.5.3. Resistance to disinfectants and antifungal agents

C. auris as a pathogen has been discovered to be resistant to a number of antifungal agents and disinfectants. Since this yeast has been found to be transmitted largely by hospital surfaces, The Centers for Disease Control and Prevention (CDC) have recommended that hospital facilities must disinfect rooms that have *C. auris* patients on a daily basis and after every type of bodily discharge. In 2017, Cadnum et al., (2017) conducted a study to test for the efficacy of 9 commercial disinfectants along with a common household disinfectant which is distilled white vinegar (>5% acetic acid, pH 2.0), against *C. auris, C. glabrata* and *C. albicans*. Five previously described strains of *C. auris* were tested, 3 of which were multidrug-resistant isolates and 2 of which were drug-susceptible. Nonsporicidal-improved hydrogen peroxide disinfectants and quaternary ammonium disinfectants were tested for effectiveness in the study, and it was uncovered that the hydrogen peroxide disinfectants were highly active against *C. auris* and the other *Candida* species; however, the quaternary ammonium disinfectants which are more widely used had a poor activity against all the *Candida* species, including *C. auris*.

C. auris has shown extensive resistance to both azoles and amphotericin B. The Molecular mechanisms that have been found to be responsible for antifungal resistance in *C. auris* include efflux pumps and mutations in the lanosterol 14-alpha demethylase (*ERG11*) gene, particularly for resistance towards fluconazole. The combination of echinocandins and voriconazole has been shown to be promising against *C. auris* in vitro. Other promising antifungals are also under development. However, the most practical way to reduce *C. auris* infections is by cleaning and decontaminating surfaces and rooms that have been in contact with *C. auris* colonized patients with disinfectants, and to use skin antiseptics for colonized patients (Arikan-Akdagli et al., 2018).

1.6. Ergosterols

The azole antifungal class has been the most utilized antifungal agent against *candida* species. This is owing to their efficacy against these species and their limited side effects. Azoles kill the fungal cells by targeting the *ERG11* gene end products, ergosterols. Ergosterols (which are important for maintaining the fluidity of the fungal cell membranes) are synthesized by demethylation of another sterol known as lanosterol. The demethylation process of lanosterol is mediated by an enzyme encoded for by the *ERG11* gene. When administered to a patient, azoles go into the fungal cells and bind to the Erg11 protein (enzyme) in the same binding pocket where the lanosterols normally bind. This inhibits lanosterols from binding, inhibiting lanosterols methylation and thus inhibiting ergosterol synthesis. This then results in the accumulation of toxic methylated sterol precursors, which are harmful to the fungi. Even though these antifungal agents are effective against *Candida* species, a repeated and lengthy use may trigger azole resistance in the treated isolates, leading to treatment failure. Studies have uncovered that those mutations in the *ERG11* gene can lead to azole resistance by causing an alteration in the Erg11 protein and so altering the ability of the azoles to bind to the protein to inhibit its function (Flowers et al., 2015).

Resistance to azoles in *C. albicans* is due to 4 known mechanisms. First mechanism is transcriptional regulation or mutations in the ergosterol biosynthetic pathway, including the up-regulation or mutations of the *ERG11* gene whose product is the direct target for azoles. Second mechanism is by the overexpression of transmembrane efflux pump proteins which can cause reduction in the cellular drug concentration. Third mechanism is cellular stress responses through changes in metabolic pathways and finally through the formation of biofilms consisting of an extracellular exopolymeric matrix, yeast cells, hyphae and pseudohyphae

(Quan-zhen et al., 2016). Most of the drug-resistant isolates have some alterations in the content and composition of membrane sterols.

Not much is known about the effect of disinfectants and the long term exposure of low concentrations of disinfectant in *C. albicans*. Presumably, this organism would develop resistance to even disinfectants through one of these drug resistance mechanisms. It is known that the mutations or disruptions in *ERG* genes lead to increased or reduced sensitivity to the antifungal agents. Mutations in this pathway, causing alterations in enzymes activity or drug binding sites, are major factors which are related with azole resistance. Mutations in the *ERG11* and *ERG3* genes or the UPC2 gene which encodes a transcription factor contribute to azole resistance. Mutations in *ERG11* are not limited to the point mutations, as the clinical isolates with 2 extra copies of Chromosome 5L, as an isochromosome, conferred increased fluconazole resistance (Quan-zhen et al., 2016). Although this mechanism of resistance towards biocide in *C. auris* is not known, the effect of antimicrobials/biocides on ergosterol biosynthesis can be studied through the measurement of quantity of ergosterol and the expression of *ERG11* gene in tolerant/resistant (drug/biocide) *C. auris*.

1.7.Rationale for the study

The increasing number of cases across continents continue to raise an alarm and awareness of the significance of *C. auris* as a pathogen and the danger it poses to human health. This pathogen has indicated its ability to survive for long periods outside of their host and this ability has been shown to relate to the high number of outbreaks caused by this pathogen. This study aims to investigate the ability of *C. auris* to survive on different environmental materials outside of host and the possible genetic responses that may result from exposure to different disinfectants that may be responsible for this persistence.

1.8. Aim: The aim of this study was to investigate the survival of *Candida auris* on various environmental surfaces under different conditions and a possible development of resistance to repeated use of disinfectants.

1.9. Objectives

- To assess the survival of planktonic *C. auris* onto plastic, stainless steel, glass, wood and fabric
- To assess the survival of biofilm form of *C. auris* onto plastic, stainless steel, glass, wood and fabric

- To examine the effect of drying on the survival of *C. auris*
- To study the effect of suboptimum concentrations of disinfectants on the survival and development of resistance through full genome sequencing
- To study the ergosterol biosynthesis in disinfectant resistant *C. albicans* isolates using ergosterol assay and *ERG11* gene expression.

1.10. Study layout

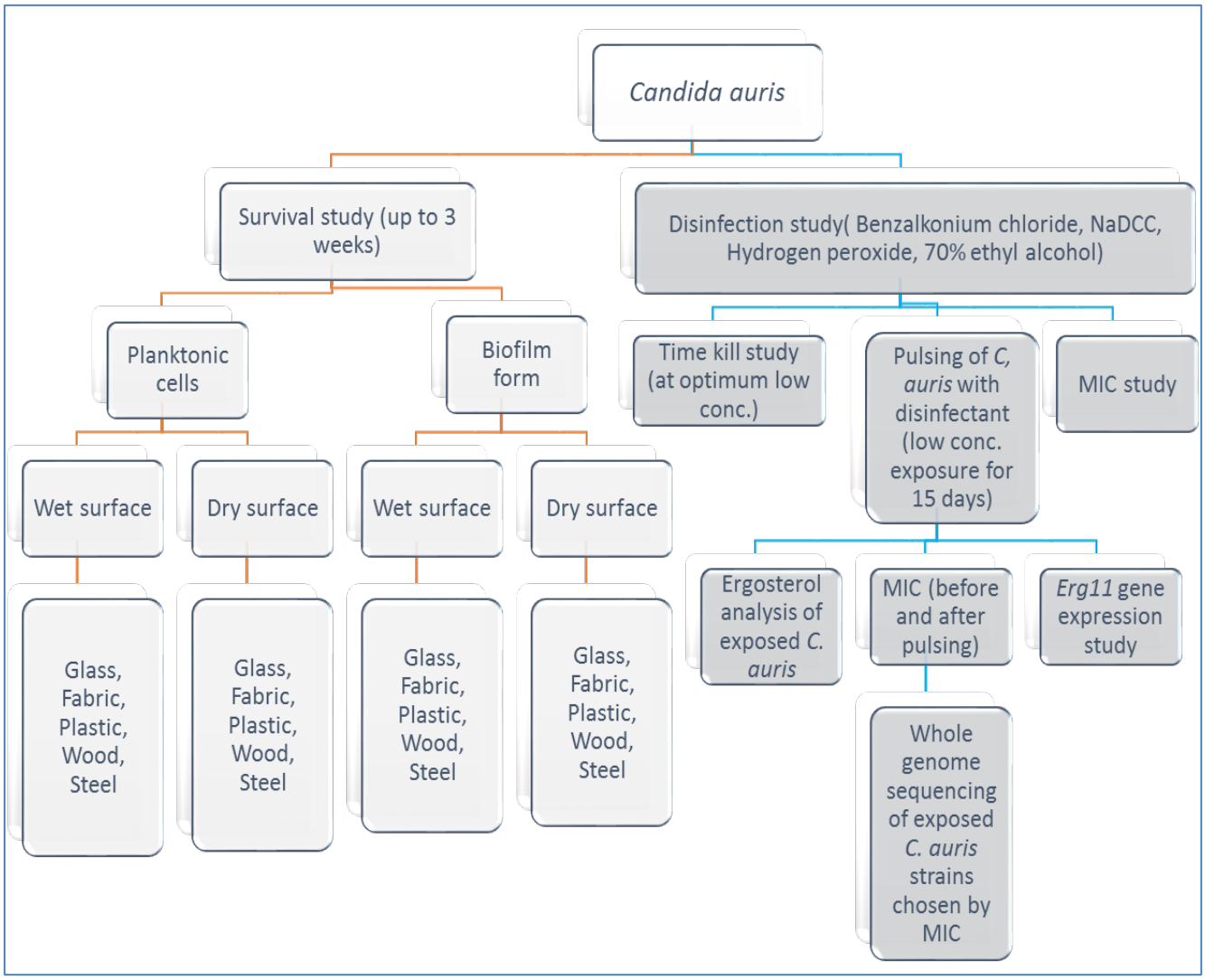


Figure 1.5: Flow diagram showing the study layout that was used to achieve these objectives.

CHAPTER 2 METHODS AND MATERIALS

2. Methods and materials

An Ethics waiver was obtained from the Human Research Ethics Committee (HREC), University of the Witwatersrand for the use of isolates and the study (Appendix 1).

All the chemicals purchased were of an analytical grade. Preparation of media and reagents are described in Appendix 2.

2.1. *C. auris* strains

Three (3) different strains of *C. auris,* namely Cau4888, Cau3499 and Cau6326 and a single *Candida albicans* strain (Cal5339), a reference strain were used in the study. These clinical strains of *C. auris* were isolated under ethical clearance and they had been obtained from the division of Mycology, National Institute for Communicable Diseases, Johannesburg, South Africa. Cultures were stored in glycerol stock at -80^{0}C in the department of Clinical Microbiology and Infectious Disease. Cultures were then grown on Sabouraud dextrose agar (SDA) and incubated at 37°C for 48 hours in preparation for tests (fresh cultures were used for each test). An Ethics waiver was obtained from the HREC, Wits for the use of isolates (Appendix 1).

2.2. Disinfectants

For the disinfection study, four disinfectants were used, namely, **Ethanol (Eth), Hydrogen peroxide (HP), Sodium dichloroisocyanurate (NaDCC) and Benzalkonium chloride (BC)**. Absolute (100%) ethanol was obtained from the National Health Laboratory Service (NHLS) and was diluted with sterile water to obtain a 70% ethanol for the test. A 3% (10 volume) Hydrogen peroxide BP solution was purchased from Clicks (Pty) Ltd. And it consisted of 3% HP in purified water. Presept™ tablets were obtained from Johnson & Johnson (Pty) Ltd. And they contained 50% (2.5g) NaDCC (active ingredient- sodium dichloroisocyanurate) in an inert effervescent base. 95% BC was obtained from Merck Life Science (Pty) Ltd.

2.3. Survival of planktonic *C. auris* on wet and dry surfaces

2.3.1. Surface material samples and test conditions

The survival study was performed using a method described by Welsh et al., (2017) with modifications.

Five different types of surface materials were used to test for the survival of planktonic *C. auris.* They were plastic, glass, wood, fabric and stainless steel. Sixteen pieces (2 x 2 cm) of each of 39 the material squares were sterilized using 70% ethanol and a 2 hour exposure to UV light on each side. In addition all the other material squares, except for plastic were autoclaved. Thereafter they were placed in empty plastic specimen bottle (40 ml capacity). There were 32 squares, of which 16 were used for the wet,

and the other 16 for the dry experimental conditions. Simulation of the moist environmental surface was done by adding a drop of purified water daily to 16 of the inoculated test materials while the other 16 test materials for the simulation of a dry environmental surface were kept dry throughout the study period.

2.3.2. Preparation of Inoculum

Each surface material piece placed in an empty plastic specimen jar was inoculated with 100ul of $1x10^6$ cells/ml *C auris* cells. To prepare the inoculum, *C. auris* cell were grown on SDA and incubated at 37°C for 48 hours. A loopful of the fresh cultures were then taken and immersed in 15 ml of 0.9% saline solution to obtain a 0.5 McFarland ($\approx 10^8$ cfu/ml). Serial dilutions were performed, and aliquots were then plated on SDA and incubated for 48 hours at 37°C. Cell counts were done and these counts, along with dilution factors, were used to determine the dilution that contained a cell concentration of $1x10^6$ cells/ml, and it was found to be the fourth (10^{-4}) dilution of the inoculum. This dilution was used in all the experiments.

2.3.3. Test Procedure

After inoculating the surface materials with *C. auris* cells, the jars were kept at room temperature and at intervals of 24 hours, 48 hours, 72 hours, 96 hours, 120 hours, 1 week, 2 weeks and 3 weeks, 2 specimen jars for each material type (in duplicates), one with wet and one with a dry test material were processed for the viability of the cells. Swabs were used to scrape the surfaces of the materials. The swabs were first moistened with sterile Phosphate buffered saline (PBS), followed by sampling. Thereafter the swabs and the test material were vortexed for 1 minute in 2 ml of sterile PBS, and serially diluted aliquots were plated on SDA. Although at the beginning of the experiment planktonic cells were applied to the surfaces; during the study periods, the counts would include surviving planktonic cells as well as any adherent cells. This experiment represents freshly contaminated and unattended surfaces. It can be considered as survival during biofilm formation; however, for this study's purposes, it was

labelled as a planktonic experiment. Colony counts were then used to determine viable cells and percentage reduction was calculated using the initial counts at 0 hours. The tests were performed in duplicate for each of the three *C. auris* strains and one strain of *C. albicans.*

2.4. Survival of biofilm form of *C. auris* on wet and dry surfaces

2.4.1. Surface material samples and test conditions

Survival study was performed using a method described by Welsh et al., (2017) with modifications.

Described in section 2.3.1, the same test materials were used to study the survival of the fully formed biofilm of *C. auris.* They were decontaminated as described above. The simulation of the moist environmental surface was done by adding a drop of purified water daily to 16 of the bottles for each material, while the other 16 were there for the simulation of a dry environmental surface and were kept dry throughout the study period.

2.4.2. Preparation of inoculum

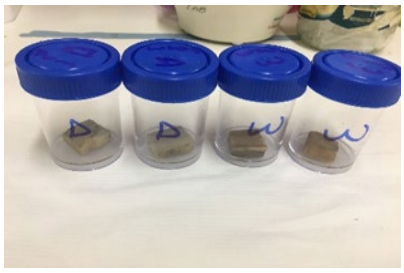
To prepare the inoculum, *C. auris* cells were grown on SDA and incubated at 37°C for 48 hours. A loopful of the fresh cultures was then taken and immersed in 15 ml of 0.9% saline solution to 0.5 McFarland ($\approx 10^8$ cfu/ml). Serial dilutions were performed, and aliquots were plated on SDA and incubated for 48 hours at 37°C. Colony counts were then used to determine viable cells and percentage reduction was calculated using the initial counts at 0 hours. The tests were performed in duplicate for each of the three *C. auris* strains and one strain of *C. albicans.*

2.4.3. Biofilm formation

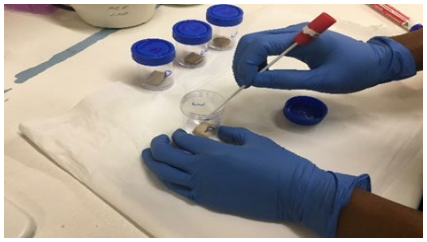
Each piece of material was then placed into a specimen jar and inoculated with 100µl of $1x10^6$ cells/ml *C. auris* cells and incubated for 2 hours at 37ºC. The pieces were then rinsed using sterile PBS to remove unattached cells (adhesion phase) and again incubated for 48 hours (biofilm development phase) with 10 µl of SD broth to encourage the biofilm formation. Thereafter, pieces were rinsed with sterile PBS to remove media and unattached cells. This was considered as day 0. This experiment was considered as survival of fully formed biofilm, representing long standing contaminated surfaces.

2.4.4. Test procedure

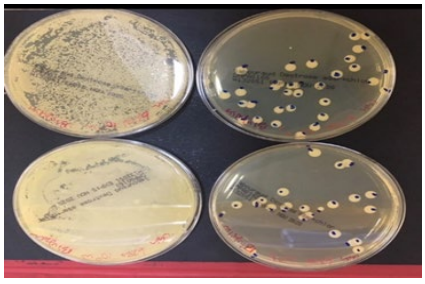
The sample jars were then kept at room temperature; and at intervals of 24 hours, 48 hours, 72 hours, 96 hours, 120 hours, 1 week, 2 weeks and 3 weeks; 2 jars for each material type, one with a wet and one with a dry surface, were processed for the viable counts of the cells. Swabs were used to scrape the surfaces of the materials. The swabs were first moistened with sterile PBS, followed by sampling. Thereafter the swabs and the test material were vortexed for 1 minute in 2 ml of sterile PBS, and serially diluted aliquots were plated on sabouraud dextrose agar. Colony counts were then used to determine viable counts of the cells. The tests were performed in duplicate for each of the three *C. auris* strains and one strain of *C. albicans*. Figure 2.1 is a simple representation of the procedure.



A. Inoculation



B. Harvesting and Culturing



C. Colony Counts

Figure 2.1: A visual representation of the test procedure carried out to determine the survival of *C. auris* cells on wet and dry environmental surfaces, with **A** being the inoculation step where the surface materials inside specimen jars were inoculated with the cells, **B** represents the harvesting of cells from the surface materials at given time intervals, and **C** shows the colony counts used to determine viability of the cells.

2.5. Disinfection study

2.5.1. Minimum Inhibitory Concentration (MIC)

Hydrogen Peroxide (HP), Sodium dichloroisocyanurate (NaDCC) (1000 parts per million), 70% Ethanol (Eth) and Benzalkonium chloride (BC) were assessed since they have been recommended by Govender et al., (2019) for use in South African hospitals. These are commercially available disinfectants with recommended concentrations for the specific purposes. The unit of measurement for each of the disinfectants are different as per recommendation. For the HP, Eth and BC the measured quantities were percentage (all supplied in a liquid form) and for the NaDCC it was ppm (supplied in a tablet form). For the chosen disinfectants the test was performed using planktonic *C. auris* cells. For each disinfectant an MIC was obtained using the micro-broth dilution technique (Patel et al., 2015). The assessment was carried out with two controls for each disinfectant (a positive and a negative control). Each of the wells except the controls consisted of a culture medium (Sabouraud dextrose broth), $1x10^6$ cells/ml *C. auris* cells and a certain concentration of the disinfectant. The positive control wells contained only the culture medium and the cell culture, while the negative control wells received the cell culture and the disinfectant. The well plates were then incubated at 37°C for 24 hours and this was followed by the use of a spectrophotometer in order to determine the difference in absorbance readings and thus determine the minimum dose of the disinfectants needed to inhibit *C. auris.* Wells were examined for visible growth (MIC) and the last concentration with no visible growth was recorded as an MIC. The test was done in duplicates.

2.5.2. Time kill study

The time kill study was performed using a method described by Cadnum et al (2017) with modifications. A 0.5 McFarland inoculum of each of the *C. auris* (Cau4888, Cau3499, and Cau6326) and *C. albicans* (Cal5339) strains were prepared in 0.9% sterile saline from cultures grown on SDA plates for 48 hours at 37°C. To simulate the presence of an organic material 100µl of horse serum was added to each inoculum. 100 µl of the inocula were then dispersed onto SDA plates that already contained 100µl of different concentrations of the disinfectants (HP, NaDCC, Ethanol and BC) at different concentrations. For each disinfectant, the MIC, along with one concentration below and one concentration above the MIC, were tested. Following disinfectant exposure, the disinfection reactions were stopped, using Dey-Engley neutralizing broth, at 30 second intervals for 5 minutes, with the initial reading being 0 seconds.

At each time interval, 100µl of the neutraliser was dispersed onto an agar plate, and the contents were then spread onto the plate and incubated at 37°C for 48 hours. After 48 hours colony counts were done in order to determine the extent of kill/disinfection. Chlorhexidine gluconate and SD broth were used as positive and negative controls, respectively.

2.5.3. Disinfection pulsing and second MIC

C. auris and *C. albicans* strains were grown in Sabouraud dextrose broth in 16 (sixteen) 15ml Eppendorf tubes ($1x10^6$ cells/ml) over a period of 15 days. The cells were kept viable in the tubes for 15 days with a constant change of media. Each of the strains was exposed to 10 ml of a specific disinfectant (Ethanol, NaDCC, BC or HP) at the obtained MIC concentrations for 1 hour on a daily basis. After 15 days, the cultures were spread on Sabouraud dextrose agar plates with different concentrations of the specific disinfectants, and incubated at 37◦C for 48 hours to investigate for resistance. Colonies (resistant colonies) were then selected from the agar plates and harvested; an MIC was performed to investigate the difference, if any, compared to the initial MIC readings obtained.

Based on the MIC results obtained before and after the strains were treated with disinfectants, two (2) disinfectants were chosen (i.e. NaDCC and BC) for further investigation (whole gene sequencing) for the Cau4888 *C. auris* strain.

2.5.4. ERG11 gene expression

2.5.4.1. RNA Extraction and cDNA Construction

An RNA extraction was done for *C. auris* (Cau4888, Cau3499, Cau6326) and *C. albicans* (Cal5339) using a Zymo-Research Miniprep RNA Extraction Kit supplied by Inqaba Biotec (Iqaba Biotechnical Indusries (Pty) Ltd). cDNA was then synthesized from the obtained RNA using a Bio-Rad iScript™ Cdna Extraction Kit.

2.5.4.2. Standard and real-time PCR

Standard PCR was performed on the obtained cDNA to study the expression of *ERG11* gene on each of the strains. The disinfectant-untreated strains were used as controls and actin encoding gene (*ACT*) was used as a reference gene. Real-time PCR was then performed, using a Roche LightCycler 480II Instrument to assess the level of *ERG11* gene expression for *C. auris* (Cau4888, Cau3499 and Cau6326) and *C. albicans* (Cal5339) treated with disinfectants, with a negative control being a sample without DNA in PCR reaction mix and *ACT* as a

reference gene. For PCR the amplification primers used for *Candida ERG11* were ERG11F-50-CGCTAAGCTTGCGGATGTTT-30 and ERG11R-50-ACTGGAGTGGTCAAGTGGG AAT-30 as obtained from literature (Chowdhary et al., 2018). This was followed by the performance of a protein assay to study the level of Ergosterol, which is the end product of *ERG11,* to see the expression differences between the treated and untreated *C. auris* strains.

2.5.4.3. Ergosterol extraction and quantification

Ergosterol extraction was performed on *C. auris* (Cau4888, Cau3499 and Cau6326) and *C. albicans* (Cal5339) strains as described by Ahmad et al., (2011) with modifications. The disinfectant-treated and untreated strains were grown in falcon tubes in 50 ml SDB for 24 hours. The cells were then harvested by centrifugation for 5 minutes at 2700 rpm and washed twice with sterile distilled water, followed by the determination of the net wet weight of the cell pellets. 3 ml of 25% alcoholic potassium hydroxide solution was prepared, containing 25 g KOH, 35 ml sterile distilled water, and brought to 100 ml with 100% ethanol and this was added to each of the pellets and mixed by vortexing for 1 min. The cell suspensions were then transferred to 16 × 100 mm sterile borosilicate glass screw-cap tubes and incubated in an 85 ºC water bath for 1 hour. Following incubation, the tubes were allowed to cool to room temperature. The sterols were then extracted by adding a mixture of 1 ml sterile distilled water and 3 ml n-heptane, followed by vigorous vortex mixing for 3 min. The heptane layer was then transferred to a clean borosilicate glass screw-cap tube. Prior to analysis, 1 ml aliquot of sterol extract was diluted three-fold in 100% ethanol and scanned spectrophotometrically between 240 and 300 nm. The presence of ergosterol and the late sterol intermediate 24(28) DHE in the extracted sample resulted in a characteristic four-peaked curve. The absence of detectable ergosterol in extracts was indicated by a flat line. Ergosterol content was calculated as a percentage of the wet weight of the cell, using the following equations:

$$\%\ \text{ergosterol} + \%\ 24(28)\text{DHE} = \frac{\left[\left(\frac{\text{A}281.5}{290}\right) \times\ \text{F}\right]}{\text{pellet weight}}$$

$$\%\ 24(28)\text{DHE}\ = \frac{[(\text{A}230/518)\ \times\ \text{F}]}{\text{pellet weight}}$$

$$\%\ \text{ergosterol}\ =\ [\%\ \text{ergosterol}\ +\ \%\ 24(28)\ \text{DHE}]\ -\ \%\ 24(28)\ \text{DHE}$$

Where F is the dilution factor in ethanol and 290 and 518 are the E values (in percentages per centimetre) determined for crystalline ergosterol and 24(28) DHE, respectively. Values were shown as mean ± SEM.

2.5.5. Genomic DNA Extraction and Whole-Genome Sequencing

Whole-genome sequencing (WGS) of the treated and untreated Can4888 strain was performed to determine the response of the genes to the disinfectant. Genomic DNA (gDNA) was extracted using the Roche High Pure PCR Template Preparation Kit following the manufacturer's instructions. The gDNA was quantified using a Nanodrop ND1000 170 spectrophotometer (Thermo Scientific, Waltham, USA) and sent to the National Institute for Communicable Diseases (NICD) sequencing core facility for WGS.

The resulting WGS raw reads were checked for quality, trimmed using Trim Galore v0.6.2 (https://github.com/FelixKrueger/TrimGalore). Aa *de novo* assembly into contigs of the untreated Can4888 was performed using the CLC Genomics Workbench and annotated using National Centre for Biotechnology Information (NCBI) prokaryotic genome annotation pipeline and Rapid Annotations using Subsystems Technology (RAST) 2.0 server. The BC and NaDCC treated Can4888 reads were then mapped to the assembled genome on the untreated Can4888 using the CLC Genomics Workbench. The assembly of the reference genome was performed using the Jekesa pipeline (https://github.com/stanikae/jekesa).The putative SNPs between the treated and untreated Can4888 assembled genomes were identified using SNIPPY (https://github.com/tseemann/snippy). In addition further annotations for the assemblies were done using KBASE (https://www.kbase.us/) in which the eukaryotic rerences and Prokka annotation tool was applied to the assemblies.The putative SNPs were filtered based on sequencing depth, type of variation, and the location of the SNPs in potential protein-coding regions on the organism was valuated. Kbase (https://www.kbase.us/) was used to determine the coordinates (start and stop positions of genes annotated on the Untreated Can4888) as well as to determine whether the SNPs are located within a predicted gene region.

2.6. Statistical analysis

Statistical analyses of all the data were performed using Stata and GraphPad Prism software. In the survival study, survival at different time intervals; survival in planktonic and biofilm; survival on wet and dry surfaces; and on different surfaces were compared using student t- test. In the disinfectant study, results were expressed using descriptive analysis such as mean and median.

CHAPTER 3 RESULTS

3. Results

3.1. Survival of planktonic form of *C. auris* on the wet and dry surfaces

3.1.1 *C. auris* counts on various surfaces

Survival of *C. auris* on various surfaces in the presence of wet and dry conditions was studied. Results are shown in table 3.1 to table 3.16

3.1.1.1 Glass surface

Results of the survival on the **glass surfaces** are shown in table 3.1 and figure 3.1. In addition, percentage reduction was calculated. The results showed that the counts of *C. auris* fluctuated over 3 weeks regardless of the wet and dry condition. A paired samples t-test was conducted to compare the counts at 0 hours to those at 1 week, 2 weeks and 3 weeks separately for the wet and dry surfaces. The reduction in the counts was significant only at 2 weeks and thereafter (*p* value – 0.007). Whereas in the dry condition the percentage reduction was significant at 1 week and thereafter (*p* value – 0.004).

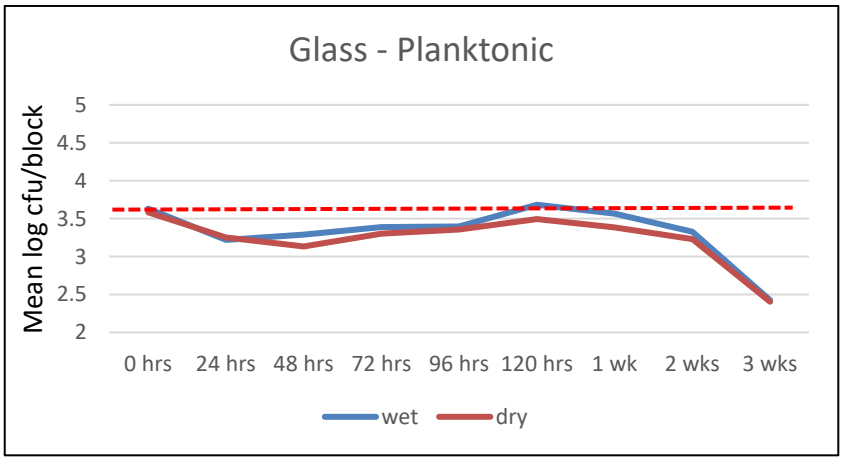


Figure 3.1 Survival of planktonic cells of C. *auris* on wet and dry glass surface. Red dotted line represents baseline counts at 0 hrs.

Table 3.1 Survival of planktonic cells of C. *auris* on wet and dry glass surface

Strains	Type	Repeat	Planktonic cells – cfu/glass block at various exposure time								
			0 hrs*	**24 hrs**	**48 hrs**	**72 hrs**	**96 hrs**	**120 hrs**	**1 Wk***	**2 Wks***	**3 Wks***
Cau 4888	Wet	1	5700	2840	4200	2820	2300	6000	4600	1770	240
		2	5740	2320	4000	3700	2820	5740	3400	2190	160
Cau 3499	Wet	1	4700	1700	1740	2360	2480	6100	4610	2230	570
		2	3320	2440	2300	3200	2120	4580	4090	2330	690
Cau 6326	Wet	1	4000	900	950	1700	2700	3960	3370	2180	90
		2	3000	840	850	1600	2660	3300	2510	2120	260
Mean			**4410**	**1840**	**2340**	**2563**	**2513**	**4946**	**3763**	**2136**	**335**
SD			**1171**	**835**	**1464**	**834**	**265**	**1173**	**822**	**192**	**239**
% reduction				**58**	**47**	**42**	**43**	**-12**	**15**	**52**	**92**
t test: *p* value									**0.189**	**0.007**	**0.001**
Cau 4888	Dry	1	4300	1450	2800	1810	2350	3600	2500	1270	140
		2	3940	1550	2340	1970	2490	2940	1800	1900	220
Cau 3499	Dry	1	4300	1820	1820	3580	2330	4700	3660	2070	400
		2	4200	2140	1900	3100	2510	3700	2340	1590	520
Cau 6326	Dry	1	3190	2040	600	1440	1970	2630	2510	1500	190
		2	3210	1820	470	1140	2030	1910	2090	2020	220
Mean			**3857**	**1803**	**1655**	**2173**	**2280**	**3247**	**2483**	**1725**	**282**
SD			**525**	**268**	**936**	**961**	**229**	**970**	**637**	**320**	**146**
% reduction				**53**	**57**	**44**	**41**	**16**	**36**	**55**	**93**
t test: *p* value									**0.004**	**0.001**	**0.000**
Cal 5339	Wet	1	4590	3680	2100	3490	3360	5980	63000	2700	1900
		2	3170	2300	2140	3010	3600	5020	58000	2200	1640
Cal 5339	Dry	1	3610	1870	2640	2500	2770	3300	57600	2980	1320
		2	3590	1830	2080	2180	2450	3500	50000	2940	800

% reduction: negative results represents % increase in counts,
Cau: *C. auris*, Cal: *C. albicans,*
*: Comparisons 0 hrs to wk1, wks 2 and wks 3

3.1.1.2 Fabric surface

Results of the survival on the **fabric surfaces** are shown in table 3.2 and figure 3.2. In addition, percentage reduction was calculated. The results showed that the counts of *C. auris* fluctuated over 3 weeks regardless of the wet and dry condition with increase of up to 230%. Decline of counts was seen only at 2 weeks in a wet condition and at 1 week in a dry condition. A paired samples t-test was conducted to compare the counts at 0 hours to those at 1 week, 2 weeks and 3 weeks separately for the wet and dry surfaces. In the wet condition, the reduction in the counts was significant only at 3 weeks and thereafter (*p* value – 0.011). Whereas in the dry condition the percentage reduction was significant at 2 week and thereafter (*p* value – 0.05 and 0.013).

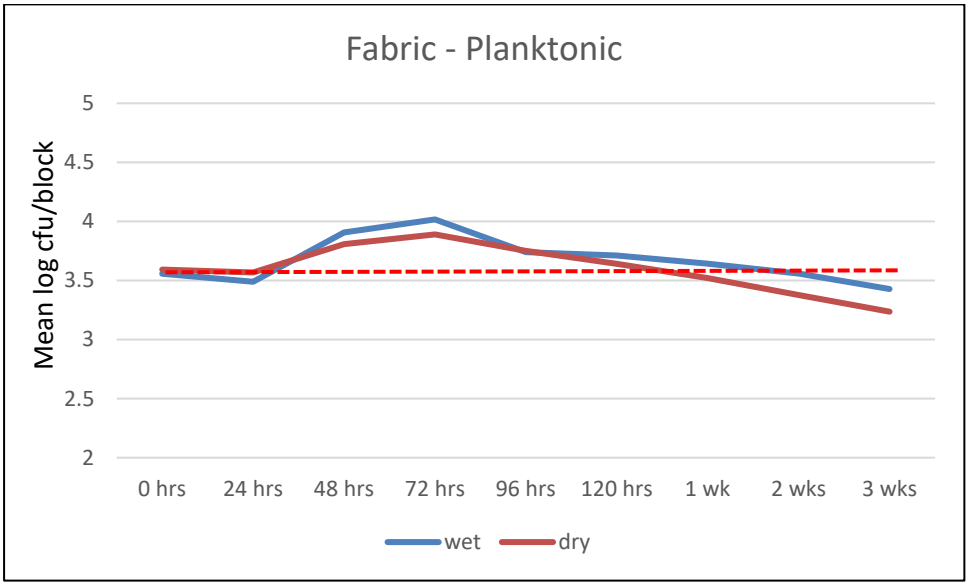


Figure 3.2 Survival of planktonic cells of *C. auris* on wet and dry fabric surface. Red dotted line represents baseline counts at 0 hrs.

Table 3.2. Survival of planktonic cells of *C. auris* on wet and dry fabric surface

Strain	Type	Repeats	Planktonic cells- cfu/fabric block at various exposure time								
			0 Hrs*	**24 hrs**	**48 hrs**	**72 Hrs**	**96 hrs**	**120 hrs**	**1 Wk***	**2 Wks***	**3 Wks***
Cau 4888	Wet	1	4000	2010	3940	5200	5890	5860	4830	4500	3410
		2	3620	2470	3760	4160	4650	5500	4670	3560	2430
Cau 3499	Wet	1	4060	5280	12600	16000	5200	5070	3460	3370	2700
		2	3440	3860	16200	19800	6640	4810	3940	3090	2040
Cau 6326	Wet	1	4190	3170	11200	14000	5230	4920	4620	4200	3010
		2	2610	2690	8200	13200	5570	4840	5080	3180	2690
Mean			**3653**	**3247**	**9317**	**12060**	**5530**	**5167**	**4433**	**3650**	**2713**
SD			**585**	**1180**	**4955**	**6164**	**684**	**423**	**609**	**574**	**471**
% reduction				**11**	**-155**	**-230**	**-51**	**-41**	**-21**	**0**	**26**
t test: *p* value									**0.115**	**0.987**	**0.011**
Cau 4888	Dry	1	3390	2400	3490	3890	4540	5430	4700	3520	2930
		2	3030	1960	2770	3350	3860	5090	3800	3180	2350
Cau 3499	Dry	1	5700	5370	11000	12200	6850	4080	3340	2080	1730
		2	4840	4490	6090	7800	11000	5260	3860	2520	2370
Cau 6326	Dry	1	3800	4500	9000	11800	5560	3740	2570	2040	1780
		2	3340	4980	12000	15000	4400	3200	2230	1520	520
Mean			**4017**	**3950**	**7392**	**9007**	**6035**	**4467**	**3417**	**2477**	**1947**
SD			**1038**	**1417**	**3876**	**4766**	**2653**	**920**	**908**	**755**	**827**
% reduction				**2**	**-84**	**-124**	**-50**	**-11**	**15**	**38**	**52**
t test: *p* value									**0.333**	**0.05**	**0.013**
Cal 5339	Wet	1	3640	4940	18200	21400	18400	15600	5690	4460	4100
		2	2760	4300	14400	18000	19400	16200	4570	3840	2920
Cal 5339	Dry	1	3620	3790	23800	20200	17400	9000	6040	4430	3430
		2	3060	3350	17400	19600	15400	7200	5000	4890	3170

% reduction: negative results represents % increase in counts

Cau: *C. auris*, Cal: *C. albicans,*

*: Comparisons 0 hrs to wk1, wks 2 and wks 3

3.1.1.3 Plastic surface

Results of the survival on the **plastic surfaces** are shown in table 3.3 and figure 3.3. In addition, percentage reduction was calculated. The results showed that the counts of *C. auris* fluctuated over 3 weeks regardless of the wet and dry condition with increase of up to 34%. A paired samples t-test was conducted to compare the counts at 0 hours to those at 1 week, 2 weeks and 3 weeks separately for the wet and dry surfaces. Significant decline of counts was seen only at 2 weeks in a wet condition (*p* value – 0.014) and at 1 week in a dry condition (*p* value – 0.002).

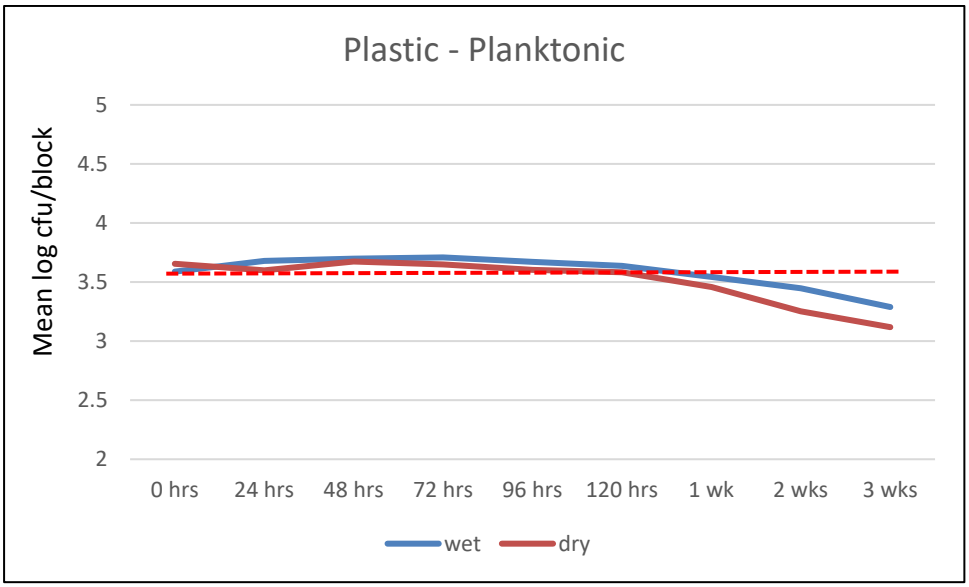


Figure 3.3 Survival of planktonic cells of *C. auris* on wet and dry plastic surface. Red dotted line represents baseline counts at 0 hrs.

Table 3.3 Survival of planktonic cells of *C. auris* on wet and dry plastic surface

Strain	Type	Repeats	Planktonic cells- cfu plastic block at various exposure time								
			0 Hrs*	**24 hrs**	**48 hrs**	**72 hrs**	**96 hrs**	**120 hrs**	**1 Wk***	**2 Wks***	**3 Wks***
Cau 4888	Wet	1	3240	3900	4400	5680	4470	5100	3440	3000	2000
		2	4100	4800	4520	3620	4350	3200	3680	3300	2680
Cau 3499	Wet	1	4000	6260	4910	5740	5240	4700	4310	2970	1710
		2	4090	5800	5950	5020	5380	6330	3750	3290	2070
Cau 6326	Wet	1	3610	4050	5500	6480	4780	3640	3350	2510	1600
		2	4310	4330	4820	4660	4020	3740	2670	1990	1780
Mean			**3892**	**4857**	**5017**	**5200**	**4707**	**4452**	**3533**	**2843**	**1973**
SD			**393**	**970**	**597**	**998**	**529**	**1161**	**540**	**508**	**389**
% reduction				**-25**	**-29**	**-34**	**-21**	**-14**	**9**	**27**	**49**
t test: *p* value									**0.262**	**0.014**	**0.0002**
Cau 4888	Dry	1	4620	5600	5380	5040	4120	3880	2650	2130	1900
		2	4180	3940	4760	4600	3480	3120	3400	1590	1540
Cau 3499	Dry	1	4400	3780	4900	4990	5030	5200	3450	2030	1010
		2	4260	3720	5920	4950	3710	4600	2850	1890	760
Cau 6326	Dry	1	4730	3700	4150	4540	4320	3630	2490	1610	1950
		2	4970	3420	3590	3040	3600	2970	2550	1550	1170
Mean			**4527**	**4027**	**4783**	**4527**	**4043**	**3900**	**2898**	**1800**	**1388**
SD			**301**	**789**	**835**	**758**	**580**	**863**	**426**	**250**	**487**
% reduction				**11**	**-6**	**0**	**11**	**14**	**36**	**60**	**69**
t test: *p* value									**0.002**	**0.000**	**0.000**
Cal 5339	Wet	1	4940	5490	4400	5130	5490	4890	4680	2730	2670
		2	4930	4490	4460	3870	4650	4050	3560	2370	2090
Cal 5339	Dry	1	3770	4040	4100	4700	4090	3940	3280	3100	1680
		2	3150	3060	3920	4380	3910	3960	3200	2460	1300

% reduction: negative results represents % increase in counts

Cau: *C. auris*, Cal: *C. albicans,*

*: Comparisons 0 hrs to wk1, wks 2 and wks 3

3.1.1.4 Wood surface

Results of the survival on the **Wood surfaces** are shown in table 3.4 and figure 3.4. In addition, percentage reduction was calculated. The results showed that the counts of *C. auris* fluctuated over 3 weeks. A paired samples t-test was conducted to compare the counts at 0 hours to those at 1 week, 2 weeks and 3 weeks separately for the wet and dry surfaces. In the wet condition, statistical significance was achieved when comparing 0 Hours to 1 week and thereafter (*p* value – 0.001) showing tremendous increase in the counts. In the dry condition as well, statistical significance was achieved but it was a decrease in counts when 0 hours was compared to 1 week and thereafter (*p* value – 0.021). In a wet condition, the counts of *C. auris* on the wood constantly and significantly increased with a maximum increase of 961% at 3 weeks.

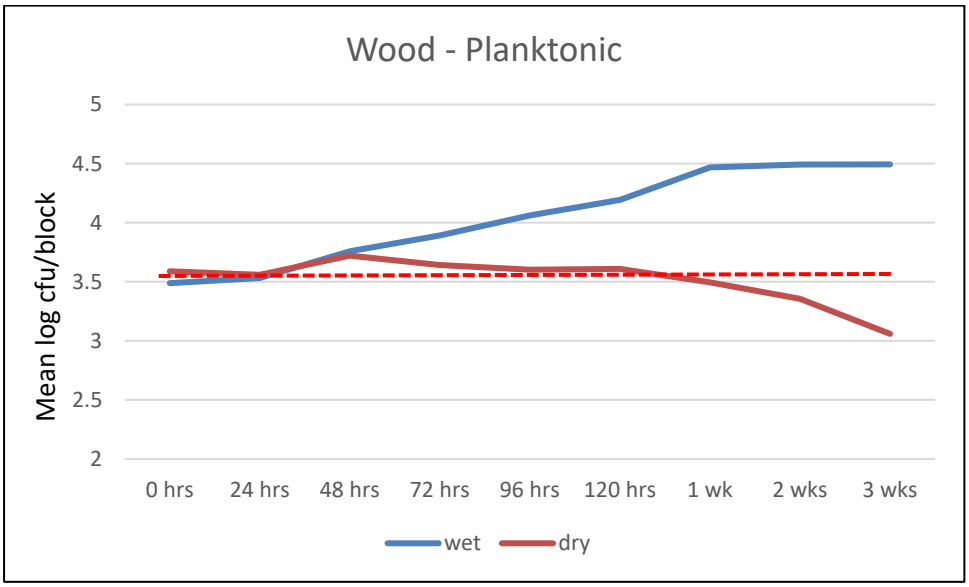


Figure 3.4 Survival of planktonic cells of *C. auris* on wet and dry wood surface. Red dotted line represents baseline counts at 0 hrs.

Table 3.4 Survival of planktonic cells of *C. auris* on wet and dry wood surface

Strain	Type	Repeats	Planktonic cells- cfu/wood block at various exposure time								
			0 Hrs*	**24 hrs**	**48 hrs**	**72 Hrs**	**96 hrs**	**120 hrs**	**1 Wk***	**2 Wks***	**3 Wks***
Cau 4888	Wet	1	2770	2600	3400	7000	10200	13600	20600	22000	16200
		2	3410	3100	3420	8200	12600	14000	24400	27400	22200
Cau 3499	Wet	1	2780	4870	9800	12600	19000	27600	45000	35200	38000
		2	2390	3350	7200	10000	15200	19800	34000	38200	44400
Cau 6326	Wet	1	3360	3720	6660	5810	10400	16000	35000	38000	42400
		2	4010	3130	6420	5360	6020	8600	23600	29200	35400
Mean			**3120**	**3462**	**6150**	**8162**	**12237**	**16600**	**30433**	**31667**	**33100**
SD			**584**	**780**	**2440**	**2750**	**4491**	**6502**	**9226**	**6527**	**11383**
% reduction				**-11**	**-97**	**-162**	**-292**	**-432**	**-875**	**-915**	**-961**
t test: *p* value									**0.001**	**0.0001**	**0.001**
Cau 4888	Dry	1	3940	3460	2850	4100	4420	4060	2940	2370	1270
		2	3780	3400	2980	5300	3480	3520	3100	1650	1540
Cau 3499	Dry	1	3460	4400	11600	5760	4250	4680	3350	2470	1080
		2	4530	4020	14200	7800	5410	5440	4250	3690	2260
Cau 6326	Dry	1	3250	3000	3600	2560	3280	3330	2540	1610	440
		2	4470	3620	4190	2800	3500	3670	2850	2360	1070
Mean			**3905**	**3650**	**6570**	**4720**	**4057**	**4117**	**3172**	**2358**	**1277**
SD			**520**	**494**	**4995**	**1982**	**806**	**806**	**593**	**755**	**603**
% reduction				**7**	**-68**	**-21**	**-4**	**-5**	**19**	**40**	**67**
t test: *p* value									**0.021**	**0.001**	**0.000**
Cal 5339	Wet	1	3110	2950	4440	6320	15400	19600	43000	45400	62000
		2	3030	3700	4660	6070	10400	16800	35800	40400	52800
Cal 5339	Dry	1	3900	2220	4450	4480	3920	4050	2580	2130	910
		2	3650	3060	4820	5060	4610	4000	2800	2660	1500

% reduction: negative results represents % increase in counts
Cau: *C. auris*, Cal: *C. albicans,* cfu: colony forming unit
*: Comparisons 0 hrs to wk1, wks 2 and wks 3

3.1.1.5 Steel surface

Results of the survival on the **steel surfaces** are shown in table 3.5 and figure 3.5. In addition, percentage reduction was calculated. The results showed that the counts of *C. auris* fluctuated over 3 weeks regardless of the wet and dry condition. A paired samples t-test was conducted to compare the counts at 0 hours to those at 1 week, 2 weeks and 3 weeks separately for the wet and dry surfaces. In the wet condition, statistical significance was achieved only when comparing 0 Hours to 1 week only with increase in counts (*p* value – 0.008). Whereas in the dry condition no statistical significance was achieved i.e., the change in fungal cell counts where all statistically non-significant.

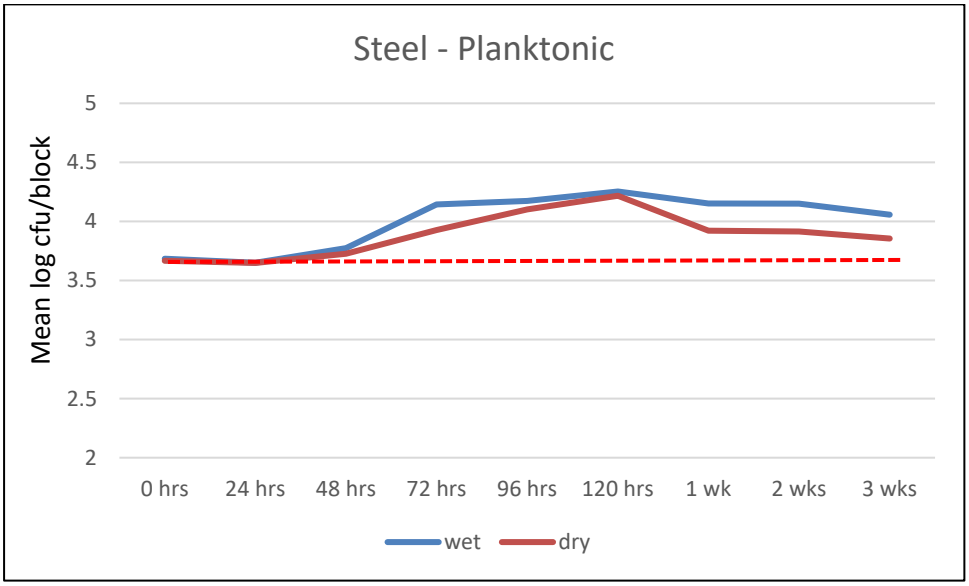


Figure 3.5 Survival of planktonic cells of *C. auris* on wet and dry steel surface. Red dotted line represents baseline counts at 0 hrs.

Table 3.5 Survival of planktonic cells of *C. auris* on wet and dry steel surface

Strain	Type	Repeats	Planktonic cells- cfu/steel block at various exposure time								
			0 Hrs*	**24 hrs**	**48 hrs**	**72 Hrs**	**96 hrs**	**120 hrs**	**1 Wk***	**2 Wks***	**3 Wks***
Cau 4888	Wet	1	5080	5040	5900	11200	13800	19600	13600	8200	6180
		2	4300	4100	4520	13400	12200	15000	10600	14000	5030
Cau 3499	Wet	1	5900	5540	9000	16600	21000	27800	25200	42000	52000
		2	4990	5400	7050	14200	17000	22000	21400	36200	46600
Cau 6326	Wet	1	4580	3900	4830	18000	14800	13200	9000	6600	5900
		2	4310	3310	5420	11400	12400	14000	11600	7000	5000
Mean			**4860**	**4548**	**6120**	**14133**	**15200**	**18600**	**15233**	**19000**	**20118**
SD			**607**	**906**	**1669**	**2744**	**3342**	**5665**	**6535**	**15901**	**22673**
% reduction				**6**	**-26**	**-191**	**-213**	**-283**	**-213**	**-291**	**-314**
t test: *p* value									**0.008**	**0.075**	**0.153**
Cau 4888	Dry	1	4510	4920	5380	7100	18000	23400	3930	2800	2060
		2	3880	3300	4060	5300	11600	17200	3220	2790	1940
Cau 3499	Dry	1	4750	5530	6700	11400	14400	23400	24000	35600	40600
		2	4320	4350	5920	7400	12000	17400	19800	28200	37200
Cau 6326	Dry	1	5770	4630	5860	14400	11400	11000	6400	5810	4060
		2	4820	4320	4490	8000	10000	11200	8800	6750	5480
Mean			**4675**	**4508**	**5402**	**8933**	**12900**	**17267**	**11025**	**13658**	**15223**
SD			**634**	**741**	**979**	**3339**	**2878**	**5502**	**8752**	**14410**	**18419**
% reduction				**4**	**-16**	**-91**	**-176**	**-269**	**-136**	**-192**	**-226**
t test: *p* value									**0.137**	**0.189**	**0.221**
Cal 5339	Wet	1	3390	3800	5820	8200	10400	11600	13000	26000	32800
		2	4410	3750	3790	6070	7800	9000	10000	22200	26000
Cal 5339	Dry	1	3460	3440	5260	5920	11800	13400	16800	22400	26000
		2	3150	3160	3820	5080	8400	14200	19600	27200	33400

% reduction: negative results represents % increase in counts
Cau: *C. auris*, Cal: *C. albicans,* cfu: colony forming unit
*: Comparisons 0 hrs to wk1, wks 2 and wks 3

3.1.2 Comparison of surfaces wet and dry combined with planktonic cells

To compare the survival of planktonic form of *C. auris* on various surfaces, the results of wet and dry surfaces were combined and the mean counts are depicted in table 3.6. The log mean counts are shown in Figure 3.6. The results showed that after 3 weeks there was a reduction in the *C. auris* counts on glass, fabric and plastic whereas there was increase on wood and steel.

Table 3.6 Comparison of planktonic *C. auris* counts on various surfaces including wet and dry conditions

Strain	Type	Surface	Mean planktonic cells- cfu/ block at various exposure time								
			0 Hrs	**24 hrs**	**48 hrs**	**72 hrs**	**96 Hrs**	**120 hrs**	**1 wk**	**2 wks**	**3 Wks**
All	Wet and dry	Glass	4133	1822	1998	2368	2397	4097	3123	1931	308
		% reduction									**92.55**
		Fabric	3835	3598	8354	10533	5783	4817	3925	3063	2330
		% reduction									**39.24**
		Plastic	4209	4442	4900	4863	4375	4176	3216	2322	1681
		% reduction									**60.06**
		Wood	3513	3556	6360	6441	8147	10358	16803	17013	17188
		% reduction									**-389.27**
		Steel	4768	4528	5761	11533	14050	17933	13129	16329	11836
		% reduction									**-148.24**

% reduction: negative results represents % increase in counts

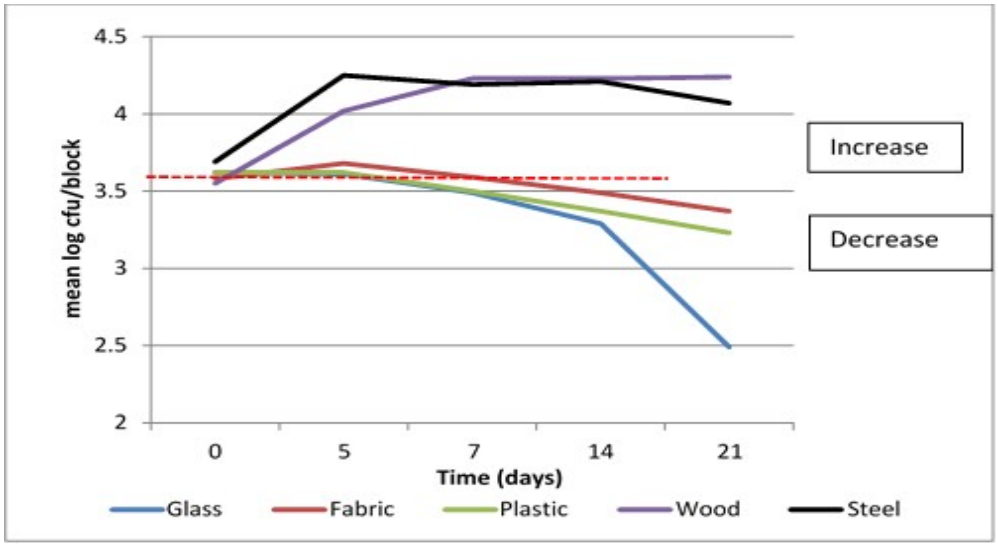


Figure 3.6: Comparison of planktonic *C. auris* counts on various surfaces including wet and dry conditions. Red dotted line represents baseline counts at 0 hrs. After 3 weeks, the counts of *C. auris i*ncreased on wood and steel surfaces and decreased on glass, plastic and fabric.

3.1.3 Comparison of wet and dry condition combining all the surfaces with planktonic cells

To compare the survival of planktonic form of *C. auris* in wet and dry conditions, the results of all the surfaces were combined and the mean counts are depicted in table 3.7. The log mean counts are shown in Figure 3.7. The results showed that after 3 weeks there was a reduction in the *C. auris* counts in dry condition whereas there was an increase in wet condition. A paired samples t-test was conducted to compare planktonic *C. auris* growth on wet and dry surfaces for various surfaces combined. The results showed that there was a significant increase in the counts in the wet condition after 1 week and thereafter whereas in the dry condition there was a significant decrease in the counts (Table 3.7).

Table 3.7 Comparison of planktonic *C. auris* counts in wet and dry conditions combining various surfaces

Strain	**Surface**	**Type**	**Mean planktonic cells- cfu/ block at various exposure time**								
			0 Hrs*	**24 hrs**	**48 hrs**	**72 hrs**	**96 hrs**	**120 hrs**	**1 Wk***	**2 Wks***	**3 Wks***
All	All	Wet	3987	3591	5789	8424	8037	9953	11479	11859	11648
		P value							0.002	0.002	0.004
		Dry	4196	3588	5160	5872	5863	6599	4599	4404	1689
		p value							0.002	0.002	0.004

*: Comparisons 0 hrs to wk1, wks 2 and wks 3, cfu: colony forming unit

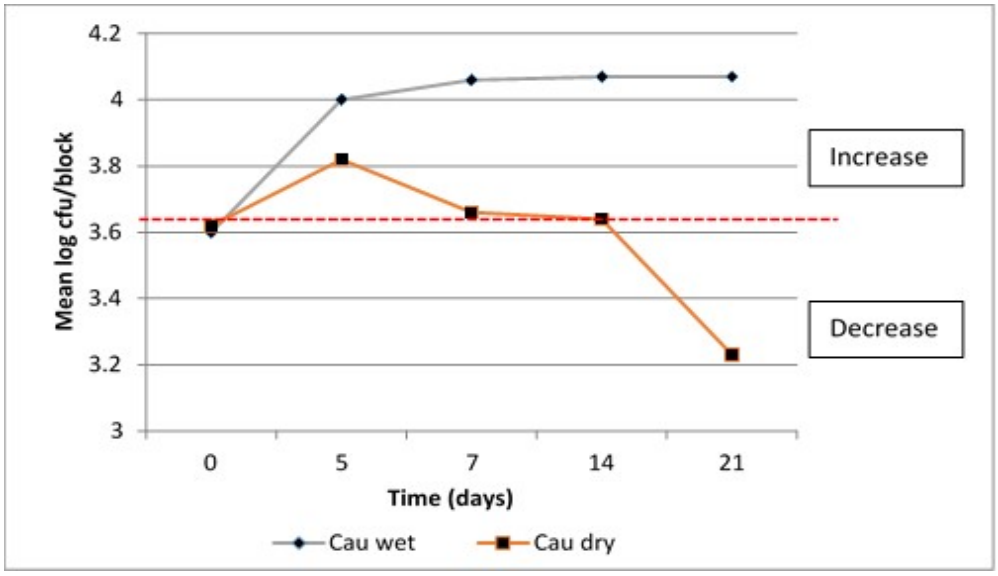


Figure 3.7 Comparison of planktonic *C. auris* counts in wet and dry conditions on various surfaces. Red dotted line represents baseline counts at 0 hrs. After 3 weeks, the counts of *C. auris i*ncreased on wet surfaces and decreased on dry surfaces.

3.2 Survival of biofilm form of cells of *C. auris*

Biofilms of *C. auris* were grown on various test surfaces and the survival period was measured, and the percentage reduction was calculated.

3.2.1 Counts of biofilm of *C. auris* on various surfaces

3.2.1.1 Glass surfaces

Results of the survival of biofilm on the **glass surfaces** are shown in table 3.8 and figure 3.8. In addition, percentage reduction was calculated. The results showed that the counts of *C. auris* increased exponentially from 48 hours to 3 weeks in wet and dry conditions. A paired samples t-test was conducted to compare the counts at 0 hours to those at 1 week, 2 weeks and 3 weeks separately for the surface when wet and when dry. In the wet condition, significant increase in counts was achieved when comparing 0 Hours to 1 week and thereafter (*p* value – 0.0001). In the dry condition as well, significant increase was achieved when 0 hours was compared to 1 week and thereafter (*p* value – 0.001).

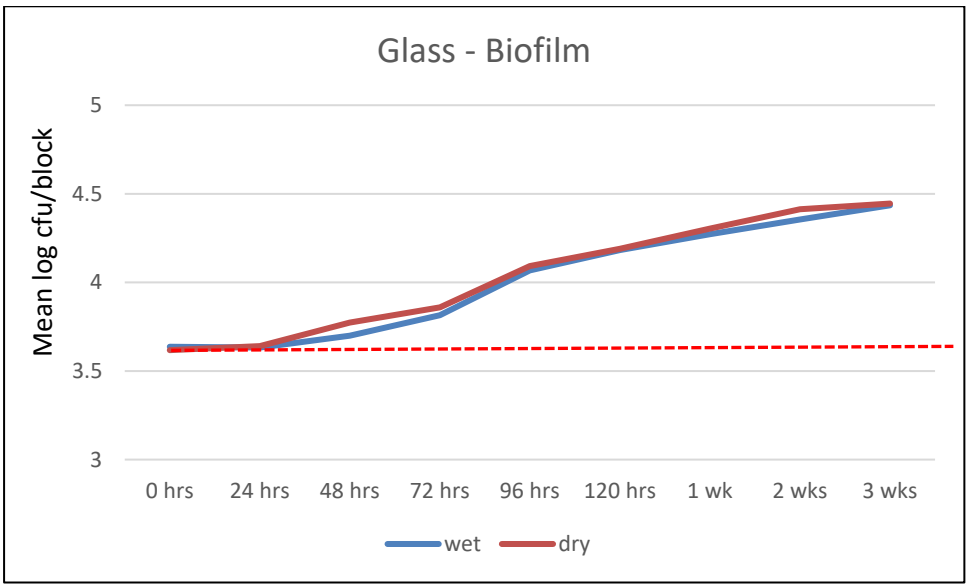


Figure 3.8 Survival of Biofilms of *C. auris* on wet and dry glass surface. Red dotted line represents baseline counts at 0 hrs.

Table 3.8 Survival of Biofilms of *C. auris* on wet and dry glass surface

Strain	Type	Repeats	Biofilm cells- cfu/glass block at various exposure time								
			0 Hrs*	**24 hrs**	**48 hrs**	**72 Hrs**	**96 hrs**	**120 hrs**	**1 Wk***	**2 Wks***	**3 Wks***
Cau 4888	Wet	1	5690	5840	7200	9400	12200	18400	24600	30000	36600
		2	6510	5310	7000	8400	17400	18800	23400	36600	38000
Cau 3499	Wet	1	3620	4900	5740	6600	12200	13600	17800	21800	27400
		2	4080	4140	4500	9000	10600	15000	18800	22800	30800
Cau 6326	Wet	1	3900	3480	4250	4600	10600	12400	13200	13800	16400
		2	3120	2840	2850	3600	8800	14200	16800	18000	21200
Mean			**4487**	**4418**	**5257**	**6933**	**11967**	**15400**	**19100**	**23833**	**28400**
SD			**1317**	**1140**	**1699**	**2416**	**2946**	**2623**	**4257**	**8257**	**8509**
% reduction				**2**	**-17**	**-55**	**-167**	**-243**	**-326**	**-431**	**-533**
t test: *p* value									**0.000**	**0.001**	**0.001**
Cau 4888	Dry	1	5400	5750	9000	11600	14200	23400	26200	33200	27000
		2	5610	6010	10200	14000	17200	18600	25800	41400	44000
Cau 3499	Dry	1	3480	5820	6310	8400	12400	14400	17600	19200	34200
		2	4250	5340	5700	9200	16800	22200	28600	37400	46400
Cau 6326	Dry	1	3490	2940	3830	3620	9000	8800	12600	14800	13200
		2	3250	2220	3470	3140	7800	11200	15400	20600	18800
Mean			**4247**	**4680**	**6418**	**8327**	**12900**	**16433**	**21033**	**27767**	**30600**
SD			**1034**	**1657**	**2716**	**4306**	**3921**	**5938**	**6653**	**10964**	**13395**
% reduction				**-10**	**-51**	**-96**	**-204**	**-287**	**-395**	**-554**	**-621**
t test: *p* value									**0.001**	**0.002**	**0.004**
Cal 5339	Wet	1	4520	5680	10200	11400	15400	23600	32400	36200	41600
		2	4570	5300	13200	14800	19800	25200	36000	42000	47800
Cal 5339	Dry	1	4810	5870	11400	16600	17800	26800	35600	43200	45800
		2	5590	7200	13800	18200	22600	31600	44600	48800	51200

% reduction: negative results represents % increase in counts
Cau: *C. auris*, Cal: *C. albicans,* cfu: colony forming unit
*: Comparisons 0 hrs to wk1, wks 2 and wks 3

3.2.1.2 Fabric surfaces

Results of the survival of biofilm on the **fabric surfaces** are shown in table 3.9 and figure 3.9. In addition, percentage reduction was calculated. The results showed that the counts of *C. auris* increased exponentially from 48 hours to 3 weeks in wet and dry conditions. A paired samples t-test was conducted to compare the counts at 0 hours to those at 1 week, 2 weeks and 3 weeks separately for the surface when wet and when dry. In the wet condition, the increase in counts was significant when comparing 0 Hours to 1 week and thereafter (*p* value – 0.003). In the dry condition as well, significant increase was achieved when 0 hours was compared to 1 week and thereafter (*p* value – 0.0001).

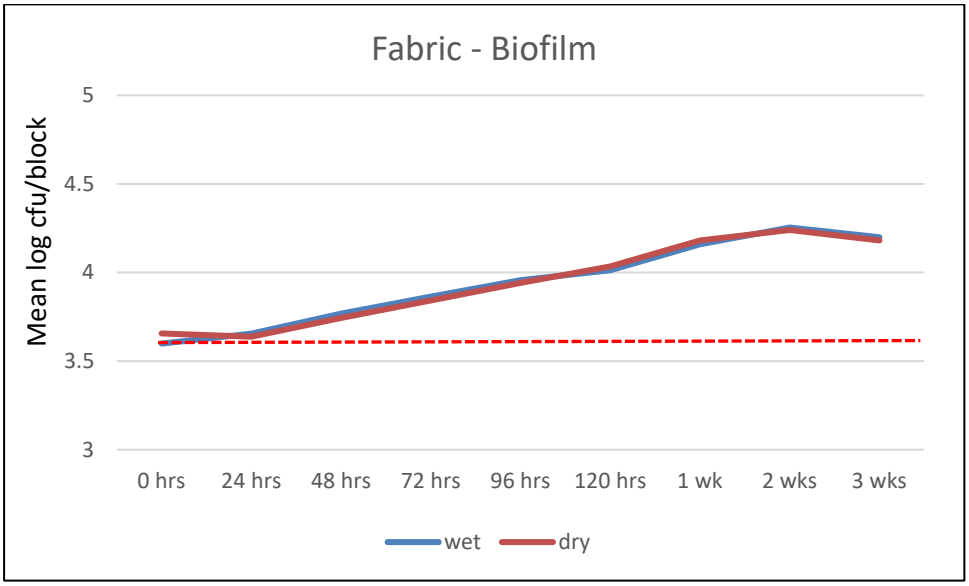


Figure 3.9 Survival of Biofilms of *C. auris* on wet and dry fabric surface. Red dotted line represents baseline counts at 0 hrs.

Table 3.9 Survival of Biofilms of *C. auris* on wet and dry fabric surface

Strain	Type	Repeats	Biofilm cells- cfu/fabric block at various exposure time								
			0 Hrs*	**24 hrs**	**48 hrs**	**72 Hrs**	**96 hrs**	**120 hrs**	**1 Wk***	**2 Wks***	**3 Wks***
Cau 4888	Wet	1	5100	5900	7400	8600	10200	11600	16600	24400	31000
		2	4220	4870	4000	11800	13400	16000	24200	29200	39000
Cau 3499	Wet	1	4290	6220	9600	8000	9200	11800	17000	14400	10000
		2	3440	4370	7200	7600	8400	8600	12200	17200	12000
Cau 6326	Wet	1	4390	3570	4720	5140	9400	9600	11800	15000	10600
		2	2810	3040	4210	4810	5550	6600	9200	12600	10200
Mean			**4042**	**4662**	**6188**	**7658**	**9358**	**10700**	**15167**	**18800**	**18800**
SD			**802**	**1258**	**2236**	**2554**	**2549**	**3242**	**5343**	**6547**	**12820**
% reduction				**-15**	**-53**	**-89**	**-132**	**-165**	**-275**	**-365**	**-365**
t test: *p* value									**0.003**	**0.002**	**0.034**
Cau 4888	Dry	1	4990	4090	6020	7000	7800	9400	12600	17200	24600
		2	5030	2960	5200	7200	9600	11000	15800	23000	22000
Cau 3499	Dry	1	5650	5770	7000	8400	9200	11000	13800	11600	9400
		2	4510	4490	5600	7800	11000	14600	18000	15400	10800
Cau 6326	Dry	1	3900	4500	5260	5720	6000	8800	14800	18200	12600
		2	3440	4750	4500	5960	9800	11000	16600	21400	17600
Mean			**4587**	**4427**	**5597**	**7013**	**8900**	**10967**	**15267**	**17800**	**16167**
SD			**810**	**915**	**851**	**1035**	**1756**	**2018**	**1950**	**4116**	**6237**
% reduction				**3**	**-22**	**-53**	**-94**	**-139**	**-233**	**-288**	**-252**
t test: *p* value									**0.0001**	**0.001**	**0.006**
Cal 5339	Wet	1	3640	4740	7000	9200	11400	15600	19800	24200	24600
		2	2860	4100	5800	7200	10400	16200	22000	33400	32400
Cal 5339	Dry	1	3820	4490	8200	10600	13200	19000	26200	29800	39600
		2	3170	3550	7400	11600	14800	25200	24400	26800	43400

% reduction: negative results represents % increase in counts

Cau: *C. auris*, Cal: *C. albicans,* cfu: colony forming unit

*: Comparisons 0 hrs to wk1, wks 2 and wks 3

3.2.1.3 Plastic surfaces

Results of the survival of biofilm on the **plastic surfaces** are shown in table 3.10 and figure 3.10. In addition, percentage reduction was calculated. The results showed that the counts of *C. auris* increased exponentially from 48 hours to 3 weeks in wet and dry conditions. A paired samples t-test was conducted to compare the counts at 0 hours to those at 1 week, 2 weeks and 3 weeks separately for the surface when wet and when dry. The increase was significant with *p*-values ranging from 0.02 to 0.001.

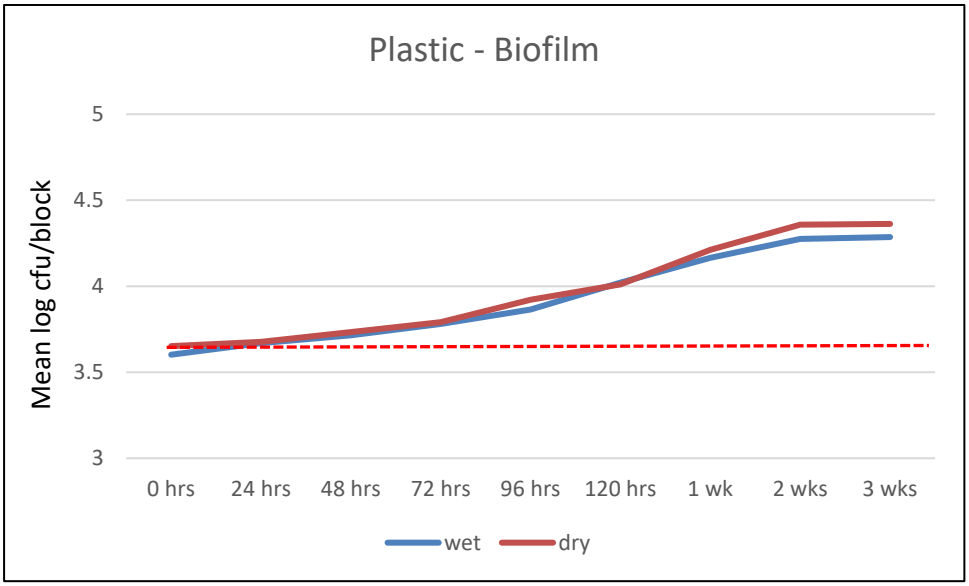


Figure 3.10. Survival of Biofilms of *C. auris* on wet and dry plastic surface. Red dotted line represents baseline counts at 0 hrs.

Table 3.10 Survival of Biofilms of *C. auris* on wet and dry plastic surface

Strain	Type	Repeats	Biofilm cells- cfu/plastic block at various exposure time								
			0 Hrs*	**24 hrs**	**48 hrs**	**72 Hrs**	**96 hrs**	**120 hrs**	**1 Wk***	**2 Wks***	**3 Wks***
Cau 4888	Wet	1	3800	4500	4840	5360	8200	11200	11000	9800	7000
		2	3960	4120	4220	5110	6250	8600	9400	8200	6030
Cau 3499	Wet	1	3910	5160	5710	6110	6600	9200	13000	23400	24000
		2	3990	5300	5910	7000	7000	9600	15400	27200	32400
Cau 6326	Wet	1	4720	4750	5870	7400	8400	11200	19200	27000	38000
		2	3700	4330	4840	5600	7800	14000	24800	32200	41600
Mean			**4013**	**4693**	**5232**	**6097**	**7375**	**10633**	**15467**	**21300**	**24838**
SD			**363**	**466**	**697**	**925**	**885**	**1961**	**5725**	**9944**	**15388**
% reduction				**-17**	**-30**	**-52**	**-84**	**-165**	**-285**	**-431**	**-519**
t test: *p* value									**0.004**	**0.008**	**0.021**
Cau 4888	Dry	1	4220	4750	5380	5740	7200	9800	13400	19000	22600
		2	4260	3940	4560	4600	5880	7000	10200	15800	17600
Cau 3499	Dry	1	4400	5280	5300	5490	8200	8800	14000	19000	21000
		2	3820	4210	5900	5650	9400	10600	16400	25600	28800
Cau 6326	Dry	1	5030	5100	5750	7800	9400	12400	22200	25800	22000
		2	5370	5420	5690	8800	11000	14600	26800	37400	28200
Mean			**4517**	**4783**	**5430**	**6347**	**8513**	**10533**	**17167**	**23767**	**23367**
SD			**574**	**599**	**483**	**1598**	**1817**	**2685**	**6188**	**7775**	**4340**
% reduction				**-6**	**-20**	**-41**	**-88**	**-133**	**-280**	**-426**	**-417**
t test: *p* value									**0.003**	**0.001**	**0.001**
Cal 5339	Wet	1	5220	5010	5600	5730	8400	9600	19600	29800	37200
		2	4460	4230	4660	4970	5450	7200	12600	25400	32000
Cal 5339	Dry	1	4770	4940	5410	5750	6400	10000	15000	29400	30600
		2	4350	3760	4320	4900	5910	7200	12400	22800	33800

% reduction: negative results represents % increase in counts
Cau: *C. auris*, Cal: *C. albicans,* cfu: colony forming unit
*: Comparisons 0 hrs to wk1, wks 2 and wks 3

3.2.1.4 Wood surfaces

Results of the survival of biofilm on the **wood surfaces** are shown in table 3.11 and figure 3.11. In addition, percentage reduction was calculated. The results showed that the counts of *C. auris* increased exponentially from 24 hours to 3 weeks in wet conditions. A paired samples t-test was conducted to compare the counts at 0 hours to those at 1 week, 2 weeks and 3 weeks separately for the surface when wet and when dry. In the wet condition, the increase in counts was significant when comparing 0 Hours to 1 week and thereafter (*p* value – 0.001). Whereas In the dry condition statistical significance was achieved when 0 hours was compared to 2 weeks and thereafter (*p* value – 0.048).

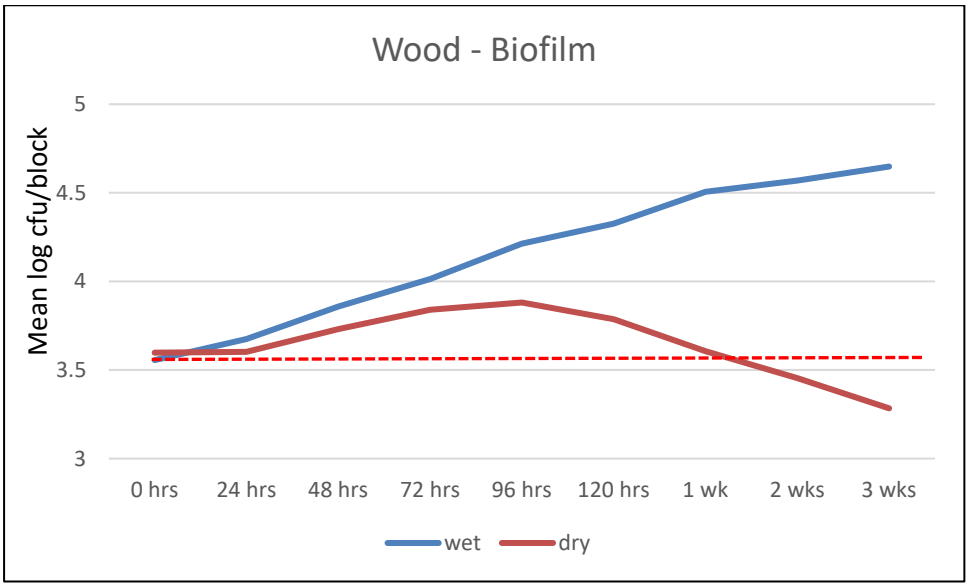


Figure 3.11 Survival of biofilms of *C. auris* on wet and dry wood surface. Red dotted line represents baseline counts at 0 hrs.

Table 3.11 Survival of biofilms of *C. auris* on wet and dry wood surface

Strain	Type	Repeats	Biofilm cells- cfu/wood block at various exposure time								
			0 Hrs*	**24 hrs**	**48 hrs**	**72 Hrs**	**96 hrs**	**120 hrs**	**1 Wk***	**2 Wks***	**3 Wks***
Cau 4888	Wet	1	3140	5300	7000	8400	11000	13400	19600	22000	32200
		2	3710	4990	7600	9200	12600	14000	24800	29400	37200
Cau 3499	Wet	1	3180	4170	7800	12600	19400	29800	44200	51200	56000
		2	4090	4350	7200	10000	14800	21800	33200	42000	44400
Cau 6326	Wet	1	3560	4820	7400	11600	18400	26000	35000	38000	47000
		2	4020	4830	6400	10600	25800	28600	43600	49200	55400
Mean			**3617**	**4743**	**7233**	**10400**	**17000**	**22267**	**33400**	**38633**	**45367**
SD			**404**	**417**	**497**	**1544**	**5392**	**7183**	**9876**	**11342**	**9564**
% reduction				**-31**	**-100**	**-188**	**-370**	**-516**	**-824**	**-968**	**-1154**
t test: *p* value									**0.001**	**0.001**	**0.001**
Cau 4888	Dry	1	3670	3460	4250	4600	4220	4060	3440	2720	1535
		2	3810	3430	4080	5100	3780	3520	3140	1650	1840
Cau 3499	Dry	1	3560	4320	7600	11000	13600	11400	5550	3570	2680
		2	4730	4070	5740	7800	5810	5360	3950	3650	1720
Cau 6326	Dry	1	3630	4000	5730	8000	13200	6530	4640	3710	3040
		2	4510	4920	5590	6800	11600	9200	3950	2460	1270
Mean			**3985**	**4033**	**5498**	**7217**	**8702**	**6678**	**4112**	**2960**	**2014**
SD			**503**	**559**	**1273**	**2314**	**4589**	**3074**	**871**	**829**	**692**
% reduction				**-1**	**-38**	**-81**	**-118**	**-68**	**-3**	**26**	**49**
t test: *p* value									**0.793**	**0.048**	**0.007**
Cal 5339	Wet	1	2780	3350	4870	9400	15600	24600	44000	50400	62000
		2	3050	3920	4760	6940	11200	18800	37800	45000	64600
Cal 5339	Dry	1	3220	3660	4450	5480	5320	4150	3010	2030	1210
		2	3550	4030	4820	5040	5410	4070	2880	2660	1500

% reduction: negative results represents % increase in counts

Cau: *C. auris*, Cal: *C. albicans,* cfu: colony forming unit

*: Comparisons 0 hrs to wk1, wks 2 and wks 3

3.2.1.5 Steel surfaces

Results of the survival of biofilm on the **steel surfaces** are shown in table 3.12 and figure 3.12. In addition, percentage reduction was calculated. A paired samples t-test was conducted to compare the counts at 0 hours to those at 1 week, 2 weeks and 3 weeks separately for the surface when wet and when dry. In the wet condition, significant increase in the counts was achieved when comparing 0 Hours to 1 week and thereafter (*p* value – 0.002). In the dry condition as well, statistical significance was achieved when 0 hours was compared to 1 week and thereafter (*p* value – 0.006).

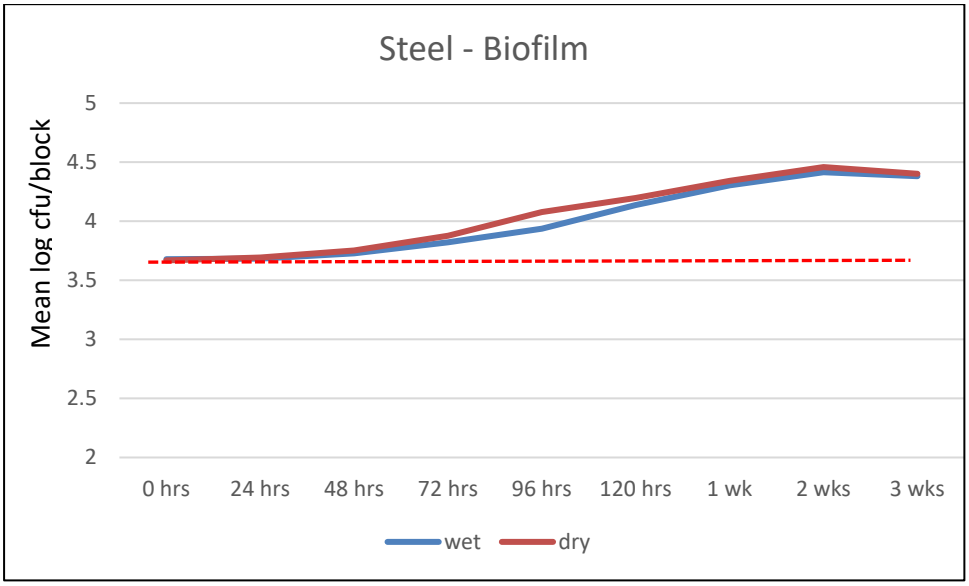


Figure 3.12 Survival of Biofilms of *C. auris* on wet and dry steel surface. Red dotted line represents baseline counts at 0 hrs.

Table 3.12 Survival of Biofilms of *C. auris* on wet and dry steel surface

Strain	Type	Repeats	Biofilm cells- cfu/steel block at various exposure time								
			0 Hrs*	**24 hrs**	**48 hrs**	**72 Hrs**	**96 hrs**	**120 Hrs**	**1 Wk***	**2 Wks***	**3 Wks***
Cau 4888	Wet	1	5370	5640	5900	6800	8200	12400	19600	28200	26400
		2	4400	4450	4520	5000	6200	8600	11800	16000	12400
Cau 3499	Wet	1	5500	5940	7400	8600	12200	23800	29600	42000	51200
		2	3990	4400	4660	6220	9400	15000	21400	32200	42200
Cau 6326	Wet	1	5410	5060	5610	8400	10800	17400	29000	26600	18000
		2	4210	3810	4520	5560	6600	10000	15600	19000	15200
Mean			**4813**	**4883**	**5435**	**6763**	**8900**	**14533**	**21167**	**27333**	**27567**
SD			**686**	**812**	**1130**	**1477**	**2359**	**5561**	**7123**	**9359**	**15802**
% reduction				**-1**	**-13**	**-41**	**-85**	**-202**	**-340**	**-468**	**-473**
t test: *p* value									**0.002**	**0.002**	**0.016**
Cau 4888	Dry	1	5410	5420	6010	7400	11600	14800	15000	25400	20000
		2	3850	4640	4560	5600	8600	11200	13000	23200	23600
Cau 3499	Dry	1	4850	5730	7800	10400	15400	26600	29000	35800	43000
		2	4340	4350	5980	7400	11800	17400	39800	48200	57200
Cau 6326	Dry	1	5410	5330	5460	7200	15400	17000	26400	22200	15800
		2	4320	4310	4690	8000	10400	11600	18800	25000	14000
Mean			**4697**	**4963**	**5750**	**7667**	**12200**	**16433**	**23667**	**29967**	**28933**
SD			**637**	**606**	**1179**	**1563**	**2728**	**5622**	**10087**	**10180**	**17324**
% reduction				**-6**	**-22**	**-63**	**-160**	**-250**	**-404**	**-538**	**-516**
t test: *p* value									**0.006**	**0.002**	**0.019**
Cal 5339	Wet	1	3670	3800	5720	8200	10400	13200	33000	52400	52800
		2	3910	3760	4890	6030	7400	9000	16000	42200	46000
Cal 5339	Dry	1	4710	3410	5260	7600	11800	14600	36800	46000	48000
		2	3250	3160	3830	5380	8400	13600	19000	35200	43000

% reduction: negative results represents % increase in counts
Cau: *C. auris*, Cal: *C. albicans,* cfu: colony forming unit
*: Comparisons 0 hrs to wk1, wks 2 and wks 3

3.2.2 Comparison of surfaces wet and dry combined with biofilm

To compare the survival of biofilm form of *C. auris* on various surfaces, the results of wet and dry surfaces were combined and the mean counts are depicted in table 3.13. The log mean counts are shown in Figure 3.13. The results showed that after 3 weeks there was an increase in the *C. auris* counts on all the surfaces.

Table 3.13 Comparison of biofilm form of *C. auris* counts on various surfaces including wet and dry conditions

Strain	Type	Surface	Mean Biofilm cells – cfu/ block at various exposure time								
			0 hrs	24 Hrs	48 hrs	72 hrs	96 hrs	120 hrs	1 wk	2 wks	3 wks
All	Wet and dry	Glass	4367	4549	5838	7630	12433	15917	20067	25800	29500
		% reduction									**-575.52**
		Fabric	4314	4544	5893	7336	9129	10833	15217	18300	17483
		% reduction									**-305.26**
		Plastic	4265	4738	5331	6222	7944	10583	16317	22533	24103
		% reduction									**-465.13**
		Wood	3801	4388	6366	8808	12851	14473	18756	20797	23690
		% reduction									**-523.25**
		Steel	4755	4923	5593	7215	10550	15483	22417	28650	28250
		% reduction									**-494.11**

% reduction: negative results represents % increase in counts, cfu: colony forming unit

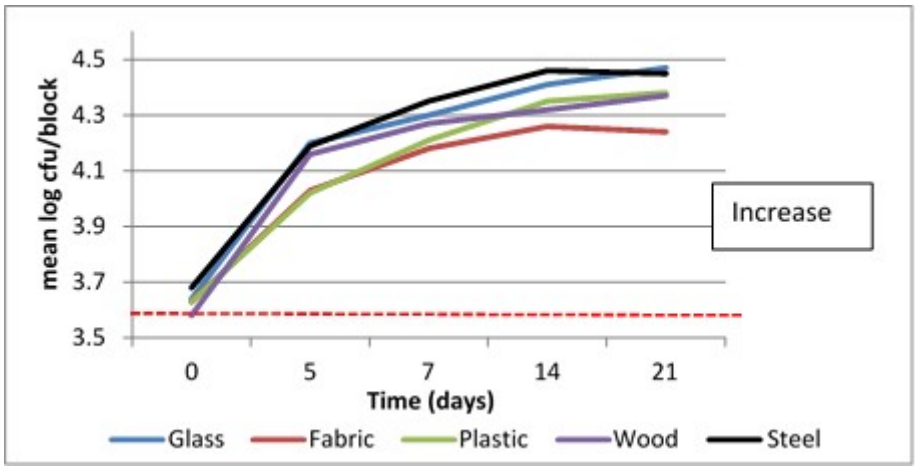


Figure 3.13 Comparison of biofilm form of *C. auris* counts on various surfaces including wet and dry conditions. Red dotted line represents baseline counts at 0 hrs. After 3 weeks, the counts of *C. auris i*ncreased on all the surfaces.

3.2.3 Comparison of wet and dry condition combining all the surfaces with biofilms

To compare the survival of biofilm form of *C. auris* in wet and dry conditions, the results of all the surfaces were combined and the mean counts are depicted in table 3.14. The log mean counts are shown in Figure 3.14. The results showed that after 3 weeks there was an increase in the *C. auris* counts in wet and dry condition. A paired samples t-test was conducted to compare biofim *C. auris* growth on wet and dry surfaces for various surfaces combined. The results showed that for both wet and dry conditions increase in counts was significant only at 3 weeks (table 3.14) with *p* value of 0.001.

Table 3.14 Comparison of biofilms of *C. auris* counts in wet and dry conditions combining various surfaces

Strain	**Surface**	**Type**	**Mean biofilm cell count in cfu/ block at various exposure time**								
			0 Hrs*	**24 hrs**	**48 hrs**	**72 Hrs**	**96 hrs**	**120 hrs**	**1 Wk***	**2 Wks***	**3 Wks***
All	All	Wet	4194	4680	5869	7570	10920	14707	20860	25980	28994
		p value							0.08	0.08	**0.001**
		Dry	4406	4577	5739	7314	10243	12209	16249	20452	20216
		p value							0.08	0.08	**0.001**

*: Comparisons 0 hrs to wk1, wks 2 and wks 3, cfu: colony forming unit

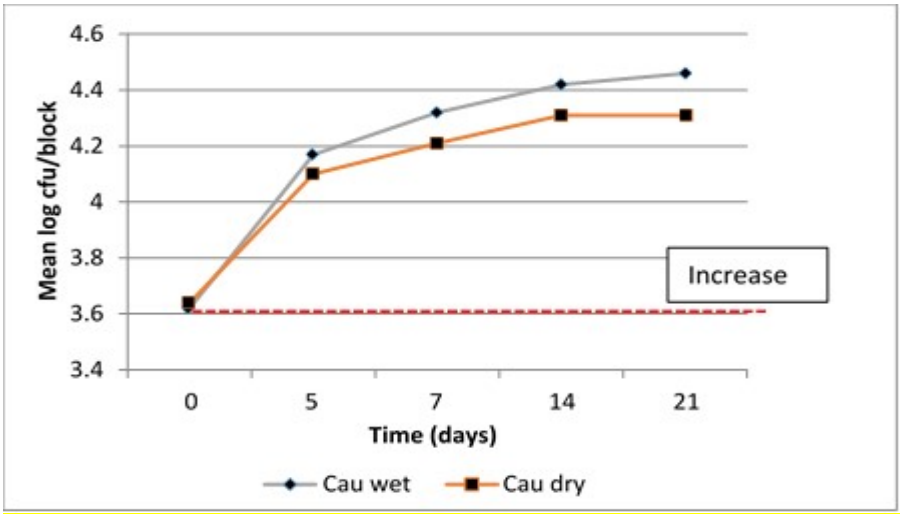


Figure 3.14 Comparison of biofilm form of *C. auris* counts in wet and dry conditions on various surfaces. Red dotted line represents baseline counts at 0 hrs. After 3 weeks, the counts of *C. auris i*ncreased on wet and dry surfaces.

3.3 Comparison of planktonic and biofilm forms of *C. auris*

3.3.1 Comparison of planktonic cells to biofilm cells combining all the surfaces

In order to compare the *C. auris* counts in the planktonic and biofilm forms, the results of all the surfaces and the two, wet and dry conditions were combined. The mean counts are shown in table 3.15 and the mean log counts are plotted in figure 3.15. The results showed that after 3 weeks the count increased in both the forms of growth. However, the increase was more in the biofilm after 3 weeks compared to the planktonic form. A paired samples t-test was conducted to compare biofilm and planktonic *C. auris* growth for all various surfaces under both wet and dry conditions. Significant increase in the counts was noted after 1 week and thereafter with *p* values of 0.000 (Table 3.15).

Table 3.15 Comparison of planktonic counts of *C. auris* to biofilm combining all the surfaces, and wet and dry conditions.

Strain, surfaces, wet and dry	Growth form	Mean cell count / wet and dry blocks at various exposure time -cfu/block								
		0 Hrs*	24 hrs	48 hrs	72 hrs	96 Hrs	120 hrs	1 Wk*	2 Wks*	3 Wks*
All	planktonic	4092	3589	5475	7148	6950	8276	8039	8132	7836
	% reduction							-96.46	-98.73	-91.5
	p value							0.000	0.000	0.000
	Biofilm	4300	4629	5804	7442	10582	13458	18555	23216	24605
	% reduction							-331.51	-439.91	-472.21
	p value							0.000	0.000	0.000

% reduction: negative results represents % increase in counts
*: Comparisons 0 hrs to wk1, wks 2 and wks 3, cfu: colony forming unit

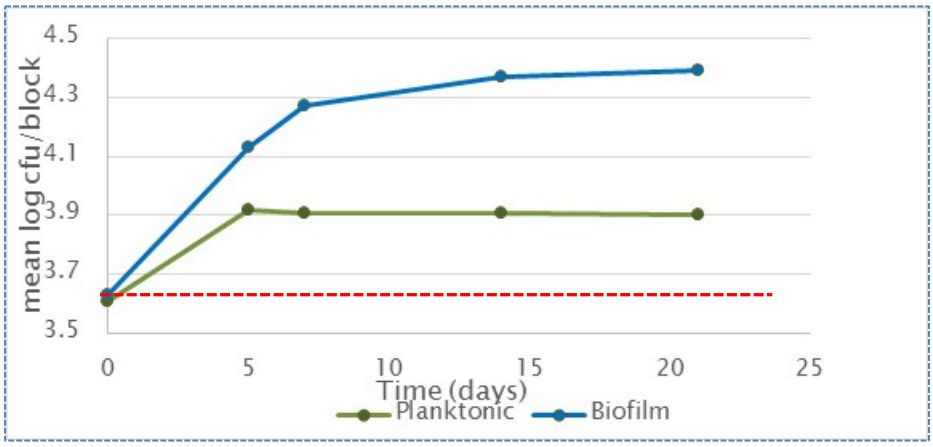


Figure 3.15: Comparison of planktonic *C. auris* counts to biofilm counts over 3 weeks combining all the surfaces under wet and dry conditions. Red dotted line represents baseline counts at 0 hour. Within the 3 weeks period, the counts of *C. auris* increased in planktonic as well as biofilm form with a greater increase in the biofilm form of *C. auris*.

3.3.2 Comparison of planktonic cells to biofilm cells in wet and dry condition combining all the surfaces

When the results of wet and dry biofilm combining all the surfaces were compared, it was shown that the counts after 3 weeks increased in the wet and dry biofilm. Whereas in dry planktonic form of *C. auris* began to decrease after 1 week (Table 3.16, Figure 3.16)

Table 3.16 Comparison of *C. auris* planktonic cells to biofilm cells in wet and dry condition combining all the surfaces

Strain, surfaces, wet and dry	**Type**	**Mean cell count/ wet and dry blocks at various exposure time-cfu/block**								
		0 hrs	**24 hrs**	**48 hrs**	**72 hrs**	**96 Hrs**	**120 hrs**	**1 wk**	**2 wks**	**3 wks**
Planktonic	Wet	3987	3591	5789	8424	8037	9953	11479	11859	11648
	% reduction									**-192.15**
	Dry	4196	3588	5160	5872	5863	6599	4599	4404	4023
	% reduction									**4.12**
Biofilm	Wet	4194	4680	5869	7570	10920	14707	20860	25980	28994
	% reduction									**-591.32**
	Dry	4406	4577	5739	7314	10243	12209	16249	20452	20216
	% reduction									**-358.83**

% reduction: negative results represents % increase in counts, cfu: colony forming unit

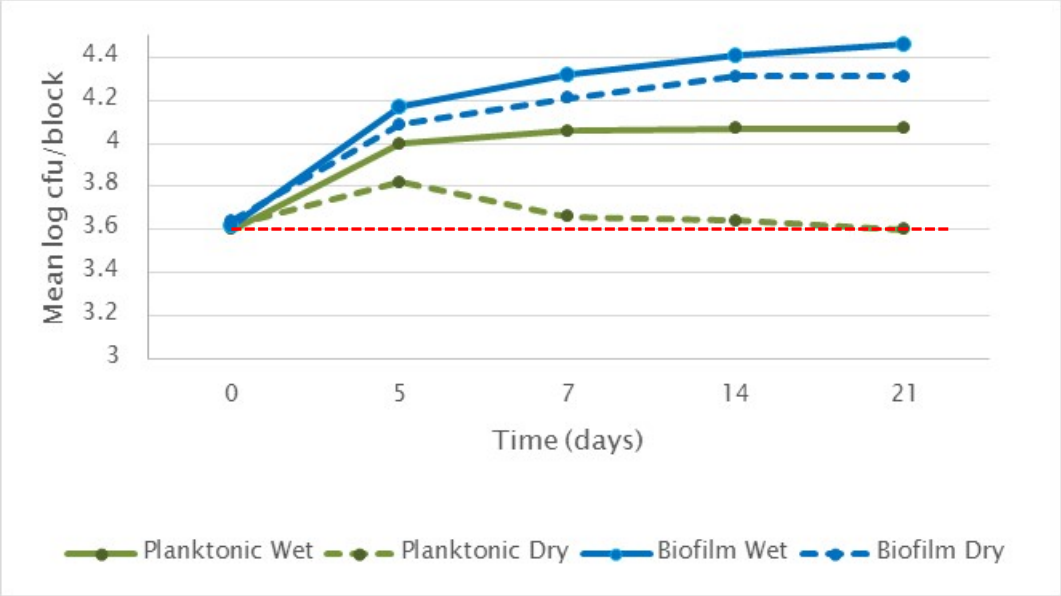


Figure 3.16: Comparison of planktonic cells to biofilm cells in wet and dry condition combining all the surfaces. Red dotted line represents baseline counts at 0 hrs. Within the 3 weeks period, the counts of *C. auris i*ncreased in planktonic cells on wet, biofilm cells on wet and dry surfaces. Whereas planktonic cells on dry surfaces started declining at day 5.

3.4 Comparison of planktonic and biofilm forms of *C. albicans* strain Cal5339

To compare the survival of biofilm and planktonic forms of *C. albicans* on various surfaces, the results of wet and dry surfaces were combined and the mean counts are depicted in table 3.17 and table 3.18. The log mean counts are shown in Figure 3.17. The results showed that after 3 weeks there was an increase in the *C. albicans* counts for both planktonic and biofilm forms, with the biofilm cells increasing more than the planktonic cells.

Table 3.17 Survival of Planktonic *C. albicans* strain Cal5339 on wet and dry glass, fabric, plastic, wood and steel surfaces

			Planktonic cell count/ wet and dry blocks at various exposure time-cfu/block								
Surface	**Type**	**Repeats**	**0hrs**	**24hrs**	**48hrs**	**72hrs**	**96hrs**	**120hrs**	**1wk**	**2wks**	**3wks**
Glass	Wet	1	4590	3680	2100	3490	3360	5980	63000	2700	1900
		2	3170	2300	2140	3010	3600	5020	58000	2200	1640
	Dry	1	3610	1870	2640	2500	2770	3300	57600	2980	1320
		2	3590	1830	2080	2180	2450	3500	50000	2940	800
Fabric	Wet	1	3640	4940	18200	21400	18400	15600	5690	4460	4100
		2	2760	4300	14400	18000	19400	16200	4570	3840	2920
	Dry	1	3620	3790	23800	20200	17400	9000	6040	4430	3430
		2	3060	3350	17400	19600	15400	7200	5000	4890	3170
Plastic	Wet	1	4940	5490	4400	5130	5490	4890	4680	2730	2670
		2	4930	4490	4460	3870	4650	4050	3560	2370	2090
	Dry	1	3770	4040	4100	4700	4090	3940	3280	3100	1680
		2	3150	3060	3920	4380	3910	3960	3200	2460	1300
Wood	Wet	1	3110	2950	4440	6320	15400	19600	43000	45400	62000
		2	3030	3700	4660	6070	10400	16800	35800	40400	52800
	Dry	1	3900	2220	4450	4480	3920	4050	2580	2130	910
		2	3650	3060	4820	5060	4610	4000	2800	2660	1500
Steel	Wet	1	3390	3800	5820	8200	10400	11600	13000	26000	32800
		2	4410	3750	3790	6070	7800	9000	10000	22200	26000
	Dry	1	3460	3440	5260	5920	11800	13400	16800	22400	26000
		2	3150	3160	3820	5080	8400	14200	19600	27200	33400
Mean			**3646.5**	**3461**	**6835**	**7783**	**8682.5**	**8764.5**	**20410**	**11374.5**	**13121.5**
SD			**628.15**	**961.89**	**6241.33**	**6344.78**	**5756.31**	**5397.34**	**21849.07**	**13887.42**	**18927.40**
% reduction				**5.09**	**-87.44**	**-113.44**	**-138.11**	**-140.35**	**-459.71**	**-211.93**	**-259.84**

% reduction: negative results represents % increase in counts, cfu: Colony forming unit

Table 3.18 Survival of Biofilm *C. albicans* strain Cal5339 on wet and dry glass, fabric, plastic, wood and steel surfaces

			Biofilm cell count/ wet and dry blocks at various exposure time-cfu/block								
Surface	**Type**	**Repeats**	**0hrs**	**24hrs**	**48hrs**	**72hrs**	**96hrs**	**120hrs**	**1wk**	**2wks**	**3wks**
Glass	Wet	1	4520	5680	10200	11400	15400	23600	32400	36200	41600
		2	4570	5300	13200	14800	19800	25200	36000	42000	47800
	Dry	1	4810	5870	11400	16600	17800	26800	35600	43200	45800
		2	5590	7200	13800	18200	22600	31600	44600	48800	51200
Fabric	Wet	1	3640	4740	7000	9200	11400	15600	19800	24200	24600
		2	2860	4100	5800	7200	10400	16200	22000	33400	32400
	Dry	1	3820	4490	8200	10600	13200	19000	26200	29800	39600
		2	3170	3550	7400	11600	14800	25200	24400	26800	43400
Plastic	Wet	1	5220	5010	5600	5730	8400	9600	19600	29800	37200
		2	4460	4230	4660	4970	5450	7200	12600	25400	32000
	Dry	1	4770	4940	5410	5750	6400	10000	15000	29400	30600
		2	4350	3760	4320	4900	5910	7200	12400	22800	33800
Wood	Wet	1	2780	3350	4870	9400	15600	24600	44000	50400	62000
		2	3050	3920	4760	6940	11200	18800	37800	45000	64600
	Dry	1	3220	3660	4450	5480	5320	4150	3010	2030	1210
		2	3550	4030	4820	5040	5410	4070	2880	2660	1500
Steel	Wet	1	3670	3800	5720	8200	10400	13200	33000	52400	52800
		2	3910	3760	4890	6030	7400	9000	16000	42200	46000
	Dry	1	4710	3410	5260	7600	11800	14600	36800	46000	48000
		2	3250	3160	3830	5380	8400	13600	19000	35200	43000
Mean			**3996**	**4398**	**6779.5**	**8751**	**11354.5**	**15961**	**24654.5**	**33384.5**	**38955.5**
SD			**818.55**	**1020.64**	**3021.59**	**4003.07**	**5052.09**	**8134.26**	**12497.05**	**13982.13**	**16310.88**
% reduction				**-10.06**	**-69.66**	**-118.99**	**-184.15**	**-299.42**	**-516.98**	**-735.45**	**-874.86**

% reduction: negative results represents % increase in counts, cfu: Colony forming unit

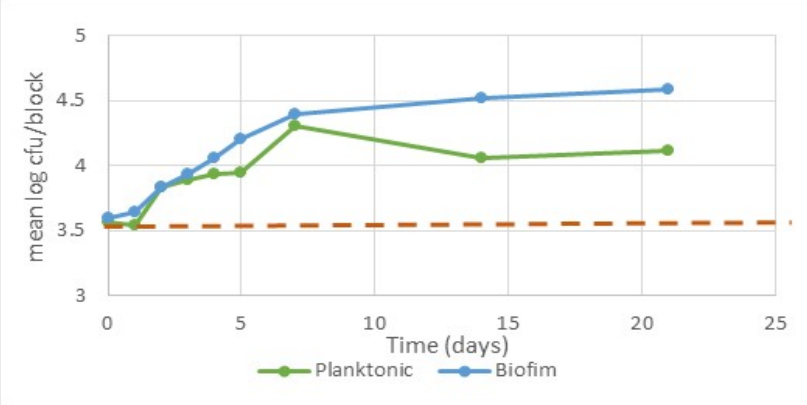


Figure 3.17: Comparison of planktonic and biofilm counts of the ***C. albicans*** strain Cal5339 combining all the surfaces, and wet and dry conditions. Red dotted line represents baseline counts at 0 hrs. After 3 weeks, the counts of *C. albicans* increased in planktonic as well as biofilm form of *C. albicans* regardless of the type of wet and dry surfaces with biofilm form increasing more than planktonic form.

3.5 Disinfectant study

3.5.1 Minimum inhibitory concentrations of disinfectants (MIC)

The MIC of four different disinfectants was tested for three *C. auris* strains (and one strain of *C. albicans*). The first disinfectant tested was **70% Ethanol** which is mostly used in the asepsis of skin, hands, goods and environmental surfaces and is at times also used as a pre-surgical antiseptic in solution with other disinfectants. This disinfectant presented an MIC range of 4.38-8.75% for the different *C. auris* strains (table 3.25). A quaternary ammonium compound **BC** which is used as an antiseptic and for surface disinfection presented MIC range of 5.94-11.88%. The third disinfectant tested was **Sodium dichloroisocyanurate (NaDCC)** which is a chlorine releasing chemical agent that is often utilizing for antisepsis, disinfection and also decontamination of surfaces in hospitals yielded MIC range of 31.25-62.50 ppm for the *C. auris* strains. The last disinfectant tested was **HP** which yielded the same MIC reading of 0.05% for all the *C. auris* strains. This disinfectant (HP) is a strong oxidant agent that showed the most disinfection activity in 24 hours as observed by MIC in comparison to the other three 3 disinfectants. HP is utilized in cleaning surfaces and the treatment of effluents among other applications.

Table 3.19 Minimum inhibitory concentrations of disinfectants (MIC) of disinfectants against *C. auris*

Disinfectant	Strain	MIC	Median
70% Ethanol	Cau 4888	8.75%	8.75%
	Cau 3499	4.38%	
	Cau 6326	8.75%	
NaDCC	Cau 4888	31.25 ppm	62.50 ppm
	Cau 3499	62.50 ppm	
	Cau 6326	62.50 ppm	
Benzalkonium Chloride	Cau 4888	5.94%	11.88%
	Cau 3499	11.88%	
	Cau 6326	11.88%	
Hydrogen Peroxide	Cau 4888	0.05%	0.05%
	Cau 3499	0.05%	
	Cau 6326	0.05%	

70% Ethanol	Cal 5339	8.75%	
NaDCC	Cal 5339	250 ppm	
Benzalkonium Chloride	Cal 5339	11.88%	
Hydrogen Peroxide	Cal 5339	0.01%	

Cau: *C. auris*, Cal: *C. albicans,* MIC: Minimum inhibitory concentrations

3.5.2 Time-kill study of disinfectants

In order to determine the percentage of *C. auris* cells killed at various time interval by these test disinfectants, a time-kill study was performed. Results of the %-kill are shown in table 3.20 – 3.23.

Ethanol at 70% concentration achieved 100% kill within 30 seconds. The %-kill was lower at lower concentrations. As the contact time increased, the %-kill also increased for all the concentrations (Table 3.20).

Sodium dichloroisocyanurate at 500 ppm concentration achieved 100% kill within 30 seconds. The %-kill was lower at lower concentrations. As the contact time increased, the %-kill also increased for all the concentrations (Table 3.21).

BC at 95% concentration achieved 100% kill within 30 seconds. The %-kill was lower at lower concentrations. As the contact time increased, the %-kill also increased for all the concentrations (Table 3.22).

HP at 3 % concentration achieved 100% kill only after 5 minutes. The %-kill was lower at lower concentrations. As the contact time increased, the %-kill also increased for all the concentrations (Table 3.23).

From these results, it was evident that some killing of *C. auris* was occurring even at subinhibitory concentrations of all the disinfectants. Chlorhexidine killed all the *C. auris* strains within 30 sec., whereas media control had growth even after 5 minutes.

Table 3.20: Mean percentage kill of *C. auris* cells obtained for different concentrations of ethanol (Eth)

Time (min)	(Starting inoculum:$2.10x10^6$/ml) Mean % kill at Concentration		
	70%	**35%**	**17.5%**
0.5	95.88	54.91	27.84
1	100	53.81	30.19
1.5	100	57.84	33.33
2	100	61.17	34.11
2.5	100	61.96	35.68
3	100	65.68	37.64
3.5	100	69.71	40.78
4	100	72.57	41.96
45	100	78.28	42.35
5	100	69.71	43.52

Table 3.21: Mean percentage kill of *C. auris* cells obtained for different concentrations of sodium dichloroisocyanurate (NaDCC)

Time (min)	(Starting inoculum:$2.36x10^6$/ml) Mean % kill at Concentration		
	500 ppm	**250 ppm**	**125 ppm**
0.5	100	53.22	36.61
1	100	57.59	47.54
1.5	100	60.0	53.87
2	100	63.72	54.53
2.5	100	66.77	58.47
3	100	67.86	60.87
3.5	100	78.80	62.19
4	100	90.16	63.93
45	100	94.97	66.99
5	100	95.25	68.74

Table 3.22: Mean percentage kill of *C. auris* cells obtained for different concentrations of benzalkonium chloride

Time (min)	(Starting inoculum:$1.64x10^6$/ml) Mean % kill at Concentration		
	95%	**47.5%**	**23.75%**
0.5	100	42.19	41.46
1	100	44.87	42.43
1.5	100	47.56	44.88
2	100	50.73	46.09
2.5	100	53.17	46.83
3	100	54.14	48.29
3.5	100	57.80	49.02
4	100	60.10	50.49
45	100	61.46	52.44
5	100	64.15	54.15

Table 3.23: Mean percentage kill of *C. auris* cells obtained for different concentrations of hydrogen peroxide

Time (min)	(Starting inoculum:$1.84x10^6$/ml) Mean % kill at Concentration		
	3%	**1.5%**	**0.75%**
0.5	68.57	29.12	17.67
1	70.20	30.33	18.11
1.5	74.69	33.0	18.65
2	77.55	34.67	19.67
2.5	78.98	39.58	20.88
3	82.04	41.32	22.47
3.5	86.12	43.33	23.0
4	91.63	45.33	25.78
45	96.12	48.42	26.56
5	100	50.56	28.33

3.5.3 Pulsing of *C. auris* with disinfectant

In order to study the effect of disinfectants on the *C. auris* cells, cells were exposed to the disinfectants daily for 15 days and then the MIC study was repeated. Table 3.24 shows the new

MIC readings in "after pulsing" column while the before exposure (initial) readings are represented in the "before pulsing" column.

The results showed that MIC values increased with the limited daily exposure of disinfectants particularly with BC and NaDCC which suggested that these disinfectants may have made genotypic or phenotypic changes in these exposed cells of *C. auris*. Strain Cau4888 was selected for further studies since the MICs increased for NaDCC and BC. Repeats were performed to establish the increase in the MIC values (Table 3.25).

Table 3.24: MIC results of *C. auris* and *C. albicans* against disinfectants before and after the 15 day limited exposure to sub-inhibitory concentrations.

Before pulsing				After pulsing	
Disinfectant	Strain	MIC	Median	MIC	Median
70% Ethanol	Cau 4888	8.75	8.75%	8.75	8.75%
	Cau 3499	4.38		8.75	
	Cau 6326	8.75		8.75	
NaDCC	Cau 4888	31.25	62.50 ppm	125	250 ppm
	Cau 3499	62.50		250	
	Cau 6326	62.50		250	
Benzalkonium Chloride	Cau 4888	5.94	11.88%	23.75	23.75%
	Cau 3499	11.88		23.75	
	Cau 6326	11.88		47.5	
Hydrogen Peroxide	Cau 4888	0.05	0.05%	0.02	0.05%
	Cau 3499	0.05		0.05	
	Cau 6326	0.05		0.05	
70% Ethanol	Cal 5339	8.75%		8.75%	
NaDCC	Cal 5339	250 ppm		500 ppm	
Benzalkonium Chloride	Cal 5339	11.88%		23.75%	
Hydrogen Peroxide	Cal 5339	0.01%		0.05%	

Cau: *C. auris*, Cal: *C. albicans,* MIC: Minimum inhibitory concentrations

Table 3.25: MIC results of *C. auris* Cau4888 against disinfectants before and after the 15 day limited exposure.

C. auris	Disinfectant		MIC			
		Repeats	Untreated		Treated	
Cau 4888	NaDCC		MIC	Median	MIC	Median
		1	31.25	31.25 ppm	62.5	125 ppm
		2	31.25		125	
		3	31.25		125	
		4	31.25		125	
		5	31.25		250	
		6	31.25		250	
		7	62.5		250	
Cau 4888	Benzalkonium Chloride					
		1	5.94	5.94%	11.88	11.88%
		2	5.94		11.88	
		3	5.94		11.88	
		4	5.94		11.88	
		5	5.94		23.76	
		6	11.88		23.76	
		7	11.88		47.52	

3.5.4 Ergosterol quantification of disinfectant exposed *C. auris*

Drug resistance mechanisms in *C. auris* varies depending on the type of therapeutic drug. Azoles resistance can be due to over expression of efflux pumps, point mutation in *ERG11* gene responsible for the Lanosterol 14-alpha-demethylase which converts lanosterol to ergosterol. Overexpression of *EGR11* gene increased production of Lanosterol 14-alpha-demethylase, which overpowers the capacity of the antifungal to inhibit the activity of the protein and results into accumulation of active protein (Chaabane et al., 2019). In *C. auris*, although, resistance to disinfectants is known, disinfectant tolerance is known (Mataraci-Kara et al., 2020, Kean et al., 2018). This resistance/tolerance to disinfectants could be due to the environmental stress or due to the biofilm formation (Kean et al., 2018, Ku et al., 2018). After

disinfection, persistence of few live *C. auris* cells have been noted suggesting some resistance to the applied disinfectant. This phenomenon was simulated by pulsing *C. auris* with disinfectants, and as shown above the MIC values changed after the exposure to the disinfectants. Therefore, although the effect of disinfectant on the synthesis of ergosterol is not known, an attempt was made to measure the quantities of ergosterol and the expression of *EGR11* gene in these disinfectant exposed strains of *C. auris*.

The ergosterol quantities of the isolates were measured by absorbance using a spectrophotometer and the 4 peak graphs were plotted- as figure 3.18. The levels of ergosterol production for the treated isolates in comparison to the untreated isolates were calculated and tabulated (table 3.26). The untreated samples are assumed to be 100% ergosterol production, and anything above or below this percentage is either an under production (decrease) or over production (increase) in ergosterol.

Results showed that there was a decrease in quantities of ergosterol when *C. auris* was treated with BC, NaDCC and ethanol. Whereas there as an increase in ergosterol quantities when *C. auris* was treated with hydrogen peroxide. Although only one strain of *C. albicans* was included in the study, similar pattern of results was seen in these organisms.

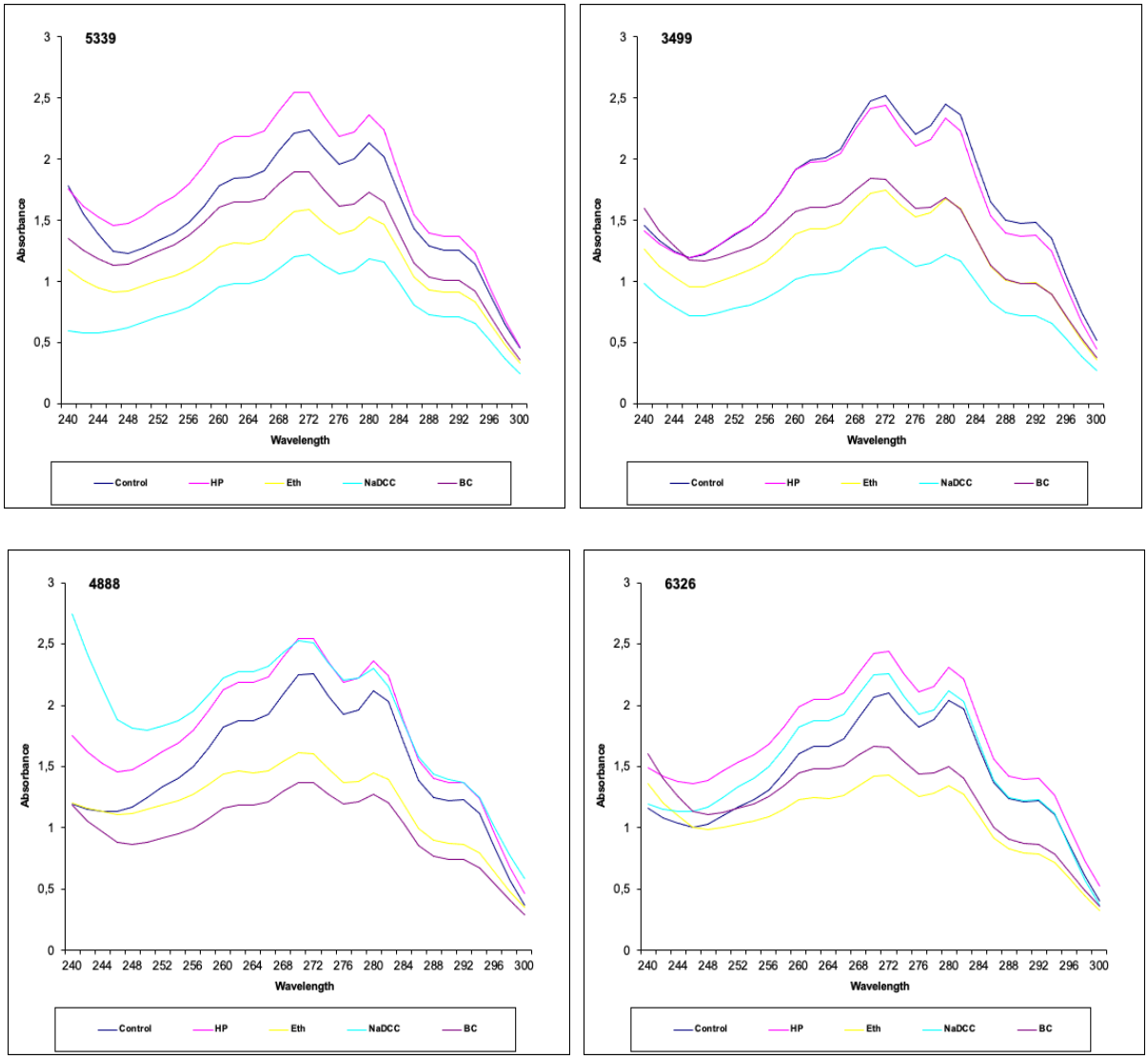


Figure 3.18: Ultra-Violet spectrophotometric sterol profile representing the quantification of ergosterol in the *C. auris* strains Cau4888, Cau6326 and Cau3499 and *C. albicans* strain Cal5339 after 15 days of disinfection pulsing with 5 different disinfectants separately. The spectra profile was determined between 240 and 300 nm. Control represents the untreated isolates, while HP represents isolates that have been treated with hydrogen peroxide, Eth represents ethanol treated isolates, NaDCC represent isolates that have been treated with sodium dichloroisocyanurate and BC represents isolates that were treated with benzalkonium chloride.

Table 3.26: Quantities of ergosterol in disinfectant treated *C. auris* and *C. albicans.*

Disinfectant	Strain	Quantity of Ergosterol (nm)	% Ergosterol production	Difference in % Ergosterol (control-Test)	Mean difference in % ergosterol
Ethanol	Cau 4888	0.00581	72.81	-27.19	-25.36 (decrease)
	Cau 3499	0.00447	57.00	-43.00	
	Cau 6326	0.00489	74.12	-5.88	
NaDCC	Cau 4888	0.00898	112.53	12.53	-14.17 (decrease)
	Cau 3499	0.00253	32.22	-67.78	
	Cau 6326	0.00743	112.74	12.74	
Benzalkonium Chloride	Cau 4888	0.00379	47.49	-52.51	-36.3 (decrease)
	Cau 3499	0.00489	62.37	-37.63	
	Cau 6326	0.00536	81.23	-18.77	
Hydrogen Peroxide	Cau 4888	0.00892	111.78	11.78	14.78 (increase)
	Cau 3499	0.00799	101.73	1.73	
	Cau 6326	0.00863	130.84	30.84	
Ethanol	Cal 5339	0.00187	77.75	-22.25	Decrease
NaDCC	Cal 5339	0.00117	48.53	-51.47	Decrease
Benzalkonium Chloride	Cal 5339	0.00211	87.63	-2.37	Decrease
Hydrogen Peroxide	Cal 5339	0.00391	162.43	62.43	Increase

3.5.5 Effect of disinfectants on the *ERG11* gene expression

3.5.5.1. Standard PCR

To further analyze the effect of disinfection on the production of ergosterol in *C. auris* (15 days pulsed), the expression of *ERG11 genes* was studied. Firstly, standard PCR was performed to assess for the presence of *ERG11* genes and the results are shown in figure 3.19 and 3.20. The level of *ERG11* expression was then tested for using quantitative/real-time PCR, and the results are shown in figure 3.11 and table 3.27.

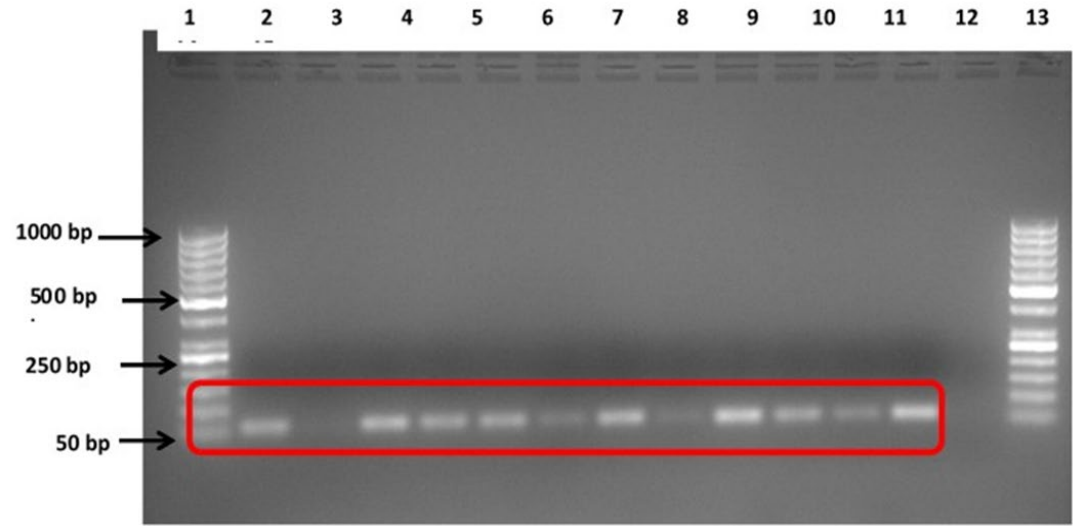


Figure 3.19. A 2% agarose gel profile following amplification of cDNA derived from *C. auris* and *C. albicans*. Lane 1: 50 bp DNA ladder, used to estimate the size of amplified PCR products. Lane 2-5: *Candida* strains (in the order Cau4888, Cau3499, Cau6326, Cal5339) treated with HP. Lane 6-9: *Candida* strains (in the order Cau4888, Cau3499, Cau6326, Cal5339) treated with Ethanol. Lane 10-13: *Candida* strains (in the order Cau4888, Cau3499, Cau6326, Cal5339) treated with BC. Lane 14: Negative control (i.e., sample without DNA in PCR reaction mix) Lane 15: 50 bp DNA ladder, used to estimate the size of amplified PCR products.

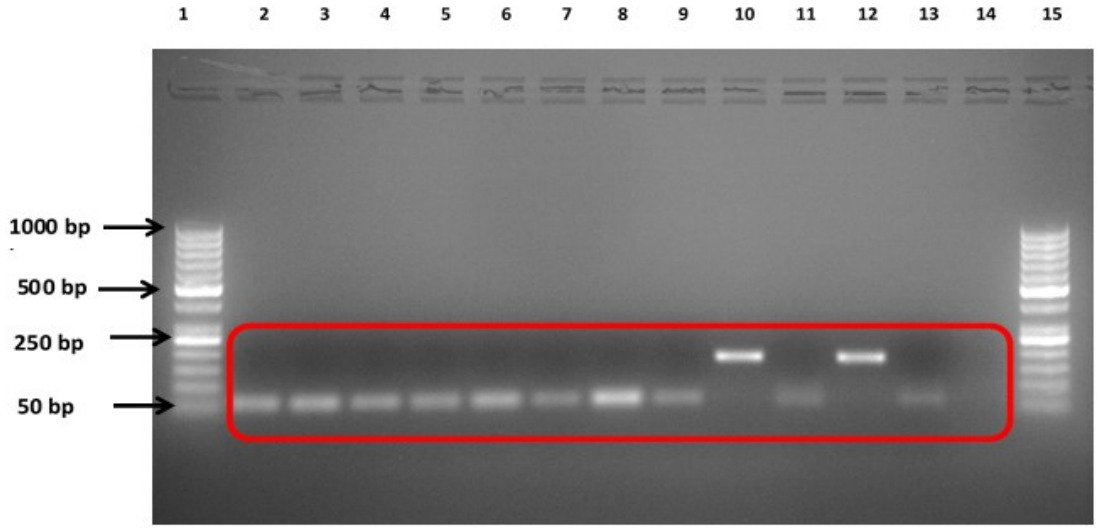


Figure 3.20. A 2% agarose gel validating amplification of *Candida* cDNA. Lane 1: 50 bp DNA ladder, used to estimate the size of amplified PCR products. Lane 2-5: *Candida* strains (in the order Cau4888, Cau3499, Cau6326, Cal5339) treated with NaDCC. Lane 6-9: Untreated *Candida* strains (in the order Cau4888, Cau3499, Cau6326, Cal5339). Lane 10-13: house-keeping genes. Lane **10:** Cal5339- actin encoding gene (*ACT*), **Lane 11:** Cau6326- actin encoding

gene (*ACT*), **Lane 12:** Cal5339- actin encoding gene (*ACT*), **Lane 13:** Cau6326- actin encoding gene (*ACT*), Lane 14: Negative control (i.e., sample without DNA in PCR reaction mix). Lane 15: 50 bp DNA ladder, used to estimate the size of amplified PCR products.

The *actin* primers (house-keeping gene is used as a control to verify the validity of the technique) that were employed in our gene expression experiments were designed to specifically target and amplify the *ACT* gene of *C. albicans* as its nucleotide has been extensively characterised relative to the recently discovered *C. auris*. However, as a result of nucleotide similarities between *C. albicans* and *C. auris* at a genus level, these primers were also able to amplify the *ACT* gene in *C. auris*. Consequently, following amplification of the *ACT* gene region by conventional PCR, the DNA fragment sizes corresponding to *C. auris and C. albicans* were different. This disparity is due to genetic variations in the *ACT* gene sequence between the two fungal pathogens at a species level.

3.5.5.2. Real time PCR

The gene expression level was calculated by applying the formula:

$$\Delta Cq = 2^{\text{Cp target gene-Cp reference gene}}$$

Where the crossing point (Cp) was the average Cp value of target gene minus the mean of the *16S* reference gene.

Fold change in gene expression was determined by 84elativizing the mean $2^{\text{Cp target gene}}$ in treated samples to the average $2^{\text{Cp target gene}}$ in untreated samples (Table 3.27).

Gene expression data were expressed as the mean ± standard error of the mean (±SEM). Statistical analysis was carried out using the non-parametric test (GraphPad Software Inc, CA, USA) and data were considered statistically significant when $p < 0.05$.

The results showed (Figure 3.21) that there was a significant overexpression of *ERG11* gene in HP treated *C. auris* (4 – 9-folds). There was a slight overexpression of *ERG11* gene in NaDCC treated *C. auris* as well and it was significant. However, there was an under expression or no

change in expression of *ERG11* gene when *C. auris* was treated with BC and ethanol. Similar pattern of results was found in *C. albicans*.

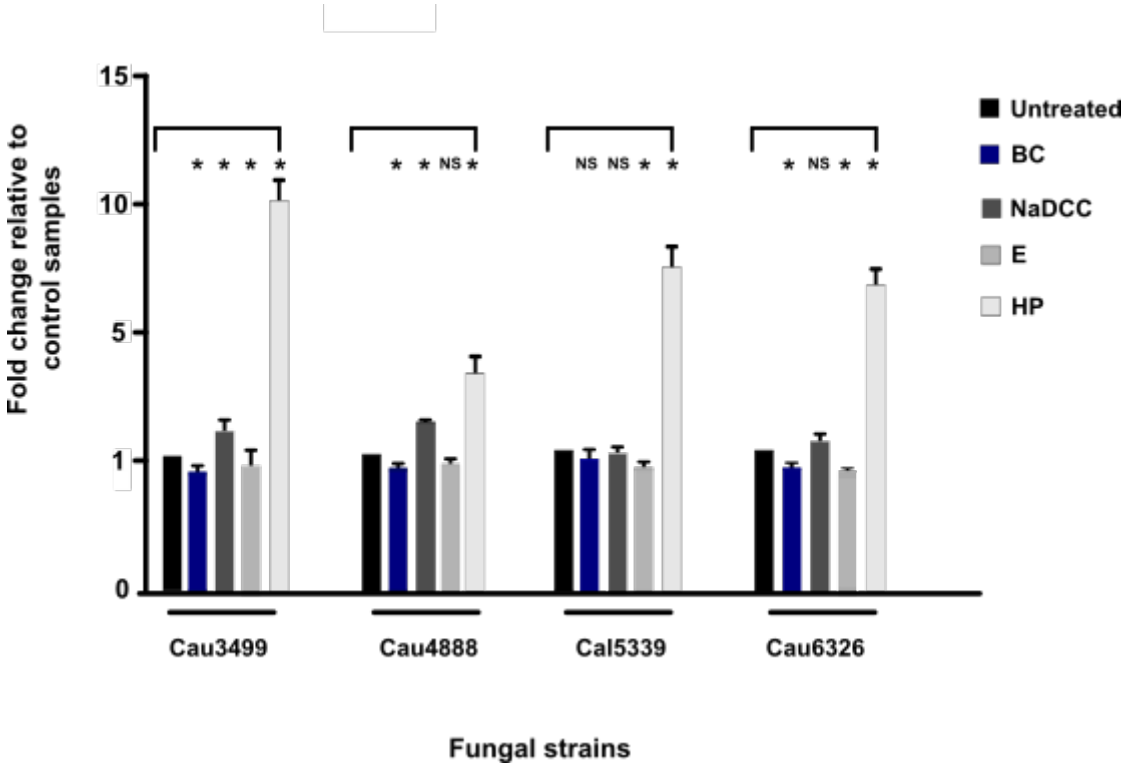


Figure 3.21: Fold change in gene expression relative to control (untreated) samples graphed for the different strains for the tested disinfectants. Statistical significance was determined and ***** indicates statistical significance, **(p <0.05)** while **NS** indicated no statistical significance **(p >0.05)**. Untreated represents untreated isolates, HP represents isolates that have been treated with Hydrogen Peroxide, E represents Ethanol treated isolates, NaDCC represent isolates that have been treated with Sodium dichloroisocyanurate and BC represents isolates that have been treated with Benzalkonium Chloride.

Table 3.27: *ERG11* expression quantity (ΔCp) results for *C. auris* and *C. albicans* as observed by real-time PCR.

Disinfectant	Strain	Repeats	*ERG11* expression (ΔCp)	Mean ΔCp
Untreated	Cau 4888	1	15483365016.14	35012569671.41
		2	66378639047.02	
		3	22356882807.57	
	Cau 3499	1	169207199709.49	244035365241.76
		2	100611202885.06	
		3	462287693130.72	
Eth	Cau 4888	1	26220933517.29	26714107678.17
		2	26586964105.81	
		3	27334425411.43	
	Cau 3499	1	68244796603.35	101762584683.08
		2	178855062684.26	
		3	58187894761.62	
NaDCC	Cau 4888	1	54291225517.54	65246176075.65
		2	107087564374.00	
		3	34359738335.41	
	Cau 3499	1	60658887984.86	82138451080.48
		2	53916208625.86	
		3	131840256630.71	
BC	Cau 4888	1	48929992110.08	52150869264.42
		2	58592623572.16	
		3	48929992111.02	
	Cau 3499	1	61505654506.24	89195286010.42
		2	114773609666.36	
		3	91306593858.67	
HP	Cau 4888	1	24128179207.79	34739628956.91
		2	28102900705.70	
		3	52806629100.74	
	Cau 3499	1	86381301313.76	93332780227.99
		2	85784622077.09	
		3	107832417293.11	
Untreated	Cal 5339	1	217164902170.77	178241994553.14
		2	120479772908.25	
		3	197081308580.39	
Eth	Cal 5339	1	67305250321.26	64722075866.94
		2	78937949845.91	
		3	47923027433.63	
NaDCC	Cal 5339	1	76249033489.21	91797107586.18
		2	99916230541.79	
		3	99226058727.55	
BC	Cal 5339	1	97184015965.70	113397567837.05
		2	128235077874.67	
		3	114773609670.76	
HP	Cal 5339	1	30540401941.67	31405526091.65
		2	32058769440.93	
		3	31617406892.35	

3.5.5.3. Whole genome sequencing of disinfectant exposed *C. auris*

Whole-genome sequencing was carried out on treated and untreated *C. auris* strain Cau4888 based on the MIC results in Table 3.24 and 3.25. The BC-treated Cau4888 strain was found to have more genetic variations (SNPs) than NaDCC-treated Cau4888. Most of the SNPs occurred upstream of the genes, possibly in the promoter regions (Table 3.28 and Table 3.29). Several mutations were identified in the assembled genomes of the treated Can4888 strain few proteins of the mutated genes were identified, this is largely because the genes in the *Candida auris* genomes are not characterized.

Some of the proteins identified included the Electron transfer flavoprotein-ubiquinone oxidoreductase, Peptidyl-prolyl cis-trans isomerase A, Sensor histidine kinase RcsC, Ribosomal RNA small subunit methyltransferase F, Bifunctional ligase/repressor BirA, Elongation factor G and putative metabolite transport protein CsbC.

Table 3.28: Results of SNPs identified in BC treated Cau4888 mapped against untreated CAU4888 *C. auris*.

Position on genome	Type of mutation	Reference (untreated Can4888)	Alteration	Evidence	Gene name	Protein name	Type of strand
53528	deletion	ACTATTG	A	A:56 ACTATTG :0		hypothetical protein	exon
224012	SNP	A	G	G:60 A:1		Electron transfer flavoprotein-ubiquinone oxidoreductase	CDS
215068	SNP	T	A	A:80 T:5		hypothetical protein	CDS
319515	SNP	G	T	T:68 G:2	*ppiA1*	Peptidyl-prolyl cis-trans isomerase A	gene
170408	SNP	T	C	C:64 T:3		hypothetical protein	CDS
55860	SNP	T	C	C:70 T:2		hypothetical protein	CDS
105927	SNP	T	G	G:55 T:2			gene
39639	insertion	T	TG	TG:53 T:2	*rcsC3*	Sensor histidine kinase RcsC	gene
197258	SNP	C	T	T:51 C:0	*rsmF1*	Ribosomal RNA small subunit methyltransferase F	gene
138609	deletion	GT	G	G:66 GT:0	*birA*	Bifunctional ligase/repressor BirA	gene
SNP	SNP	C	T	T:60 C:2	*Fus*	Elongation factor G	gene
8619	deletion	ACAGT	A	A:76 ACAGT:0	*csbC*	putative metabolite transport protein CsbC	gene

Table 3.29: Results of SNPs identified in NaDCC treated Cau4888 mapped against untreated CAU4888 *C. auris*.

Position on genome	Type of mutation	Reference	Alteration	Evidence	Gene name	Protein name	Type of strand
311627	SNP	T	C	C:25 T:2		hypothetical protein	CDS
58947	SNP	C	A	A:11 C:0		hypothetical protein	mRNA

CHAPTER 4 DISCUSSION

4. Discussion

Many different types of surfaces such as steel, wood, plastic, glass and fabric are used in the healthcare setting and they are necessary for the type of functions. For example, bed cover can only be fabric and in-dwelling devices can only be plastic. And therefore, studies such as this has to include all types of surfaces. These surfaces come across many pathogens and become contaminated. As also shown in this study, if these surfaces are not disinfected, pathogens can either diminish or flourish over a time period depending on the type of surface, contamination (planktonic or biofilm) and wet/dry conditions.

In a planktonic forms, regardless of wet or dry, wood and steel supported growth of *C. auris* (Table 3.6). However, when the biofilm was allowed to develop, all the surfaces supported growth of *C. auris* (Table 3.13). This results highlights the importance of immediate thorough cleaning and disinfection of surfaces. It is known that dry surfaces cannot support growth of microorganisms for a long time, unless they have stress regulatory mechanisms or produce spores. This study has shown a similar pattern. In a wet condition, planktonic *C. auris* cell counts increased whereas in a dry condition, the counts decreased by 60% (Table 3.7). However, in the biofilm form whether the surfaces were dry or wet, there was an increase in *C. auris* counts which further highlights the importance of continuous disinfection using appropriate disinfectant and methods (Table 3.7 and 3.14).

In this study the *C. auris* counts on surfaces were determined daily for a week because it was not known whether these organisms were going survive longer than a week. The daily results were not analysed because the organism survived longer than a week and the time period between week 1 and 3 showed meaningful results and comparisons of surfaces and conditions. Nevertheless, from observation, it can be seen that the survival was surface dependent. Wood and steel supported growth of *C. auris* (planktonic) with an increase in counts in first 5 days whereas, plastic, glass and fabric showed stationary growth (Figure 3.6). However in the biofilm form, there was an exponential increase in counts even in first 5 days regardless of the type of surface (Figure 3.3).

Candida auris has been found to cause outbreaks mainly in hospital facilities and elderly nursing homes. This pathogen spreads through surface contamination, medical devices, patient to patient infections and also between patients to or from health care workers (Sabino et al.,

2020). In a study to compare the human immune response to *C. auris* and *C. albicans* strains, it was found that *C. auris* cells have the ability to invade neutrophil attack along with the innate immune response (Nett, 2019). This thus explains why *C. auris* infections results in high mortality rates, particularly among immune compromised individuals. Even though the expression of virulence factors is weaker for *C. auris* when compared to other *Candida* species, this pathogen has the ability to develop resistance to a number of antifungal agents and which makes more important compared to the other species. Unlike other *Candida* species which are mostly commensals of the human skin and gastrointestinal tract (which only cause infections when balance is disturbed), *C. auris* has been found to thrive on the skin and colonize patients within a few hours of exposure and is typically associated with nosocomial transmission (Nett, 2019). Its ability to survive on surfaces further enhances the nosocomial transmission.

4.1. Survival of planktonic and biofilm *C. auris* and the effects of wet and dry surfaces

Environmental surfaces play a vital role in human life, particularly indoor environmental surfaces and this is because we are in touch with these surfaces daily. As such when these environments become contaminated with pathogens, they pose a serious health hazard (Haleem Khan & Mohan Karuppayil., 2012). This is important in health care facilities where patients and health care workers are constantly moving between different hospital rooms, thereby spreading pathogens among other occupants of the health care facilities, including the high-risk immune compromised patients. It has been previously reported that there is a direct correlation between indoor fungal concentration and public health problems and the occurrence of human diseases, this means that contamination of health care facilities can lead to increased occurrence of infections and health complications, which may result in more contamination of the facilities' equipment and environmental surfaces and thus an endless circle of health care complications (Tamburini et al., 2015).

Although planktonic experiments may have some adherent cells of *C. auris*, the results were different from mature biofilm associated *C. auris* counts (Figure 3.15 and 3.16). This suggest that during the study time period of 3 weeks, initially applied planktonic cells did not form as much biofilm as mature biofilm. Perhaps there were aggregated cells rather than a biofilm because the reduction in the counts after 3 weeks was more in the plantktonic experiments. Biofilm would have formed if there was some available nutrient, which was not provided in the experiment. These results highlights the importance of immediate disinfection after contamination of surfaces in the hospital environment. In addition, presence of organic material

such as blood, serum and saliva can enhance the biofilm formation (Short et al., 2019) suggesting of importance of thorough cleaning and disinfection, immediately after contamination of surfaces.

4.1.1. Glass Vs. Steel surface

In a study done by Tamburini et al., *(*2015) six surfaces were tested for microoganisms' deposition and contamination after a period of 30 days of placing them in a laboratory working bay used for normal lab activities, of the six environmental surfaces tested, five were dominated by fungal contamination except for glass. They found that glass was in fact contaminated more with bacteria than fungi. Among the other five surfaces they tested, was stainless steel which had more fungal contamination compared to glass. The results in this study are incongruent with these findings. In the planktonic cells study, we found that there were more viable cells surviving on stainless steel surfaces (148.24% increase from 0 day to 3 weeks) compared to glass surfaces after 3 weeks (figure 3.6). In fact, glass had the least *C. auris* counts for planktonic cells with a 92.55% reduction in cell counts. This may be due to the difference in surface roughness of the materials (By observation, stainless steel and glass were the smoothest surface materials we tested). An increase in surface roughness results in an increased ability of microorganisms to bind and adhere to the surface. As such, Fungi cells are large and can hardly adhere to the smooth glass surface, and so this can be explained by dimension interaction in between surface pores and microbial cell size. The reduced adhesion of fungi then reduces the probability of permanent adhesion and thus reducing the chances of cell survival. Stainless steel has a surface film that protects the steel from corrosion and an unstable surface charge, these can play a role in the adhesion of the fungi. Even though stainless steel has antimicrobial activities the surface antimicrobial agents are biodegradable and so the fungus may have been able to degrade these compounds (Tamburini et al*.,* 2015). For biofilm cells however, glass had more *C. auris* growth than steel and this thus shows that when adhesion is induced (as biofilms) the chances of cell survival on this surface material (glass) are increased. Both glass and steel had the most surface cell growth for biofilm cells as compared to the other tested surface materials.

4.1.2. Wood surfaces

Although some wood species are known to have antimicrobial activity, the organic and porous nature of wood may allow penetration and proliferation of microorganisms (Munir et al., 2020). In our study, the wet wood surfaces appeared to promote the growth of the fungi regardless of

the form of the fungi (whether planktonic or biofilm) in comparison to when dry wood surfaces, which did not promote growth. Wood is a cellulosic material, consisting of 40 – 44 % of cellulose arranged in very discrete units that are known as elementary fibrils. Although cellulose can be degraded/ digested by a number of microorganisms, it is reinforced by lignin polymers which makes it difficult to degrade/colonise/invade. This lignocellulose matrix behaves as a rigid structural system that prevents microbial invasion. In addition, to these cell wall polymers, components such as fatty acids, proteins, starch, pectin, along with other sugars and polysaccharides which form on average 6% of the wood mass have dramatic effects with regard to wood resisting microbial adhesion and invasion (Goodell et al., 2020).

Even though the structure and make up of wood makes it difficult for fungal adhesion and invasion, moisture affects the wood properties, causing dimensional changes (Goodell et al., 2020). This thus allows invasion of the wood by the fungi and since wood is made of sugars and polysaccharides it is possible that once the fungi is able to invade the polymer matrix it then begins to feed on some of the sugars of the wood, thus the continued rise of *C. auris* growth on wet wood surfaces. From figure 3.6 and Table 3.6 it can be seen that wood had the most *C. auris* in planktonic experiments as compared to all the other surface, with a percentage increase of 389.27%. Even though wood did not have the highest *C. auris* counts in the biofilm experiments, it still had a notable 523.25% increase in *C. auris* cell count (Table 3.13).

D-xylose and cellobiose –fermenting and xylanase-producing yeasts have been found in decaying wood (Morais et al., 2013, Ali et al., 2017, Lara et al., 2014, lopes et al., 2018) which contributes towards the wood degradation. Up to 10^6 cfu/cm^2 of yeasts have been isolated from benches and wooden mates in spa establishments which suggests that wood allows the adherence and establishment of yeast even in a starved conditions (Svorcová, 1982). *Candida albicans* and *C. tropicalis* can produce xylanase and/or β-xylosidase and utilize D-xylose as a carbon source which suggests that wood can provide some nutrient to these yeasts (Cadete et al., 2017). Not much is known about biochemical properties of *C. auris*, perhaps it produces some enzymes that can cause microscopic degradation and obtain some nutrient showing tremendous rise in counts in this study. Wet wood may have further facilitated this process. Further research is required to understand this phenomenon. Similar phenomenon has been seen in a study conducted on wooden water storage containers and the survival of cholera causing bacterium *Vibrio cholera* (Patel and Isaacson, 1989). In this study it was shown that *V. cholera* survived for 21 days in water stored in wooden containers without any nutrient and during this time the counts increased from the initial counts of 13000 cfu/ml to 24000 cfu/ml).

They suggested that the presence of organic matter in the wooden containers may have been responsible for this survival of *V. cholera*.

4.1.3. Fabric surfaces

The findings of this investigation showed that *C. auris* is able to survive on both dry and wet fabrics for at least 21 days. Nosocomial pathogens including Candida species are known to survive on different types of fabric for more than a month (Koca et al., 2012). Critically ill patients spend most of their time in bed, and are thoroughly in contact with the bedding and also the hospital clothing. Since *C. auris* is able to persist and survive on fabrics for such a notable period of time, if these hospital linen is not disinfected properly, or not changed regularly, cross contamination can occur. Similarly, the hospital clothing worn by the health-care workers can become contaminated, and spread *C. auris* throughout health care facilities (Biswal et al., 2017). Our study has thus shown that the tested *C. auris* (and *C. albicans*) is able to remain viable for at least 21 days on all the tested surface environmental material including plastic which are very common in hospital facilities. This shows that the chances of spread of *C. auris* in the hospital environment is high. This thus emphasizes the importance of *C. auris* (and *C. albicans*) infection prevention through infection control as well as rapid detection in health care facilities (Welsh et al., 2017).

4.1.4. Wet Vs dry surfaces

There was a significant difference between the survival of *C. auris* on wet and dry surfaces. Figure 3.7 shows that on wet surfaces planktonic *C. auris* had a percentage increase of 13.06% in cell growth while on dry surfaces there was a percentage reduction of 3.23%. For biofilm cells on the other hand (figure 3.4), there was a percentage increase in cell counts on both wet (23.20%) and dry (18.41) surfaces. It has been previously reported that the dependence of fungi on moisture for growth depends on the genus or species (Haleem Khan & Mohan Karuppayil., 2012). From the findings of this study, it is clear that the tested *C. auris* strains (along with the *C. albicans* strain) do actually require moisture in order to thrive. This means that keeping the hospital surfaces dry at all times after contacts with liquids is important in minimizing transmission of *C. auris.*

The biofilm forms of the cells appeared to thrive more than the planktonic cells as observed in figure 3.5, with biofilm cells having a 20.95% increase in cell count while planktonic cells only had 8.03% percentage increase in cell counts. This is owed to the fact that binding of

microorganism to a surface increases the chances of cell survival. In addition, attachment to surfaces can regulate the secretion or production of certain bio products that increases the cells' chances to survive, for example the upregulation of alginate synthesis by *Pseudomonas spp* to strengthen cell-substrate binding (Tamburini et al., 2015). The biofilm form of this pathogen thrives even better in the presence of moisture, thus emphasizing the importance of keeping environmental surfaces dry, particularly in health-care facilities where this pathogen causes outbreaks.

4.1.5. Survival of *C. auris* under 1 week

Since the survival of *C. auris* beyond 1 week was not known, the counts were determined daily for a week, then weekly for 3 weeks. The findings of this investigation showed that the first 7 days of surface contamination are crucial, since the the growth is exponential (figure 3.13-3.16). This is regardless of the type of surface and whether the surface is wet or dry. The cell counts only start either declining or stationary after 5-7 days of contamination. This suggests that it is of utmost importance that within the first 7 days the contaminated surface should be cleaned thoroughly and preferably daily to ensure complete decontamination of the surface. Other findings of this study have also shown that the type of disinfectant used, exposure time and concentration are also very important and should be taken into consideration when decontaminating surfaces.

4.1.6. Survival of *C. albicans*

Table 3.17 and 3.18 along with figure 3.17 shows the survival of biofilm and planktonic *C. albicans* cells over a 3-week period for all surfaces combined. From the results, there was an increase in both the amount of planktonic and biofilm cells, with biofilm cells increasing more than planktonic cells. Like *C. auris, C. albicans* cells also show an exponential growth in the first 7 days of contact with surface, followed by a constant growth after the 7 days. This further shows how dire the first 7 days of *Candida* surface contamination are in terms of the rate of possible infection and the need to disinfect surfaces as early as possible. When compared to *C. auris,* the rate of growth depicted by *C. albicans* is much higher. Planktonic *C. auris* cells had an average percentage increase of 91.5% after 3 weeks while biofilm *C. auris* cells had an average increase of 472.21% (table 3.15). On the other hand, planktonic *C. albicans* cells had an average percentage increase of 259.84% and biofilm cells had an increase of 874.86% (tables 3.17 and 3.18). This shows that *C. albicans* persist and thrive better on surfaces than *C. auris* and this may be due to the rarity of pseudohyphae in *C. auris* as compared to *C. albicans,*

as a result *C. albicans* adhere better to surfaces and have an increased ability to form biofilms (Nett, 2019).

4.1.7. Increase of *C. auris* on certain surfaces

In dry condition, on glass and plastic, if the experiment began with planktonic cells, no increase in counts was noted. On the other hand, in the similar dry condition, on fabric, wood and steel, increase in the counts was noted. This phenomenon is rare however, three strains of *C. auris* with two repeats and all experiments performed on different days and months showed the same pattern and therefore these results can be taken as valid results. There are two explanations for these increases in counts. Although planktonic form of cells were placed onto these surfaces, the duration of these experiments was 3 weeks therefore the adherent cells may have multiplied depending on the type of surfaces and adherence ability of *C. auris* (in this experiment fabric, wood and steel). Atmospheric moisture may have played a role too because in these experiments, desiccants were not placed into the jars containing surface blocks to simulate a day-to-day situation. Through esterase activity, Welsh et al., (2017) have also shown the survival of *C. auris* on plastic surface to be for 28 day with a slight increase in counts on day one. Nevertheless, microbial kinetics of re-contamination of surfaces after cleaning has shown that fungal contamination and establishment on surfaces is greater than bacteria suggesting the attachment abilities of fungi (Tamburini et al., 2015).

In wet condition, only the glass surface did not support growth. On the rest of the test surfaces up to 7 days there was an increase in the counts (Fabric and plastic) and even up to 3 weeks (wood and steel). Although no data is available for *C. auris*, *C. albicans* is known to survive in water without any nutrient for more than 196 days and the counts were found to be $>10^4$ cfu/ml even after 6 months (Richards et al., 2010). Similarly, starved *C. albicans* has shown to survive for more than 240 days with a less than 1 log reduction (from log 7 to 6.2) in counts (Ning et al., 2013). Study performed by Piedrahita et al., (2017) also showed that wet steel surface supported growth of *C. auris*. They measured the survival for 7 days with an increase at day one and nonsignificant change in counts at 7 days compared to the inoculum. Studies reporting the survival of *C. auris* on steel surface longer than 7 days could not be found and therefore our results could not be compared. Nevertheless, this study has shown that there is a greater influence of the surface material and the wet/dry conditions.

4.2.Disinfection Study (Effect of suboptimum concentrations of disinfectants)

4.2.1. Minimum Inhibitory Concentration (MIC)

The disinfectants tested in this study are used in health care facilities for sterilization and disinfection of different environmental surfaces as well as some of the equipment and resources used in these facilities (Mazzola et al., 2009). The MIC method allows the selection of a better performing commercial disinfectant that may be used for disinfection against the investigated pathogen or microorganism based on comparison with other disinfectants tested for the same pathogen. For this particular investigation, the better performing disinfectant in comparison to the other tested disinfectants after a 24 hour exposure against *C. auris* is HP. Even though the MIC method allows the determination of the minimum concentration of the disinfectants needed to inhibit the growth of pathogens, this method does not allow the analog comparison between the activities of the different disinfectants, and it also shows inhibition of the pathogens' growth after a 24 hour exposure, which is impractical with regard to how the disinfectants are utilized for surface disinfection in the health care facilities (Mazzola et al., 2009). Thus, it became necessary to do a time kill analysis for the four (4) investigated disinfectants over a 5 minute time period exposure in order to simulate a more practical setting for surface disinfection in health care facilities. MIC results were also necessary for the comparison of efficacy of disinfectants before and after pulsing. This could provide indication of tolerance and effective concentrations.

4.2.2. Time Kill Study

The time kill study showed that the tested disinfectants are effective against *C. auris* (and *C. albicans*), however disinfectant concentration and exposure times are vital. Two of the disinfectants at their highest tested concentrations (i.e., 500 ppm NaDCC and 95% BC) were able to kill 100% of the pathogens within 30 seconds exposure time while 70% Ethanol was only able to kill 100% of the pathogens after 1 minute exposure time. With regards to HP, when an MIC study was conducted at a 24-hour exposure time this disinfectant had the lowest MIC compared to the other disinfectants however with the time kill study it was the least effective of all disinfectants, thus requiring a longer contact time period (more than 5 minutes) to kill 100% of this pathogen. 500 ppm NaDCC and 95% BC did the most killing in the least time. Thus, among the four tested disinfectants, the above two are worth recommending for use to disinfect hospital facilities. While 70% ethanol is also effective, it requires a longer contact time (1 minute) and this might be impractical since ethanol evaporates very quickly.

Disinfectants must meet certain requirements which are: practical use, quick action, surface compatibility, odor lessness, non-toxic and have residual effect etc. Alcohol does not meet the requirement of quick action and a minute of exposure is impractical in a work situation. NaDCC is a chlorinated compound and at 500 ppm concentration could be used with effectiveness in 30 seconds. However, it can corrode metals and therefore can only be used on certain surfaces. BC, at a recommended concentration of 95% can be used with an efficacy within 30 seconds.

Time-kill study was conducted using lower concentrations of disinfectants to show the percentage-kill of *C. auris*. This simulates the situation where "short cuts" are taken, and proper recommended concentrations are not used. In this situation, certain percentage of *C. auris* cells will die but some will still survive. The fate of these surviving cells will depend on the type of surface and wet/dry condition as shown in this study in terms of growth and death. These organisms can survive for 3 weeks and perhaps even longer.

In addition, if these disinfectants have a residual effect, these surviving *C. auris* cells can be exposed to these residues of sublethal concentrations of disinfectant for a time period. In this situation, the effect of these disinfectants on these surviving cells, their cell wall and genome are not known. Therefore, further investigation was carried out to understand the mechanism of action of disinfectants after pulsing with sublethal concentrations. The results showed that with NaDCC and BC, the MIC values increased after 15 days intermittent exposure. This may have been due to a phenotypic changes or genotypic changes. Phenotypic changes could be reversible but genotypic changes can be permanent. Either way, in this situation, some degree of resistance has developed showing increased MIC values especially in one strain of *C. auris* against two (NaDCC and BC) disinfectants. In general, drug resistance can be due to the efflux pump where the organism pumps the drug out of the cell, degradation of drug and/or binding of the drugs with cellular material which render the drug inactive. In *Candida* species azole group of drugs that are commonly used that target cell membrane associated ergosterol. Gene *ERG11* is one of the genes responsible for the synthesis of ergosterol. Therefore, the effect on the quantity of ergosterol and the gene expression was studied.

4.2.3. Ergosterol quantification and *ERG11* gene expression in disinfectant exposed *C. auris*

Ergosterol has become a target for most antifungal agents, particularly azoles (Flowers et al., 2015). These antifungal agents inhibit ergosterol synthesis which is important for cell wall fluidity, this then results in the accumulation of toxic methylated sterol precursors which are

harmful to the fungi leading to cell death. The ergosterol assay was performed on disinfectant treated and untreated strains of *C. auris* (table 3.26). The results showed that *C. auris* treated strains had a decrease in ergosterol production for all disinfectants except for HP. While NaDCC induced an increase in ergosterol production in two of the treated strains (Cau4888 and Cau6326), HP induced an increase in ergosterol production in all the treated samples, including the *C. albicans*. The change in the MIC results after the disinfectant exposure (NaDCC and BC) cannot be explained from these ergosterol results. One of the limitations of this assay is the quantification of the pellet of the cells after centrifugation and the calculations are based on these readings. If the pellet is not drained off completely, remaining fluid may increase the quantity of pellet. Nevertheless, this method is still useful and, in our experiments, overall mean ergosterol results showed that only HP treated *C. auris* cells had increased ergosterol quantity.

When the *ERG11* gene expression was studied, the results showed that in HP exposed *C. auris*, the gene expression was significantly increased. This correlates to the increase in the ergosterol quantities (Table 4.1). This increase in ergosterol quantity did not change the MIC results meaning the pulsing did not confer resistance however, the changes might be related to the external stress and a mechanism to deal with it. Perhaps the required pulsing time period was longer than 15 days in order to see any changes in the MIC values. In future this can be explored. Over expression of *ERG11* gene is also known in azole resistant *Candida species,* which may have occurred in HP exposed *C. auris and C. albicans*. However, it was not the aim of this study and therefore was not studies.

Although not as much an HP, NaDCC-exposed two strains of *C. auris,* showed increase in ergosterol quantities and overexpressed *ERG11* gene. These results correlates with the increase in the MIC readings. These results require further investigations to understand the development of disinfectant tolerance and resistance (to recommended concentrations). Furin et al., (2021) has shown that *C. auris* persisting on the health-care surfaces may enter a viable but nonculturable (VBNC) state. These VBNC organisms are generally metabolically inactive but still alive and on resuscitations they begin normal growth pattern (Xie et al., 2021). Perhaps similar phenomenon is occurring in disinfectant exposed *C. auris*. This VBNC state after disinfectant exposure is also known in bacteria from enterobacteriacae group (Alvear-Daza et al., 2021).

Table 4.1 Summary results: MIC assay, ergosterol quantification and *ERG11* gene expression

Organism	Disinfectant	Change in MIC	Ergosterol quantity	*ERG11* expression
C. auris	Ethanol	No change	Decrease	Slight decrease
	NaDCC	Increase	Decrease (increase in 2 strains)	Slight increase
	BC	Increase	Decrease	Slight decrease
	HP	No change	Increase	Increase
C. albicans	Ethanol	No change	Decrease	No change
	NaDCC	No change	Decrease	No change
	BC	No change	Decrease	Decrease
	HP	No change	Increase	Increase

4.2.4. Effects of disinfectant on the *ERG11* gene expression

4.2.4.1.Whole gene Sequencing

A few of the proteins of the mutated genes were identified, this includes Electron transfer flavoprotein-ubiquinone oxidoreductase, Peptidyl-prolyl cis-trans isomerase A, Sensor histidine kinase RcsC, Ribosomal RNA small subunit methyltransferase F, Bifunctional ligase/repressor BirA, Elongation factor G and putative metabolite transport protein CsbC and these were all identified on BC treated Cau4888.

Electron transfer flavoprotein-ubiquinone oxidoreductase is a monotopic membrane protein that is found in the mitochondria, and it forms part of the mitochondrial respiratory chain along with electron transport flavoprotein (Watmough & Frerman, 2010). This protein transfers electrons from 11 mitochondrial flavoprotein dehydrogenases to the ubiquinone pool (Watmough & Frerman, 2010). This enzyme is therefore involved in the energy production of the pathogenic cells and a mutation on the gene that codes for this enzyme suggests that BC may be affected this gene hindering energy levels in the treated cells. This has been observed in humans, whereby the deficiency of Electron transfer flavoprotein-ubiquinone oxidoreductase leads to a disorder known as multiple acyl-CoA dehydrogenase deficiency which results in the body not being able to meet the energy needs required, particularly for

major organs such as the (Watmough & Frerman, 2010). Another mutation in Peptidyl-prolyl cis-trans isomerase A coding gene was noted. Peptidyl-prolyl cis-trans isomerase A is an enzyme that catalyzes the cis-trans isomerization of proline imidic peptide bonds in oligopeptides, thus accelerating the folding of proteins (Takahashi et al, 1989). This enzyme also controls the activity, transport, and subunit assembly of some of the protein. A mutation in this enzyme can lead to conformational changes in some of the protein for which this enzyme catalyzes protein folding and subunit conformation which can cause a change in the transportation of other proteins (Takahashi et al, 1989).

Another mutation was found on a gene that codes for Sensor histidine kinase RcsC. This enzyme is a membrane-associated protein kinase that phosphorylates RcsD in response to environmental stresses or signals which in turn triggers the response regulator RcsB, thus inducing adaptive responses (Santos & Shiozaki, 2001). Since Sensor histidine kinase RcsC is a stress response protein, the mutation on the gene that codes for this protein (which was an insertion) was most likely in response to the stress that was placed on the cells by the disinfectant BC.

A mutation was also found on Ribosomal RNA small subunit methyltransferase F encoding the gene *Rsmf*. This enzyme is responsible for the methylation of cytosine of the 18S rRNA. A mutation in in this gene can lead to errors in translation and elongation phases of protein synthesis and this has previously been associated with resistance to antibiotics such as kasugamycin in bacteria (Demirci et al, 2010). The Elongation factor G gene was also mutated, which can also lead to errors in the elongation phase of protein synthesis in disinfectant treated *C. auris*.

Bifunctional ligase/repressor BirA regulates the synthesis and uptake of biotin. Biotin is a micronutrient that is essential for metabolic enzymes that are biotin dependent. The inability of the pathogens to fulfill their cellular biotin demand can have dire consequences on their cell viability and virulence. A mutation in Bifunctional ligase/repressor BirA, therefore, suggests a metabolic adaptation to the stress placed on the cells by the disinfectant and since this protein serves both as a transcriptional repressor regulating biotin synthesis genes, as well as a ligase that catalyzes biotinylation of biotin dependent enzymes, the mutation might have yielded either suppressed or accelerated function of the biotic dependent enzymes (Satiaputra et al., 2016).

Another mutation was found on a putative metabolite transport protein CsbC encoding gene. Putative metabolite transport protein CsbC is a transmembrane sugar transporting protein that is a member of the major facilitating superfamily (MFS) (Del Sorbo et al, 2000). Two major classes of transporters have been discovered to excrete toxic substances (including antifungals) out of fungal cell. These are the ATP- binding cassette (ABC) and MFS. Since putative metabolite transport protein CsbC is a member of the MFS, a mutation in the gene encoding this protein may mean that efflux pump may have been affected. This requires further investigations. In *Listeria monocytogenes* efflux pump dependent resistance to BC has been known (Meier et al, 2017, Xu et al., 2019, Martinez-Suárez et al., 2016). Further research is required to establish this mechanism in *C. auris*.

In general, due to the disinfectant (BC) exposure, non-specific mutations occurred in *C. auris* which may have made metabolic changes in the cell. These changes may have allowed the tolerance to the recommended concentrations showing increased MIC values. Phenol exposed *Candida species*, apart from physiological changes have shown to have upregulation and down regulation of genes responsible for the oxidative stress response, RNA polymerase, amino metabolism and DNA replication and repair (Xie et al., 2021). This was also shown in our study.

4.2.4.2. *C. auris* adaptation, survival and spread

The environmental source and origin of *C. auris* has been established (Arora et al., 2021). It has also been proposed that this environmental fungi may have evolved to survive in hotter temperatures and tolerate osmotic stress and salinity. In addition, it has spread from rural environments to urban regions through birds and human movement (Casadevall et al., 2019, Jackson et al., 2019) and global warming may have contributed to its evolution and spread.

Nonetheless, once it comes into contact with humans, particularly immunocompromised, it can colonise the skin (Horton et al., 2020) and flourish in a harsh environment through activation of Hog1-related stress-activated protein kinase (SAPK) signalling pathway (Day *et al.,* 2018). Once it gains access to the human body, it evades the immune response and escapes the first line of immune response by resisting engulfment by neutrophils (Johnson et al., 2018). In addition, viable *C. auris* shows significantly reduced release of inflammatory cytokines such as TNF-α, IL-6 and IL1-β compared to the *C. tropicalis, C. krusei* and *C. guiliermondii* (Navarro-Arias et al., 2019). Together with these unique characteristics, and the persistence in

the hospital environment despite disinfection, as also shown in this study makes *C. auris* a unique pathogenic yeast that requires many years of investigations.

CHAPTER 5

CONCLUSIONS, LIMITATIONS AND FUTURE RESEARCH

5. Conclusions, limitations and future research

5.1. Conclusions

In the hospital environment, if surfaces are contaminated with *C. auris* from infected patients, it can allow cross contamination between patients and cause outbreaks. Thorough cleaning and disinfection is important. This study showed that *C. auris* can survive on steel, glass, fabric, wood and plastic for more than 3 weeks. The survival was significantly higher on wood and steel surfaces when the planktonic form was studied. However, in the biofilm form, no difference was found in the survival on all the surfaces. Survival of *C. auris* was significant in the form of biofilm with increase in counts throughout the study time period of 3 weeks compare to the planktonic form. Wet surfaces significantly supported the growth of *C. auris* cells compared to the dry surfaces. Therefore decontamination of these surfaces with appropriate disinfectants is important.

When the four commonly used disinfectants (Sodium dichloroisocyanurate - NADCC, Benzylkonium chloride - BC, 70% alcohol and Hydrogen peroxide - HP) were studied against *C. auris*, it was found that at recommended concentration NADCC and BC killed 100% of *C. auris* within 30 seconds where are 70% alcohol required a minute and 3% HP was found to be very weak requiring 5 minutes to kill 100% of *C. auris*. When *C. auris* was pulsed for 10 minutes daily with disinfectants for 15 days, it was found that for NADCC and BC the minimum inhibitory concentrations increased showing some tolerance/resistance development in the exposed cells compared to the unexposed cells. When the ergosterol in disinfectant exposed cells was studied, it was found that NADCC and HP exposed cells had increased level of ergosterol and the HP induced a significant level of *ERG11* gene expression. This results showed that the exposed *C. auris* cells were resisting the effect of disinfectant. Genome sequencing of the disinfectant exposed and unexposed cells showed that BC treated cells was found to have more genetic variations. Most of the changes/variations were made upstream of the genes, possibly the promoter regions. Even though some of the mutated genes were identified, most are unannotated genes. This suggest that although there were some genotypic changes due to the disinfectant, the cells were still surviving and resisting disinfection.

This study highlights the importance to drying, cleaning, and disinfecting surfaces immediately after contamination and exposure to *C. auris.* Appropriate disinfectants should be used at recommended concentrations because the use of subinhibitory concentrations may allow development of tolerance/resistance to these disinfectants in *C. auris*.

5.2. Limitations

- Only 3 strains of *C. auris* and 1 strain of *C. albicans* were used due to the time and financial constraints. It would have been ideal to include at least 5 strains of each of the pathogen.
- Only 5 common surfaces were studied. However, other different types of surfaces can be present such as painted iron and melamine surfaces that can also become contaminated.
- Only 4 disinfectants were studied. However, disinfectants such as chlorhexidine gluconate and phenolic disinfectants are also used at certain places in hospitals.

5.3. Future research

- This study can be repeated using many strains of *C. auris* particularly the disinfectant study and the development of resistance.
- The mechanisms of resistance can be studied, for example efflux pump.
- Penetration of *C. auris* into rough surfaces such as wood and their survival can be studies.
- Biofilms can be challenged with disinfectants and the effect on the *C. auris* cells at the phenotypic and genotypic levels can be studied.

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CHAPTER 7 APPENDICES

7.1. **Appendix 1: Ethics Waiver**

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

Ref: W-CP-200320-1

20 March 2020

TO WHOM IT MAY CONCERN:

Waiver: This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical).

Investigator:	**Mr Orefemetse Dire (student no 1111596) and Dr Aijaz Ahmad**
Supervisor:	**Prof Mrudula Patel**
Faculty:	Health Sciences
School:	Therapeutic Sciences
Department:	Clinical Microbiology and Infectious Diseases
Project title:	**Survival of candida auris in the environment and the development of resistance to infection.**
Reason:	For an in vitro laboratory study for MSc in Medicine, not part of a project that already has ethics approval. Using fungal culture of candida auris isolates along with a control laboratory candida albicans SC5314 strain obtained from Division of Mycology, NICD.

Dr Clement Penny

Chair: Human Research Ethics Committee (Medical)

Copy – HREC (Medical) Secretariat: Zanele Ndlovu, Rhulani Mkansi,Iain Burns & Mapula Ramaila

Research Office Secretariat: Faculty of Health Sciences, Phillip Tobias Building, 3rd Floor, Office 301, 29 Princess of Wales Terrace, Parktown, 2193 Tel +27 (0)11-717-1252 /1234/2656/2700 Private Bag 3, Wits 2050, email: zanele.ndlovu@wits.ac.za Office email: hrec-medical.researchoffice@wits.ac.zaWebsite:

7.2. Appendix 2 Composition and preparation of media and reagents

Disinfectants

700 ml Absolute (100%) Eth (NHLS) diluted with 300 ml distilled water to obtain 70% Eth.

3% (10 volume) HP BP solution (Clicks (Pty) Ltd.). Consisted of 3% HP in purified water.

Presept™ tablets (Johnson & Johnson (Pty) Ltd.) Contained 50% (2.5g) NaDCC in an inert effervescent base.

95% BC (Merck Life Science (Pty) Ltd.) in distilled water.

All were diluted with distilled water to different concentrations as desired using the equation $C1V1 = C2V2$

4% Sabouraud dextrose agar (SDA)

60g	SDA (Merck Life Science (Pty) Ltd)
1000 ml	distilled water

60 g of SDA powder was suspended in 1000 ml distilled water and the mixture was dissolved by boiling and sterilized by autoclaving at 121 °C for 15 minutes. After cooling to below 50 °C the media was then poured into petri dishes and allowed to set. The plates were then refrigerated.

2% Sabouraud dextrose broth (SDB)

30g	SDB (Merck Life Science (Pty) Ltd)
1000 ml	distilled water

30 g of SDB powder was suspended in 1000 ml distilled water and the mixture was dissolved by boiling and sterilized by autoclaving at 121 °C for 15 minutes. The broth solution was then stored at room temperature.

0.9% saline solution

9 g sodium chloride (NaCl)
1000 ml distilled water

9 g of NaCl was dissolved in 700 ml distilled water and more water was added to bring the total volume of the solution to 1000 ml. Solution was then stored at room temperature in 20 ml aliquots to avoid cross contamination.

Phosphate buffered saline

4.2 g sodium chloride (NaCl)
0.078 g sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$)
0.64 g sodium hydrogen phosphate ($NaHPO_4$)
500 ml distilled water
The salts were suspended in distilled water and autoclaved at 121 °C for 15 minutes and stored at room temperature.

Dey-Engley neutralizing broth

54.02 g Dey-Engley neutralizing broth (Merck Life Science (Pty) Ltd)
1000 ml distilled water

54.02 grams of the Dey-Engley broth powder was dissolved in 1000 ml distilled water and autoclaved at 121 °C for 15 minutes. The solution was then stored at 4 °C.

3 ml 25% alcoholic potassium hydroxide solution
25 g potassium hydroxide (KOH)
35 ml sterile distilled water
Brought to 100 ml with 100% ethanol

25 g KOH was dissolved in 35 ml sterile distilled water and brought to 100 ml with 100% ethanol.

7.3. Appendix 3 Statistics

Fabric-Planktonic Wet

Ohours vs 1 week

```
Paired t test
─────────┬──────────────────────────────────────────────────────────────────────
> ──
Variable │     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interva
> l]
─────────┼──────────────────────────────────────────────────────────────────────
> ──
     ohr │       6    3653.333    238.6583     584.591    3039.843    4266.8
> 24
    week │       6    4433.333     248.806    609.4478    3793.757     5072.
> 91
─────────┼──────────────────────────────────────────────────────────────────────
> ──
    diff │       6        -780    409.7479    1003.673    -1833.29    273.29
> 05
─────────┴──────────────────────────────────────────────────────────────────────
> ──
     mean(diff) = mean(ohr - week)                                 t =  -1.90
> 36
 Ho: mean(diff) = 0                              degrees of freedom =
>  5

 Ha: mean(diff) < 0           Ha: mean(diff) != 0           Ha: mean(diff) >
>  0
 Pr(T < t) = 0.0577         Pr(|T| > |t|) = 0.1153          Pr(T > t) = 0.94
> 23

.
```

Ohours vs 2 week

```
Paired t test
─────────┬──────────────────────────────────────────────────────────────────────
> ──
Variable │     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interva
> l]
─────────┼──────────────────────────────────────────────────────────────────────
> ──
     ohr │       6    3653.333    238.6583     584.591    3039.843    4266.8
> 24
   weeks │       6        3650    234.2363    573.7595    3047.876    4252.1
> 24
─────────┼──────────────────────────────────────────────────────────────────────
> ──
    diff │       6    3.333333    198.0853    485.2079   -505.8611    512.52
> 78
─────────┴──────────────────────────────────────────────────────────────────────
> ──
     mean(diff) = mean(ohr - weeks)                                t =   0.01
> 68
 Ho: mean(diff) = 0                              degrees of freedom =
>  5

 Ha: mean(diff) < 0           Ha: mean(diff) != 0           Ha: mean(diff) >
>  0
 Pr(T < t) = 0.5064         Pr(|T| > |t|) = 0.9872          Pr(T > t) = 0.49
> 36

.
```

Ohours vs 3 week

```
Paired t test
─────────┬──────────────────────────────────────────────────────────────────────
> ──
Variable │     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interva
> l]
─────────┼──────────────────────────────────────────────────────────────────────
> ──
     ohr │       6    3653.333    238.6583     584.591    3039.843    4266.8
> 24
       F │       6    2713.333    192.1573    470.6874    2219.377     3207.
> 29
─────────┼──────────────────────────────────────────────────────────────────────
> ──
    diff │       6         940    236.0085    578.1003    333.3209    1546.6
> 79
─────────┴──────────────────────────────────────────────────────────────────────
> ──
     mean(diff) = mean(ohr - F)                                    t =   3.98
> 29
 Ho: mean(diff) = 0                               degrees of freedom =
>  5

 Ha: mean(diff) < 0           Ha: mean(diff) != 0           Ha: mean(diff) >
>  0
 Pr(T < t) = 0.9947         Pr(|T| > |t|) = 0.0105          Pr(T > t) = 0.00
> 53

.
```

Fabric Planktonic Dry

Ohours vs 1 week

```
Paired t test
─────────┬──────────────────────────────────────────────────────────────────────
> ──
Variable │     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interva
> l]
─────────┼──────────────────────────────────────────────────────────────────────
> ──
     ohr │       6    4016.667    423.5774    1037.548    2927.826    5105.5
> 07
    week │       6    3416.667    370.6451    907.8913    2463.893     4369.
> 44
─────────┼──────────────────────────────────────────────────────────────────────
> ──
    diff │       6         600     560.476     1372.88   -840.7494    2040.7
> 49
─────────┴──────────────────────────────────────────────────────────────────────
> ──
     mean(diff) = mean(ohr - week)                                 t =   1.07
> 05
 Ho: mean(diff) = 0                               degrees of freedom =
>  5

 Ha: mean(diff) < 0           Ha: mean(diff) != 0           Ha: mean(diff) >
>  0
 Pr(T < t) = 0.8333         Pr(|T| > |t|) = 0.3333          Pr(T > t) = 0.16
> 67
```

Ohours vs 2 week

```
Paired t test
─────────┬──────────────────────────────────────────────────────────────────────
> ──
Variable │     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interva
> l]
─────────┼──────────────────────────────────────────────────────────────────────
> ──
     ohr │       6    4016.667    423.5774    1037.548    2927.826    5105.5
> 07
   weeks │       6    2476.667    308.1306    754.7627    1684.592    3268.7
> 41
─────────┼──────────────────────────────────────────────────────────────────────
> ──
    diff │       6        1540    597.3999    1463.325    4.334612    3075.6
> 65
─────────┴──────────────────────────────────────────────────────────────────────
> ──
     mean(diff) = mean(ohr - weeks)                                t =   2.57
> 78
 Ho: mean(diff) = 0                             degrees of freedom =
>  5

 Ha: mean(diff) < 0           Ha: mean(diff) != 0           Ha: mean(diff) >
>  0
 Pr(T < t) = 0.9752         Pr(|T| > |t|) = 0.0496          Pr(T > t) = 0.02
> 48
```

Ohours vs 3 week

```
Paired t test
─────────┬──────────────────────────────────────────────────────────────────────
> ──
Variable │     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interva
> l]
─────────┼──────────────────────────────────────────────────────────────────────
> ──
     ohr │       6    4016.667    423.5774    1037.548    2927.826    5105.5
> 07
       F │       6    1946.667    337.5862    826.9139    1078.874     2814.
> 46
─────────┼──────────────────────────────────────────────────────────────────────
> ──
    diff │       6        2070    543.4213    1331.105    673.0912    3466.9
> 09
─────────┴──────────────────────────────────────────────────────────────────────
> ──
     mean(diff) = mean(ohr - F)                                    t =   3.80
> 92
 Ho: mean(diff) = 0                                degrees of freedom =
>  5

 Ha: mean(diff) < 0           Ha: mean(diff) != 0           Ha: mean(diff) >
>  0
 Pr(T < t) = 0.9937         Pr(|T| > |t|) = 0.0125          Pr(T > t) = 0.00
> 63
```

Glass-Planktonic Wet

Ohours vs 1 week

```
Paired t test
─────────┬──────────────────────────────────────────────────────────────────────
> ──
Variable │     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interva
> l]
─────────┼──────────────────────────────────────────────────────────────────────
> ──
    ohr  │       6        4410    478.2398    1171.444    3180.645    5639.3
> 55
   week  │       6    3763.333    335.7148      822.33    2900.351    4626.3
> 16
─────────┼──────────────────────────────────────────────────────────────────────
> ──
   diff  │       6    646.6667    424.8346    1040.628   -445.4055    1738.7
> 39
─────────┴──────────────────────────────────────────────────────────────────────
> ──
    mean(diff) = mean(ohr - week)                                 t =   1.52
> 22
 Ho: mean(diff) = 0                              degrees of freedom =
>  5

 Ha: mean(diff) < 0           Ha: mean(diff) != 0           Ha: mean(diff) >
>  0
 Pr(T < t) = 0.9058         Pr(|T| > |t|) = 0.1885          Pr(T > t) = 0.09
> 42
```

Ohours vs 2 week

```
Paired t test
─────────┬──────────────────────────────────────────────────────────────────────
> ──
Variable │     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interva
> l]
─────────┼──────────────────────────────────────────────────────────────────────
> ──
    ohr  │       6        4410    478.2398    1171.444    3180.645    5639.3
> 55
  weeks  │       6    2136.667    78.64124    192.6309    1934.513     2338.
> 82
─────────┼──────────────────────────────────────────────────────────────────────
> ──
   diff  │       6    2273.333    523.0339    1281.166    928.8319    3617.8
> 35
─────────┴──────────────────────────────────────────────────────────────────────
> ──
    mean(diff) = mean(ohr - weeks)                                t =   4.34
> 64
 Ho: mean(diff) = 0                              degrees of freedom =
>  5

 Ha: mean(diff) < 0           Ha: mean(diff) != 0           Ha: mean(diff) >
>  0
 Pr(T < t) = 0.9963         Pr(|T| > |t|) = 0.0074          Pr(T > t) = 0.00
> 37
```

Ohours vs 3 week

```
Paired t test
------------------------------------------------------------------------------
> ---
Variable |      Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interva
> l]
---------+--------------------------------------------------------------------
> ---
    ohr |        6        4410    478.2398    1171.444    3180.645    5639.3
> 55
      F |        6         335    97.73263    239.3951    83.77028    586.22
> 97
---------+--------------------------------------------------------------------
> ---
   diff |        6        4075    519.0938    1271.515    2740.627    5409.3
> 73
------------------------------------------------------------------------------
> ---
    mean(diff) = mean(ohr - F)                                    t =   7.85
> 02
 Ho: mean(diff) = 0                                degrees of freedom =
>  5

 Ha: mean(diff) < 0            Ha: mean(diff) != 0            Ha: mean(diff) >
>  0
 Pr(T < t) = 0.9997          Pr(|T| > |t|) = 0.0005          Pr(T > t) = 0.00
> 03

.
```

Glass Planktonic Dry

Ohours vs 1 week

```
Paired t test
------------------------------------------------------------------------------
> ---------------
Variable |      Obs        Mean    Std. Err.   Std. Dev.   [95%
> Conf. Interval]
---------+--------------------------------------------------------------------
> ---------------
     ohr |        6    3856.667    214.5175    525.4585    3305.
> 232
>         4408.102
    week |        6    2483.333    259.9829    636.8255    1815.
> 026
>         3151.641
---------+--------------------------------------------------------------------
> ---------------
    diff |        6    1373.333    263.9023    646.4261    694.9
> 508
>         2051.716
------------------------------------------------------------------------------
> ---------------
     mean(diff) = mean(ohr - week)
>     t =   5.2039
 Ho: mean(diff) = 0                                 degrees of fre
> edom =        5

 Ha: mean(diff) < 0            Ha: mean(diff) != 0            Ha:
>  mean(diff) > 0
 Pr(T < t) = 0.9983          Pr(|T| > |t|) = 0.0035          Pr(
> T > t) = 0.0017
```

Ohours vs 2 weeks

```
Paired t test
─────────┬──────────────────────────────────────────────────────────────────────
> ──
Variable │     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interva
> l]
─────────┼──────────────────────────────────────────────────────────────────────
> ──
     ohr │       6    3856.667    214.5175    525.4585    3305.232    4408.1
> 02
   weeks │       6        1725    130.7096    320.1718        1389         20
> 61
─────────┼──────────────────────────────────────────────────────────────────────
> ──
    diff │       6    2131.667    266.8135    653.5569    1445.801    2817.5
> 33
─────────┴──────────────────────────────────────────────────────────────────────
> ──
     mean(diff) = mean(ohr - weeks)                                t =   7.98
> 94
 Ho: mean(diff) = 0                               degrees of freedom =
>  5

 Ha: mean(diff) < 0           Ha: mean(diff) != 0           Ha: mean(diff) >
>  0
 Pr(T < t) = 0.9998         Pr(|T| > |t|) = 0.0005          Pr(T > t) = 0.00
> 02
```

Ohours vs 3 week

```
Paired t test
─────────┬──────────────────────────────────────────────────────────────────────
> ──
Variable │     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interva
> l]
─────────┼──────────────────────────────────────────────────────────────────────
> ──
     ohr │       6    3856.667    214.5175    525.4585    3305.232    4408.1
> 02
       F │       6    281.6667    59.68901    146.2076    128.2312    435.10
> 21
─────────┼──────────────────────────────────────────────────────────────────────
> ──
    diff │       6        3575     196.023    480.1562    3071.107    4078.8
> 93
─────────┴──────────────────────────────────────────────────────────────────────
> ──
     mean(diff) = mean(ohr - F)                                    t =  18.23
> 77
 Ho: mean(diff) = 0                               degrees of freedom =
>  5

 Ha: mean(diff) < 0           Ha: mean(diff) != 0           Ha: mean(diff) >
>  0
 Pr(T < t) = 1.0000         Pr(|T| > |t|) = 0.0000          Pr(T > t) = 0.00
> 00
```

Plastic Planktonic Wet

Ohours vs 1 week

```
Paired t test
─────────┬──────────────────────────────────────────────────────────────────────
> ──
Variable │     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interva
> l]
─────────┼──────────────────────────────────────────────────────────────────────
> ──
     ohr │       6    3891.667    160.5702     393.315    3478.908    4304.4
> 25
    week │       6    3533.333    220.5549    540.2469    2966.379    4100.2
> 88
─────────┼──────────────────────────────────────────────────────────────────────
> ──
    diff │       6    358.3333    283.7184    694.9652   -370.9879    1087.6
> 55
─────────┴──────────────────────────────────────────────────────────────────────
> ──
    mean(diff) = mean(ohr - week)                                 t =   1.26
> 30
 Ho: mean(diff) = 0                               degrees of freedom =
>  5

 Ha: mean(diff) < 0           Ha: mean(diff) != 0           Ha: mean(diff) >
>  0
 Pr(T < t) = 0.8689         Pr(|T| > |t|) = 0.2623          Pr(T > t) = 0.13
> 11

.
```

Ohours vs 2 week

```
Paired t test
─────────┬──────────────────────────────────────────────────────────────────────
> ──
Variable │     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interva
> l]
─────────┼──────────────────────────────────────────────────────────────────────
> ──
     ohr │       6    3891.667    160.5702     393.315    3478.908    4304.4
> 25
   weeks │       6    2843.333    207.1982      507.53    2310.713    3375.9
> 53
─────────┼──────────────────────────────────────────────────────────────────────
> ──
    diff │       6    1048.333     282.659    692.3703    321.7352    1774.9
> 31
─────────┴──────────────────────────────────────────────────────────────────────
> ──
    mean(diff) = mean(ohr - weeks)                                t =   3.70
> 88
 Ho: mean(diff) = 0                               degrees of freedom =
>  5

 Ha: mean(diff) < 0           Ha: mean(diff) != 0           Ha: mean(diff) >
>  0
 Pr(T < t) = 0.9931         Pr(|T| > |t|) = 0.0139          Pr(T > t) = 0.00
> 69

.
```

Ohours vs 3 week

```
Paired t test
------------------------------------------------------------------------------
> ---
Variable |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interva
> l]
---------+--------------------------------------------------------------------
> ---
     ohr |       6    3891.667    160.5702     393.315    3478.908    4304.4
> 25
       F |       6    1973.333    158.6961    388.7244    1565.392    2381.2
> 75
---------+--------------------------------------------------------------------
> ---
    diff |       6    1918.333    203.2636    497.8922    1395.827    2440.8
> 39
---------+--------------------------------------------------------------------
> ---
     mean(diff) = mean(ohr - F)                                    t =   9.43
> 77
 Ho: mean(diff) = 0                              degrees of freedom =
>  5

 Ha: mean(diff) < 0           Ha: mean(diff) != 0           Ha: mean(diff) >
>  0
 Pr(T < t) = 0.9999         Pr(|T| > |t|) = 0.0002          Pr(T > t) = 0.00
> 01

.
```

Plastic Planktonic Dry

Ohours vs 1 week

```
Paired t test
------------------------------------------------------------------------------
> ---
Variable |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interva
> l]
---------+--------------------------------------------------------------------
> ---
     ohr |       6    4526.667    123.0086    301.3083    4210.463     4842.
> 87
    week |       6    2898.333    173.9812    426.1651    2451.101    3345.5
> 66
---------+--------------------------------------------------------------------
> ---
    diff |       6    1628.333     279.588    684.8479    909.6295    2347.0
> 37
---------+--------------------------------------------------------------------
> ---
     mean(diff) = mean(ohr - week)                                 t =   5.82
> 40
 Ho: mean(diff) = 0                              degrees of freedom =
>  5

 Ha: mean(diff) < 0           Ha: mean(diff) != 0           Ha: mean(diff) >
>  0
 Pr(T < t) = 0.9989         Pr(|T| > |t|) = 0.0021          Pr(T > t) = 0.00
> 11

.
```

Ohours vs 2 week

```
Paired t test
------------------------------------------------------------------------------
> ---
Variable |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interva
> l]
---------+--------------------------------------------------------------------
> ---
     ohr |       6    4526.667    123.0086    301.3083    4210.463     4842.
> 87
   weeks |       6        1800    102.0784      250.04    1537.599    2062.4
> 01
---------+--------------------------------------------------------------------
> ---
    diff |       6    2726.667    179.3073    439.2114    2265.743    3187.5
> 91
---------+--------------------------------------------------------------------
> ---
     mean(diff) = mean(ohr - weeks)                                t =  15.20
> 67
 Ho: mean(diff) = 0                              degrees of freedom =
>  5

 Ha: mean(diff) < 0           Ha: mean(diff) != 0           Ha: mean(diff) >
>  0
 Pr(T < t) = 1.0000         Pr(|T| > |t|) = 0.0000          Pr(T > t) = 0.00
> 00

.
```

Ohours vs 3 week

```
Paired t test
------------------------------------------------------------------------------
> ---
Variable |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interva
> l]
---------+--------------------------------------------------------------------
> ---
     ohr |       6    4526.667    123.0086    301.3083    4210.463     4842.
> 87
       F |       6    1388.333    198.8201    487.0079    877.2499    1899.4
> 17
---------+--------------------------------------------------------------------
> ---
    diff |       6    3138.333    198.6356    486.5559    2627.724    3648.9
> 42
---------+--------------------------------------------------------------------
> ---
     mean(diff) = mean(ohr - F)                                    t =  15.79
> 94
 Ho: mean(diff) = 0                              degrees of freedom =
>  5

 Ha: mean(diff) < 0           Ha: mean(diff) != 0           Ha: mean(diff) >
>  0
 Pr(T < t) = 1.0000         Pr(|T| > |t|) = 0.0000          Pr(T > t) = 0.00
> 00

.
```

Steel Planktonic Wet

Ohours vs 1 week

```
. ttest ohr= week if surface==4 & wetdry==2 & method==2

Paired t test
─────────┬────────────────────────────────────────────────────────────────────────
> ──
Variable │      Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interva
> l]
─────────┼────────────────────────────────────────────────────────────────────────
> ──
     ohr │        6        4860    247.7499    606.8608    4223.139    5496.8
> 61
    week │        6    15233.33    2668.041     6535.34    8374.915    22091.
> 75
─────────┼────────────────────────────────────────────────────────────────────────
> ──
    diff │        6   -10373.33    2456.981    6018.351   -16689.21   -4057.4
> 61
─────────┴────────────────────────────────────────────────────────────────────────
> ──
     mean(diff) = mean(ohr - week)                                 t =  -4.22
> 20
 Ho: mean(diff) = 0                              degrees of freedom =
>  5

 Ha: mean(diff) < 0           Ha: mean(diff) != 0           Ha: mean(diff) >
>  0
 Pr(T < t) = 0.0042         Pr(|T| > |t|) = 0.0083          Pr(T > t) = 0.99
> 58
```

Ohours vs 2 week

```
. ttest ohr= weeks if surface==4 & wetdry==2 & method==2

Paired t test
─────────┬────────────────────────────────────────────────────────────────────────
> ──
Variable │      Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interva
> l]
─────────┼────────────────────────────────────────────────────────────────────────
> ──
     ohr │        6        4860    247.7499    606.8608    4223.139    5496.8
> 61
   weeks │        6       19000    6491.636    15901.19    2312.719    35687.
> 28
─────────┼────────────────────────────────────────────────────────────────────────
> ──
    diff │        6      -14140    6305.493    15445.24   -30348.79    2068.7
> 87
─────────┴────────────────────────────────────────────────────────────────────────
> ──
     mean(diff) = mean(ohr - weeks)                                t =  -2.24
> 25
 Ho: mean(diff) = 0                              degrees of freedom =
>  5

 Ha: mean(diff) < 0           Ha: mean(diff) != 0           Ha: mean(diff) >
>  0
 Pr(T < t) = 0.0375         Pr(|T| > |t|) = 0.0750          Pr(T > t) = 0.96
> 25
```

Ohours vs 3 week

```
. ttest ohr= F if surface==4 & wetdry==2 & method==2

Paired t test
─────────┬────────────────────────────────────────────────────────────────────────
> ──
Variable │      Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interva
> l]
─────────┼────────────────────────────────────────────────────────────────────────
> ──
     ohr │        6        4860    247.7499    606.8608    4223.139    5496.8
> 61
       F │        6    20118.33    9256.312    22673.24   -3675.774    43912.
> 44
─────────┼────────────────────────────────────────────────────────────────────────
> ──
    diff │        6   -15258.33    9062.125    22197.58   -38553.27       8036
> .6
─────────┴────────────────────────────────────────────────────────────────────────
> ──
    mean(diff) = mean(ohr - F)                                    t =  -1.68
> 37
 Ho: mean(diff) = 0                               degrees of freedom =
>  5

 Ha: mean(diff) < 0            Ha: mean(diff) != 0            Ha: mean(diff) >
>  0
 Pr(T < t) = 0.0765          Pr(|T| > |t|) = 0.1531          Pr(T > t) = 0.92
> 35
```

Steel Planktonic Dry

Ohours vs 1 week

```
. ttest ohr= week if surface==4 & wetdry==1 & method==2

Paired t test
─────────┬────────────────────────────────────────────────────────────────────────
> ──
Variable │      Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interva
> l]
─────────┼────────────────────────────────────────────────────────────────────────
> ──
     ohr │        6        4675    258.9047    634.1845    4009.464    5340.5
> 36
    week │        6       11025    3572.812    8751.566    1840.795    20209.
> 21
─────────┼────────────────────────────────────────────────────────────────────────
> ──
    diff │        6       -6350    3583.607    8778.009   -15561.96    2861.9
> 55
─────────┴────────────────────────────────────────────────────────────────────────
> ──
    mean(diff) = mean(ohr - week)                                 t =  -1.77
> 20
 Ho: mean(diff) = 0                               degrees of freedom =
>  5

 Ha: mean(diff) < 0            Ha: mean(diff) != 0            Ha: mean(diff) >
>  0
 Pr(T < t) = 0.0683          Pr(|T| > |t|) = 0.1366          Pr(T > t) = 0.93
> 17
```

Ohours vs 2 week

```
. ttest ohr= weeks if surface==4 & wetdry==1 & method==2

Paired t test
─────────┬──────────────────────────────────────────────────────────────────────
> ──
Variable │     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interva
> l]
─────────┼──────────────────────────────────────────────────────────────────────
> ──
     ohr │       6        4675    258.9047    634.1845    4009.464    5340.5
> 36
   weeks │       6    13658.33    5882.863    14410.01   -1464.047    28780.
> 71
─────────┼──────────────────────────────────────────────────────────────────────
> ──
    diff │       6   -8983.333    5903.711    14461.08    -24159.3    6192.6
> 38
─────────┴──────────────────────────────────────────────────────────────────────
> ──
     mean(diff) = mean(ohr - weeks)                                t =  -1.52
> 16
 Ho: mean(diff) = 0                             degrees of freedom =
>  5

 Ha: mean(diff) < 0           Ha: mean(diff) != 0           Ha: mean(diff) >
>  0
 Pr(T < t) = 0.0943         Pr(|T| > |t|) = 0.1886          Pr(T > t) = 0.90
> 57
```

Ohours vs 3 week

```
. ttest ohr= F if surface==4 & wetdry==1 & method==2

Paired t test
─────────┬──────────────────────────────────────────────────────────────────────
> ──
Variable │     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interva
> l]
─────────┼──────────────────────────────────────────────────────────────────────
> ──
     ohr │       6        4675    258.9047    634.1845    4009.464    5340.5
> 36
       F │       6    15223.33    7519.357    18418.59   -4105.789    34552.
> 46
─────────┼──────────────────────────────────────────────────────────────────────
> ──
    diff │       6   -10548.33    7553.892    18503.18   -29966.23    8869.5
> 65
─────────┴──────────────────────────────────────────────────────────────────────
> ──
     mean(diff) = mean(ohr - F)                                    t =  -1.39
> 64
 Ho: mean(diff) = 0                             degrees of freedom =
>  5

 Ha: mean(diff) < 0           Ha: mean(diff) != 0           Ha: mean(diff) >
>  0
 Pr(T < t) = 0.1107         Pr(|T| > |t|) = 0.2214          Pr(T > t) = 0.88
> 93
```

Wood Planktonic Wet

Ohours vs 1 wee, 2 weeks, 3 weeks

```
. ttest ohr= week if surface==5 & wetdry==2 & method==2

Paired t test
------------------------------------------------------------------------------
> ---
Variable |      Obs          Mean     Std. Err.    Std. Dev.    [95% Conf. Interva
> l]
---------+--------------------------------------------------------------------
> ---
     ohr |        6          3120     238.3834     583.9178     2507.216     3732.7
> 84
    week |        6     30433.33     3766.313     9225.544     20751.72     40114.
> 95
---------+--------------------------------------------------------------------
> ---
    diff |        6    -27313.33     3868.497     9475.844    -37257.62    -17369.
> 04
------------------------------------------------------------------------------
> ---
    mean(diff) = mean(ohr - week)                                 t =  -7.06
> 05
 Ho: mean(diff) = 0                                     degrees of freedom =
>  5

 Ha: mean(diff) < 0            Ha: mean(diff) != 0            Ha: mean(diff) >
>  0
 Pr(T < t) = 0.0004          Pr(|T| > |t|) = 0.0009          Pr(T > t) = 0.99
> 96

.
. ttest ohr= weeks if surface==5 & wetdry==2 & method==2

Paired t test
------------------------------------------------------------------------------
> ---
Variable |      Obs          Mean     Std. Err.    Std. Dev.    [95% Conf. Interva
> l]
---------+--------------------------------------------------------------------
> ---
     ohr |        6          3120     238.3834     583.9178     2507.216     3732.7
> 84
   weeks |        6     31666.67     2664.666     6527.072     24816.92     38516.
> 41
---------+--------------------------------------------------------------------
> ---
    diff |        6    -28546.67       2730.8     6689.068    -35566.41    -21526.
> 92
------------------------------------------------------------------------------
> ---
    mean(diff) = mean(ohr - weeks)                                t = -10.45
> 36
 Ho: mean(diff) = 0                                     degrees of freedom =
>  5

 Ha: mean(diff) < 0            Ha: mean(diff) != 0            Ha: mean(diff) >
>  0
 Pr(T < t) = 0.0001          Pr(|T| > |t|) = 0.0001          Pr(T > t) = 0.99
> 99

.
. ttest ohr= F if surface==5 & wetdry==2 & method==2

Paired t test
------------------------------------------------------------------------------
> ---
Variable |      Obs          Mean     Std. Err.    Std. Dev.    [95% Conf. Interva
> l]
---------+--------------------------------------------------------------------
> ---
     ohr |        6          3120     238.3834     583.9178     2507.216     3732.7
> 84
       F |        6         33100     4647.221     11383.32     21153.94     45046.
> 06
---------+--------------------------------------------------------------------
> ---
    diff |        6        -29980     4673.931     11448.74    -41994.72    -17965.
> 28
------------------------------------------------------------------------------
> ---
    mean(diff) = mean(ohr - F)                                    t =  -6.41
> 43
 Ho: mean(diff) = 0                                     degrees of freedom =
>  5

 Ha: mean(diff) < 0            Ha: mean(diff) != 0            Ha: mean(diff) >
>  0
 Pr(T < t) = 0.0007          Pr(|T| > |t|) = 0.0014          Pr(T > t) = 0.99
> 93

.
```

Wood Planktonic Dry

Ohours vs 1 wee, 2 weeks, 3 weeks

```
. ttest ohr= week if surface==5 & wetdry==1 & method==2

Paired t test
------------------------------------------------------------------------------
> ---
Variable |      Obs          Mean     Std. Err.    Std. Dev.    [95% Conf. Interva
> l]
---------+--------------------------------------------------------------------
> ---
     ohr |        6          3905     212.4265     520.3364      3358.94      4451.
> 06
    week |        6     3171.667     241.9286     592.6016     2549.769     3793.5
> 64
---------+--------------------------------------------------------------------
> ---
    diff |        6     733.3333      220.207     539.3947     167.2733     1299.3
> 93
------------------------------------------------------------------------------
> ---
     mean(diff) = mean(ohr - week)                                 t =    3.33
> 02
 Ho: mean(diff) = 0                                     degrees of freedom =
>  5

 Ha: mean(diff) < 0            Ha: mean(diff) != 0            Ha: mean(diff) >
>  0
 Pr(T < t) = 0.9896          Pr(|T| > |t|) = 0.0208          Pr(T > t) = 0.01
> 04

.
. ttest ohr= weeks if surface==5 & wetdry==1 & method==2

Paired t test
------------------------------------------------------------------------------
> ---
Variable |      Obs          Mean     Std. Err.    Std. Dev.    [95% Conf. Interva
> l]
---------+--------------------------------------------------------------------
> ---
     ohr |        6          3905     212.4265     520.3364      3358.94      4451.
> 06
   weeks |        6     2358.333     308.0954     754.6765     1566.349     3150.3
> 18
---------+--------------------------------------------------------------------
> ---
    diff |        6     1546.667     221.8207     543.3476     976.4583     2116.8
> 75
------------------------------------------------------------------------------
> ---
     mean(diff) = mean(ohr - weeks)                                t =    6.97
> 26
 Ho: mean(diff) = 0                                     degrees of freedom =
>  5

 Ha: mean(diff) < 0            Ha: mean(diff) != 0            Ha: mean(diff) >
>  0
 Pr(T < t) = 0.9995          Pr(|T| > |t|) = 0.0009          Pr(T > t) = 0.00
> 05

.
. ttest ohr= F if surface==5 & wetdry==1 & method==2

Paired t test
------------------------------------------------------------------------------
> ---
Variable |      Obs          Mean     Std. Err.    Std. Dev.    [95% Conf. Interva
> l]
---------+--------------------------------------------------------------------
> ---
     ohr |        6          3905     212.4265     520.3364      3358.94      4451.
> 06
       F |        6     1276.667     246.1526     602.9483     643.9112     1909.4
> 22
---------+--------------------------------------------------------------------
> ---
    diff |        6     2628.333     179.9151     440.7002     2165.847      3090.
> 82
------------------------------------------------------------------------------
> ---
     mean(diff) = mean(ohr - F)                                    t =   14.60
> 87
 Ho: mean(diff) = 0                                     degrees of freedom =
>  5

 Ha: mean(diff) < 0            Ha: mean(diff) != 0            Ha: mean(diff) >
>  0
 Pr(T < t) = 1.0000          Pr(|T| > |t|) = 0.0000          Pr(T > t) = 0.00
> 00

.
```

Fabric Biofilm Wet

Ohours vs 1 wee, 2 weeks, 3 weeks

```
. ttest ohr= week if surface==1 & wetdry==2 & method==1

Paired t test
─────────┬──────────────────────────────────────────────────────────────────────────
> ──
Variable │     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interva
> l]
─────────┼──────────────────────────────────────────────────────────────────────────
> ──
     ohr │       6    4041.667    327.3063    801.7335    3200.299    4883.0
> 34
    week │       6    15166.67    2181.386    5343.282    9559.236     20774
> .1
─────────┼──────────────────────────────────────────────────────────────────────────
> ──
    diff │       6      -11125    2023.846    4957.389   -16327.46   -5922.5
> 39
─────────┴──────────────────────────────────────────────────────────────────────────
> ──
     mean(diff) = mean(ohr - week)                                 t =  -5.49
> 70
 Ho: mean(diff) = 0                              degrees of freedom =
>  5

 Ha: mean(diff) < 0           Ha: mean(diff) != 0           Ha: mean(diff) >
>  0
 Pr(T < t) = 0.0014         Pr(|T| > |t|) = 0.0027          Pr(T > t) = 0.99
> 86

.
. ttest ohr= weeks if surface==1 & wetdry==2 & method==1

Paired t test
─────────┬──────────────────────────────────────────────────────────────────────────
> ──
Variable │     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interva
> l]
─────────┼──────────────────────────────────────────────────────────────────────────
> ──
     ohr │       6    4041.667    327.3063    801.7335    3200.299    4883.0
> 34
   weeks │       6       18800    2672.826     6547.06    11929.28    25670.
> 72
─────────┼──────────────────────────────────────────────────────────────────────────
> ──
    diff │       6   -14758.33        2514    6158.017   -21220.78   -8295.8
> 91
─────────┴──────────────────────────────────────────────────────────────────────────
> ──
     mean(diff) = mean(ohr - weeks)                                t =  -5.87
> 05
 Ho: mean(diff) = 0                              degrees of freedom =
>  5

 Ha: mean(diff) < 0           Ha: mean(diff) != 0           Ha: mean(diff) >
>  0
 Pr(T < t) = 0.0010         Pr(|T| > |t|) = 0.0020          Pr(T > t) = 0.99
> 90

.
. ttest ohr= F if surface==1 & wetdry==2 & method==1

Paired t test
─────────┬──────────────────────────────────────────────────────────────────────────
> ──
Variable │     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interva
> l]
─────────┼──────────────────────────────────────────────────────────────────────────
> ──
     ohr │       6    4041.667    327.3063    801.7335    3200.299    4883.0
> 34
       F │       6       18800    5233.737    12819.98    5346.251    32253.
> 75
─────────┼──────────────────────────────────────────────────────────────────────────
> ──
```

Fabric Biofilm Dry

Ohours vs 1 wee, 2 weeks, 3 weeks

```
. ttest ohr= week if surface==1 & wetdry==1 & method==1

Paired t test
------------------------------------------------------------------------------
> ---
Variable |      Obs          Mean     Std. Err.    Std. Dev.    [95% Conf. Interva
> l]
---------+--------------------------------------------------------------------
> ---
     ohr |        6     4586.667     330.7634     810.2016     3736.412     5436.9
> 21
    week |        6     15266.67     796.1016     1950.043     13220.22     17313.
> 11
---------+--------------------------------------------------------------------
> ---
    diff |        6       -10680     998.8126     2446.581    -13247.53     -8112.
> 47
------------------------------------------------------------------------------
> ---
     mean(diff) = mean(ohr - week)                                 t = -10.69
> 27
 Ho: mean(diff) = 0                                     degrees of freedom =
>  5

 Ha: mean(diff) < 0            Ha: mean(diff) != 0            Ha: mean(diff) >
>  0
 Pr(T < t) = 0.0001          Pr(|T| > |t|) = 0.0001          Pr(T > t) = 0.99
> 99

.
. ttest ohr= weeks if surface==1 & wetdry==1 & method==1

Paired t test
------------------------------------------------------------------------------
> ---
Variable |      Obs          Mean     Std. Err.    Std. Dev.    [95% Conf. Interva
> l]
---------+--------------------------------------------------------------------
> ---
     ohr |        6     4586.667     330.7634     810.2016     3736.412     5436.9
> 21
   weeks |        6        17800     1680.476     4116.309      13480.2      22119
> .8
---------+--------------------------------------------------------------------
> ---
    diff |        6    -13213.33     1875.261     4593.433    -18033.85    -8392.8
> 21
------------------------------------------------------------------------------
> ---
     mean(diff) = mean(ohr - weeks)                                t =  -7.04
> 61
 Ho: mean(diff) = 0                                     degrees of freedom =
>  5

 Ha: mean(diff) < 0            Ha: mean(diff) != 0            Ha: mean(diff) >
>  0
 Pr(T < t) = 0.0004          Pr(|T| > |t|) = 0.0009          Pr(T > t) = 0.99
> 96

.
. ttest ohr= F if surface==1 & wetdry==1 & method==1

Paired t test
------------------------------------------------------------------------------
> ---
Variable |      Obs          Mean     Std. Err.    Std. Dev.    [95% Conf. Interva
> l]
---------+--------------------------------------------------------------------
> ---
     ohr |        6     4586.667     330.7634     810.2016     3736.412     5436.9
> 21
       F |        6     16166.67     2546.326       6237.2     9621.126     22712.
> 21
---------+--------------------------------------------------------------------
> ---
    diff |        6       -11580     2567.598     6289.305    -18180.22    -4979.7
> 79
------------------------------------------------------------------------------
> ---
     mean(diff) = mean(ohr - F)                                    t =  -4.51
> 01
 Ho: mean(diff) = 0                                     degrees of freedom =
>  5

 Ha: mean(diff) < 0            Ha: mean(diff) != 0            Ha: mean(diff) >
>  0
 Pr(T < t) = 0.0032          Pr(|T| > |t|) = 0.0063          Pr(T > t) = 0.99
> 68

.
```

Glass Biofilm Wet

Ohours vs 1 wee, 2 weeks, 3 weeks

```
. ttest ohr= week if surface==2 & wetdry==2 & method==1

Paired t test
------------------------------------------------------------------------------
> ---
Variable |      Obs          Mean     Std. Err.    Std. Dev.    [95% Conf. Interva
> l]
---------+--------------------------------------------------------------------
> ---
     ohr |        6     4486.667     537.5975      1316.84     3104.728     5868.6
> 05
    week |        6        19100     1738.007     4257.229     14632.31     23567.
> 69
---------+--------------------------------------------------------------------
> ---
    diff |        6    -14613.33     1328.487     3254.115    -18028.32    -11198.
> 35
------------------------------------------------------------------------------
> ---
    mean(diff) = mean(ohr - week)                                 t = -11.00
> 00
 Ho: mean(diff) = 0                                     degrees of freedom =
>  5

 Ha: mean(diff) < 0            Ha: mean(diff) != 0            Ha: mean(diff) >
>  0
 Pr(T < t) = 0.0001          Pr(|T| > |t|) = 0.0001          Pr(T > t) = 0.99
> 99

.
. ttest ohr= weeks if surface==2 & wetdry==2 & method==1

Paired t test
------------------------------------------------------------------------------
> ---
Variable |      Obs          Mean     Std. Err.    Std. Dev.    [95% Conf. Interva
> l]
---------+--------------------------------------------------------------------
> ---
     ohr |        6     4486.667     537.5975      1316.84     3104.728     5868.6
> 05
   weeks |        6     23833.33      3371.02      8257.28     15167.85     32498.
> 82
---------+--------------------------------------------------------------------
> ---
    diff |        6    -19346.67     2892.509     7085.172    -26782.1    -11911.
> 23
------------------------------------------------------------------------------
> ---
    mean(diff) = mean(ohr - weeks)                                t =  -6.68
> 85
 Ho: mean(diff) = 0                                     degrees of freedom =
>  5

 Ha: mean(diff) < 0            Ha: mean(diff) != 0            Ha: mean(diff) >
>  0
 Pr(T < t) = 0.0006          Pr(|T| > |t|) = 0.0011          Pr(T > t) = 0.99
> 94

.
. ttest ohr= F if surface==2 & wetdry==2 & method==1

Paired t test
------------------------------------------------------------------------------
> ---
Variable |      Obs          Mean     Std. Err.    Std. Dev.    [95% Conf. Interva
> l]
---------+--------------------------------------------------------------------
> ---
     ohr |        6     4486.667     537.5975      1316.84     3104.728     5868.6
> 05
       F |        6        28400     3473.711     8508.819     19470.54     37329.
> 46
---------+--------------------------------------------------------------------
> ---
    diff |        6    -23913.33      3047.11     7463.864    -31746.18    -16080.
> 49
------------------------------------------------------------------------------
> ---
    mean(diff) = mean(ohr - F)                                    t =  -7.84
> 79
 Ho: mean(diff) = 0                                     degrees of freedom =
>  5

 Ha: mean(diff) < 0            Ha: mean(diff) != 0            Ha: mean(diff) >
>  0
 Pr(T < t) = 0.0003          Pr(|T| > |t|) = 0.0005          Pr(T > t) = 0.99
> 97

.
```

Glass Biofilm Dry

Ohours vs 1 wee, 2 weeks, 3 weeks

```
. ttest ohr= week if surface==2 & wetdry==1 & method==1

Paired t test
------------------------------------------------------------------------------
> ---
Variable |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interva
> l]
---------+--------------------------------------------------------------------
> ---
     ohr |       6    4246.667    422.0005    1033.686     3161.88    5331.4
> 54
    week |       6    21033.33    2716.084    6653.019    14051.42    28015.
> 25
---------+--------------------------------------------------------------------
> ---
    diff |       6   -16786.67    2397.461    5872.555   -22949.54    -10623
> .8
---------+--------------------------------------------------------------------
> ---
     mean(diff) = mean(ohr - week)                                 t =  -7.00
> 19
 Ho: mean(diff) = 0                              degrees of freedom =
>  5

 Ha: mean(diff) < 0           Ha: mean(diff) != 0           Ha: mean(diff) >
>  0
 Pr(T < t) = 0.0005         Pr(|T| > |t|) = 0.0009          Pr(T > t) = 0.99
> 95

.
. ttest ohr= weeks if surface==2 & wetdry==1 & method==1

Paired t test
------------------------------------------------------------------------------
> ---
Variable |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interva
> l]
---------+--------------------------------------------------------------------
> ---
     ohr |       6    4246.667    422.0005    1033.686     3161.88    5331.4
> 54
   weeks |       6    27766.67    4476.134    10964.24     16260.4    39272.
> 94
---------+--------------------------------------------------------------------
> ---
    diff |       6      -23520    4121.493    10095.56   -34114.64   -12925.
> 36
---------+--------------------------------------------------------------------
> ---
     mean(diff) = mean(ohr - weeks)                                t =  -5.70
> 67
 Ho: mean(diff) = 0                              degrees of freedom =
>  5

 Ha: mean(diff) < 0           Ha: mean(diff) != 0           Ha: mean(diff) >
>  0
 Pr(T < t) = 0.0012         Pr(|T| > |t|) = 0.0023          Pr(T > t) = 0.99
> 88

.
. ttest ohr= F if surface==2 & wetdry==1 & method==1

Paired t test
------------------------------------------------------------------------------
> ---
Variable |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interva
> l]
---------+--------------------------------------------------------------------
> ---
     ohr |       6    4246.667    422.0005    1033.686     3161.88    5331.4
> 54
       F |       6       30600    5468.455    13394.92    16542.89    44657.
> 11
---------+--------------------------------------------------------------------
> ---
    diff |       6   -26353.33    5259.248    12882.47   -39872.66   -12834.
> 01
---------+--------------------------------------------------------------------
> ---
     mean(diff) = mean(ohr - F)                                    t =  -5.01
> 09
 Ho: mean(diff) = 0                              degrees of freedom =
>  5

 Ha: mean(diff) < 0           Ha: mean(diff) != 0           Ha: mean(diff) >
>  0
 Pr(T < t) = 0.0020         Pr(|T| > |t|) = 0.0041          Pr(T > t) = 0.99
> 80

.
```

Plastic Biofilm Wet

Ohours vs 1 wee, 2 weeks, 3 weeks

```
. ttest ohr= week if surface==3 & wetdry==2 & method==1

Paired t test
------------------------------------------------------------------------------
> ——
Variable |      Obs          Mean    Std. Err.   Std. Dev.   [95% Conf. Interva
> l]
---------+--------------------------------------------------------------------
> ——
     ohr |        6     4013.333    148.0015    362.5282    3632.883    4393.7
> 83
    week |        6     15466.67     2337.33    5725.266    9458.369     21474.
> 96
---------+--------------------------------------------------------------------
> ——
    diff |        6    -11453.33      2324.2    5693.103   -17427.88   -5478.7
> 88
------------------------------------------------------------------------------
> ——
     mean(diff) = mean(ohr - week)                                 t =  -4.92
> 79
 Ho: mean(diff) = 0                                     degrees of freedom =
>  5

 Ha: mean(diff) < 0             Ha: mean(diff) != 0             Ha: mean(diff) >
>  0
 Pr(T < t) = 0.0022          Pr(|T| > |t|) = 0.0044          Pr(T > t) = 0.99
> 78

.
. ttest ohr= weeks if surface==3 & wetdry==2 & method==1

Paired t test
------------------------------------------------------------------------------
> ——
Variable |      Obs          Mean    Std. Err.   Std. Dev.   [95% Conf. Interva
> l]
---------+--------------------------------------------------------------------
> ——
     ohr |        6     4013.333    148.0015    362.5282    3632.883    4393.7
> 83
   weeks |        6        21300    4059.475    9943.641    10864.79     31735.
> 21
---------+--------------------------------------------------------------------
> ——
    diff |        6    -17286.67    4034.093    9881.469   -27656.63   -6916.7
> 01
------------------------------------------------------------------------------
> ——
     mean(diff) = mean(ohr - weeks)                                t =  -4.28
> 51
 Ho: mean(diff) = 0                                     degrees of freedom =
>  5

 Ha: mean(diff) < 0             Ha: mean(diff) != 0             Ha: mean(diff) >
>  0
 Pr(T < t) = 0.0039          Pr(|T| > |t|) = 0.0078          Pr(T > t) = 0.99
> 61

.
. ttest ohr= F if surface==3 & wetdry==2 & method==1

Paired t test
------------------------------------------------------------------------------
> ——
Variable |      Obs          Mean    Std. Err.   Std. Dev.   [95% Conf. Interva
> l]
---------+--------------------------------------------------------------------
> ——
     ohr |        6     4013.333    148.0015    362.5282    3632.883    4393.7
> 83
       F |        6     24838.33    6282.314    15388.46    8689.131     40987.
> 54
---------+--------------------------------------------------------------------
> ——
    diff |        6       -20825    6237.376    15278.39   -36858.69   -4791.3
> 15
------------------------------------------------------------------------------
> ——
     mean(diff) = mean(ohr - F)                                    t =  -3.33
> 87
 Ho: mean(diff) = 0                                     degrees of freedom =
>  5

 Ha: mean(diff) < 0             Ha: mean(diff) != 0             Ha: mean(diff) >
>  0
 Pr(T < t) = 0.0103          Pr(|T| > |t|) = 0.0206          Pr(T > t) = 0.98
> 97

.
```

Plastic Biofilm Dry

Ohours vs 1 wee, 2 weeks, 3 weeks

```
. ttest ohr= week if surface==3 & wetdry==1 & method==1

Paired t test
------------------------------------------------------------------------------
> ---
Variable |      Obs          Mean     Std. Err.    Std. Dev.    [95% Conf. Interva
> l]
---------+--------------------------------------------------------------------
> ---
     ohr |        6     4516.667      234.132     573.5039     3914.811     5118.5
> 22
    week |        6     17166.67      2526.35     6188.268     10672.48     23660.
> 86
---------+--------------------------------------------------------------------
> ---
    diff |        6       -12650     2337.315     5725.228    -18658.26    -6641.7
> 41
------------------------------------------------------------------------------
> ---
     mean(diff) = mean(ohr - week)                                 t =   -5.41
> 22
 Ho: mean(diff) = 0                                     degrees of freedom =
>  5

 Ha: mean(diff) < 0             Ha: mean(diff) != 0             Ha: mean(diff) >
>  0
 Pr(T < t) = 0.0015          Pr(|T| > |t|) = 0.0029           Pr(T > t) = 0.99
> 85

.
. ttest ohr= weeks if surface==3 & wetdry==1 & method==1

Paired t test
------------------------------------------------------------------------------
> ---
Variable |      Obs          Mean     Std. Err.    Std. Dev.    [95% Conf. Interva
> l]
---------+--------------------------------------------------------------------
> ---
     ohr |        6     4516.667      234.132     573.5039     3914.811     5118.5
> 22
   weeks |        6     23766.67     3174.237      7775.26     15607.03      31926
> .3
---------+--------------------------------------------------------------------
> ---
    diff |        6       -19250     3017.102     7390.361    -27005.71    -11494.
> 29
------------------------------------------------------------------------------
> ---
     mean(diff) = mean(ohr - weeks)                                t =   -6.38
> 03
 Ho: mean(diff) = 0                                     degrees of freedom =
>  5

 Ha: mean(diff) < 0             Ha: mean(diff) != 0             Ha: mean(diff) >
>  0
 Pr(T < t) = 0.0007          Pr(|T| > |t|) = 0.0014           Pr(T > t) = 0.99
> 93

.
. ttest ohr= F if surface==3 & wetdry==1 & method==1

Paired t test
------------------------------------------------------------------------------
> ---
Variable |      Obs          Mean     Std. Err.    Std. Dev.    [95% Conf. Interva
> l]
---------+--------------------------------------------------------------------
> ---
     ohr |        6     4516.667      234.132     573.5039     3914.811     5118.5
> 22
       F |        6     23366.67     1771.942     4340.353     18811.75     27921.
> 59
---------+--------------------------------------------------------------------
> ---
    diff |        6       -18850     1756.846     4303.375    -23366.12    -14333.
> 88
------------------------------------------------------------------------------
> ---
     mean(diff) = mean(ohr - F)                                    t = -10.72
> 95
 Ho: mean(diff) = 0                                     degrees of freedom =
>  5

 Ha: mean(diff) < 0             Ha: mean(diff) != 0             Ha: mean(diff) >
>  0
 Pr(T < t) = 0.0001          Pr(|T| > |t|) = 0.0001           Pr(T > t) = 0.99
> 99

.
```

Steel Biofilm Wet

Ohours vs 1 wee, 2 weeks, 3 weeks

```
. ttest ohr= week if surface==4 & wetdry==2 & method==1

Paired t test
─────────┬──────────────────────────────────────────────────────────────────────
> ──
Variable │     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interva
> l]
─────────┼──────────────────────────────────────────────────────────────────────
> ──
     ohr │       6    4813.333    279.8889    685.5849    4093.856    5532.8
> 11
    week │       6    21166.67    2908.111    7123.389    13691.13     28642
> .2
─────────┼──────────────────────────────────────────────────────────────────────
> ──
    diff │       6   -16353.33    2725.204     6675.36   -23358.69   -9347.9
> 73
─────────┴──────────────────────────────────────────────────────────────────────
> ──
     mean(diff) = mean(ohr - week)                                 t =  -6.00
> 08
 Ho: mean(diff) = 0                              degrees of freedom =
>  5

 Ha: mean(diff) < 0           Ha: mean(diff) != 0           Ha: mean(diff) >
>  0
 Pr(T < t) = 0.0009         Pr(|T| > |t|) = 0.0018          Pr(T > t) = 0.99
> 91

.
. ttest ohr= weeks if surface==4 & wetdry==2 & method==1

Paired t test
─────────┬──────────────────────────────────────────────────────────────────────
> ──
Variable │     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interva
> l]
─────────┼──────────────────────────────────────────────────────────────────────
> ──
     ohr │       6    4813.333    279.8889    685.5849    4093.856    5532.8
> 11
   weeks │       6    27333.33    3820.878    9359.202    17511.45    37155.
> 21
─────────┼──────────────────────────────────────────────────────────────────────
> ──
    diff │       6      -22520    3690.893    9040.805   -32007.74   -13032.
> 26
─────────┴──────────────────────────────────────────────────────────────────────
> ──
     mean(diff) = mean(ohr - weeks)                                t =  -6.10
> 15
 Ho: mean(diff) = 0                              degrees of freedom =
>  5

 Ha: mean(diff) < 0           Ha: mean(diff) != 0           Ha: mean(diff) >
>  0
 Pr(T < t) = 0.0009         Pr(|T| > |t|) = 0.0017          Pr(T > t) = 0.99
> 91

.
```

Steel Biofilm Dry

Ohours vs 1 wee, 2 weeks, 3 weeks

```
. ttest ohr= week if surface==4 & wetdry==1 & method==1

Paired t test
------------------------------------------------------------------------------
> ---
Variable |      Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interva
> l]
---------+--------------------------------------------------------------------
> ---
     ohr |        6    4696.667    259.9444    636.7312    4028.458    5364.8
> 75
    week |        6    23666.67    4118.144    10087.35    13080.64    34252.
> 69
---------+--------------------------------------------------------------------
> ---
    diff |        6      -18970    4112.622    10073.83   -29541.83   -8398.1
> 69
------------------------------------------------------------------------------
> ---
     mean(diff) = mean(ohr - week)                                 t =  -4.61
> 26
 Ho: mean(diff) = 0                                     degrees of freedom =
>  5

 Ha: mean(diff) < 0            Ha: mean(diff) != 0            Ha: mean(diff) >
>  0
 Pr(T < t) = 0.0029          Pr(|T| > |t|) = 0.0058          Pr(T > t) = 0.99
> 71

.
. ttest ohr= weeks if surface==4 & wetdry==1 & method==1

Paired t test
------------------------------------------------------------------------------
> ---
Variable |      Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interva
> l]
---------+--------------------------------------------------------------------
> ---
     ohr |        6    4696.667    259.9444    636.7312    4028.458    5364.8
> 75
   weeks |        6    29966.67    4155.772    10179.52    19283.91    40649.
> 42
---------+--------------------------------------------------------------------
> ---
    diff |        6      -25270    4218.032    10332.03    -36112.8    -14427
> .2
------------------------------------------------------------------------------
> ---
     mean(diff) = mean(ohr - weeks)                                t =  -5.99
> 09
 Ho: mean(diff) = 0                                     degrees of freedom =
>  5

 Ha: mean(diff) < 0            Ha: mean(diff) != 0            Ha: mean(diff) >
>  0
 Pr(T < t) = 0.0009          Pr(|T| > |t|) = 0.0019          Pr(T > t) = 0.99
> 91

.
. ttest ohr= F if surface==4 & wetdry==1 & method==1

Paired t test
------------------------------------------------------------------------------
> ---
Variable |      Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interva
> l]
---------+--------------------------------------------------------------------
> ---
     ohr |        6    4696.667    259.9444    636.7312    4028.458    5364.8
> 75
       F |        6    28933.33    7072.513    17324.05    10752.86    47113.
> 81
---------+--------------------------------------------------------------------
> ---
    diff |        6   -24236.67    7140.708    17491.09   -42592.44   -5880.8
> 94
------------------------------------------------------------------------------
> ---
     mean(diff) = mean(ohr - F)                                    t =  -3.39
> 42
 Ho: mean(diff) = 0                                     degrees of freedom =
>  5

 Ha: mean(diff) < 0            Ha: mean(diff) != 0            Ha: mean(diff) >
>  0
 Pr(T < t) = 0.0097          Pr(|T| > |t|) = 0.0194          Pr(T > t) = 0.99
> 03

.
```

Wood Biofilm Wet

Ohours vs 1 wee, 2 weeks, 3 weeks

```
. ttest ohr= week if surface==5 & wetdry==2 & method==1

Paired t test
------------------------------------------------------------------------------
> ---
Variable |      Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interva
> l]
---------+--------------------------------------------------------------------
> ---
     ohr |        6    3616.667    164.9781    404.1122    3192.577    4040.7
> 56
    week |        6       33400    4031.873    9876.032    23035.74    43764.
> 26
---------+--------------------------------------------------------------------
> ---
    diff |        6   -29783.33    3993.831    9782.848    -40049.8   -19516.
> 86
------------------------------------------------------------------------------
> ---
    mean(diff) = mean(ohr - week)                                 t =  -7.45
> 73
 Ho: mean(diff) = 0                                     degrees of freedom =
>  5

 Ha: mean(diff) < 0            Ha: mean(diff) != 0            Ha: mean(diff) >
>  0
 Pr(T < t) = 0.0003          Pr(|T| > |t|) = 0.0007          Pr(T > t) = 0.99
> 97

.
. ttest ohr= weeks if surface==5 & wetdry==2 & method==1

Paired t test
------------------------------------------------------------------------------
> ---
Variable |      Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interva
> l]
---------+--------------------------------------------------------------------
> ---
     ohr |        6    3616.667    164.9781    404.1122    3192.577    4040.7
> 56
   weeks |        6    38633.33    4630.455    11342.25    26730.37      50536
> .3
---------+--------------------------------------------------------------------
> ---
    diff |        6   -35016.67    4579.332    11217.03   -46788.22   -23245.
> 12
------------------------------------------------------------------------------
> ---
    mean(diff) = mean(ohr - weeks)                                t =  -7.64
> 67
 Ho: mean(diff) = 0                                     degrees of freedom =
>  5

 Ha: mean(diff) < 0            Ha: mean(diff) != 0            Ha: mean(diff) >
>  0
 Pr(T < t) = 0.0003          Pr(|T| > |t|) = 0.0006          Pr(T > t) = 0.99
> 97

.
. ttest ohr= F if surface==5 & wetdry==2 & method==1

Paired t test
------------------------------------------------------------------------------
> ---
Variable |      Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interva
> l]
---------+--------------------------------------------------------------------
> ---
     ohr |        6    3616.667    164.9781    404.1122    3192.577    4040.7
> 56
       F |        6    45366.67     3904.67    9564.448    35329.39    55403.
> 94
---------+--------------------------------------------------------------------
> ---
    diff |        6       -41750    3870.724    9481.299   -51700.01   -31799.
> 99
------------------------------------------------------------------------------
> ---
    mean(diff) = mean(ohr - F)                                    t = -10.78
> 61
 Ho: mean(diff) = 0                                     degrees of freedom =
>  5

 Ha: mean(diff) < 0            Ha: mean(diff) != 0            Ha: mean(diff) >
>  0
 Pr(T < t) = 0.0001          Pr(|T| > |t|) = 0.0001          Pr(T > t) = 0.99
> 99

.
```

Wood Biofilm Dry

Ohours vs 1 wee, 2 weeks, 3 weeks

```
. ttest ohr= week if surface==5 & wetdry==1 & method==1

Paired t test
------------------------------------------------------------------------------
> ---
Variable |      Obs         Mean    Std. Err.   Std. Dev.   [95% Conf. Interva
> l]
---------+--------------------------------------------------------------------
> ---
     ohr |        6         3985    205.5197    503.4183    3456.695    4513.3
> 05
    week |        6     4111.667     355.691    871.2615    3197.334         50
> 26
---------+--------------------------------------------------------------------
> ---
    diff |        6    -126.6667    458.5387    1123.186   -1305.378    1052.0
> 45
------------------------------------------------------------------------------
> ---
    mean(diff) = mean(ohr - week)                                 t =  -0.27
> 62
 Ho: mean(diff) = 0                                     degrees of freedom =
>  5

 Ha: mean(diff) < 0            Ha: mean(diff) != 0            Ha: mean(diff) >
>  0
 Pr(T < t) = 0.3967          Pr(|T| > |t|) = 0.7934          Pr(T > t) = 0.60
> 33

.
. ttest ohr= weeks if surface==5 & wetdry==1 & method==1

Paired t test
------------------------------------------------------------------------------
> ---
Variable |      Obs         Mean    Std. Err.   Std. Dev.   [95% Conf. Interva
> l]
---------+--------------------------------------------------------------------
> ---
     ohr |        6         3985    205.5197    503.4183    3456.695    4513.3
> 05
   weeks |        6         2960    338.3489    828.7822    2090.246    3829.7
> 54
---------+--------------------------------------------------------------------
> ---
    diff |        6         1025    393.2662    963.3016    14.07695    2035.9
> 23
------------------------------------------------------------------------------
> ---
    mean(diff) = mean(ohr - weeks)                                t =   2.60
> 64
 Ho: mean(diff) = 0                                     degrees of freedom =
>  5

 Ha: mean(diff) < 0            Ha: mean(diff) != 0            Ha: mean(diff) >
>  0
 Pr(T < t) = 0.9761          Pr(|T| > |t|) = 0.0479          Pr(T > t) = 0.02
> 39

.
. ttest ohr= F if surface==5 & wetdry==1 & method==1

Paired t test
------------------------------------------------------------------------------
> ---
Variable |      Obs         Mean    Std. Err.   Std. Dev.   [95% Conf. Interva
> l]
---------+--------------------------------------------------------------------
> ---
     ohr |        6         3985    205.5197    503.4183    3456.695    4513.3
> 05
       F |        6     2014.167    282.6022    692.2313    1287.714    2740.6
> 19
---------+--------------------------------------------------------------------
> ---
    diff |        6     1970.833    440.2356    1078.353    839.1718    3102.4
> 95
------------------------------------------------------------------------------
> ---
    mean(diff) = mean(ohr - F)                                    t =   4.47
> 68
 Ho: mean(diff) = 0                                     degrees of freedom =
>  5

 Ha: mean(diff) < 0            Ha: mean(diff) != 0            Ha: mean(diff) >
>  0
 Pr(T < t) = 0.9967          Pr(|T| > |t|) = 0.0065          Pr(T > t) = 0.00
> 33

.
```

Wet vs Dry Planktonic

All data Combined

```
. ttest Wet= Dry

Paired t test
```

Variable	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
Wet	4	3077.5	1256.864	2513.727	-922.4011	7077.401
Dry	4	2052.5	890.7616	1781.523	-782.301	4887.301
diff	4	1025	449.7685	899.5369	-406.364	2456.364

```
    mean(diff) = mean(Wet - Dry)                                  t =   2.2790
Ho: mean(diff) = 0                           degrees of freedom =        3

 Ha: mean(diff) < 0           Ha: mean(diff) != 0           Ha: mean(diff) > 0
 Pr(T < t) = 0.9465         Pr(|T| > |t|) = 0.1070          Pr(T > t) = 0.0535
```

0 hrs

```
Paired t test
```

Variable	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
Wet	30	3987	165.2891	905.3258	3648.946	4325.054
Dry	30	4196	126.8827	694.9651	3936.496	4455.504
diff	30	-209	173.1688	948.4846	-563.17	145.17

```
    mean(diff) = mean(Wet - Dry)                                  t =  -1.2069
Ho: mean(diff) = 0                           degrees of freedom =       29

 Ha: mean(diff) < 0           Ha: mean(diff) != 0           Ha: mean(diff) > 0
 Pr(T < t) = 0.1186         Pr(|T| > |t|) = 0.2372          Pr(T > t) = 0.8814
```

1 week

```
Paired t test
```

Variable	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
Wet	30	11479.33	2122.624	11626.09	7138.079	15820.59
Dry	30	4599	899.7729	4928.259	2758.758	6439.242
diff	30	6880.333	2046.569	11209.52	2694.631	11066.04

```
    mean(diff) = mean(Wet - Dry)                                  t =   3.3619
Ho: mean(diff) = 0                           degrees of freedom =       29

 Ha: mean(diff) < 0           Ha: mean(diff) != 0           Ha: mean(diff) > 0
 Pr(T < t) = 0.9989         Pr(|T| > |t|) = 0.0022          Pr(T > t) = 0.0011
```

2weeks

Paired t test

Variable	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
Wet	30	11859.33	2536.982	13895.62	6670.623	17048.04
Dry	30	4403.667	1393.649	7633.327	1553.335	7253.998
diff	30	7455.667	2135.495	11696.59	3088.089	11823.24

```
    mean(diff) = mean(Wet - Dry)                                  t =   3.4913
Ho: mean(diff) = 0                              degrees of freedom =       29

 Ha: mean(diff) < 0           Ha: mean(diff) != 0           Ha: mean(diff) > 0
 Pr(T < t) = 0.9992         Pr(|T| > |t|) = 0.0016          Pr(T > t) = 0.0008
```

.

3 Weeks

Paired t test

Variable	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
Wet	30	11648	3073.9	16836.44	5361.17	17934.83
Dry	30	4023.333	1745.994	9563.204	452.3741	7594.293
diff	30	7624.667	2455.065	13446.95	2603.494	12645.84

```
    mean(diff) = mean(Wet - Dry)                                  t =   3.1057
Ho: mean(diff) = 0                              degrees of freedom =       29

 Ha: mean(diff) < 0           Ha: mean(diff) != 0           Ha: mean(diff) > 0
 Pr(T < t) = 0.9979         Pr(|T| > |t|) = 0.0042          Pr(T > t) = 0.0021
```

Wet vs dry Biofilm

All data Combined

```
. ttest Wet= Dry

Paired t test
```

Variable	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
Wet	4	24222.5	6646.917	13293.83	3069.043	45375.96
Dry	4	22950	6055.507	12111.01	3678.674	42221.33
diff	4	1272.5	2865.994	5731.988	-7848.373	10393.37

```
    mean(diff) = mean(Wet - Dry)                                     t =   0.4440
Ho: mean(diff) = 0                                     degrees of freedom =        3

 Ha: mean(diff) < 0           Ha: mean(diff) != 0           Ha: mean(diff) > 0
 Pr(T < t) = 0.6564         Pr(|T| > |t|) = 0.6871          Pr(T > t) = 0.3436
```

0 Hours

Paired t test

Variable	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
Wet	30	4194.333	154.8389	848.0878	3877.652	4511.015
Dry	30	4406.333	133.7157	732.3909	4132.854	4679.813
diff	30	-212	104.2239	570.8578	-425.1618	1.161803

```
    mean(diff) = mean(Wet - Dry)                                     t =  -2.0341
Ho: mean(diff) = 0                                     degrees of freedom =       29

 Ha: mean(diff) < 0           Ha: mean(diff) != 0           Ha: mean(diff) > 0
 Pr(T < t) = 0.0256         Pr(|T| > |t|) = 0.0512          Pr(T > t) = 0.9744
```

1 Week

Paired t test

Variable	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
Wet	30	20860	1685.07	9229.51	17413.64	24306.36
Dry	30	16249	1628.26	8918.347	12918.83	19579.17
diff	30	4611	2545.345	13941.43	-594.8146	9816.815

```
    mean(diff) = mean(Wet - Dry)                                     t =   1.8115
Ho: mean(diff) = 0                                     degrees of freedom =       29

 Ha: mean(diff) < 0           Ha: mean(diff) != 0           Ha: mean(diff) > 0
 Pr(T < t) = 0.9598         Pr(|T| > |t|) = 0.0804          Pr(T > t) = 0.0402
```

2weeks

Paired t test

Variable	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
Wet	30	25980	2025.932	11096.49	21836.5	30123.5
Dry	30	20452	2227.765	12201.97	15895.71	25008.29
diff	30	5528	3103.033	16996.01	-818.4153	11874.42

mean(diff) = mean(Wet - Dry) t = 1.7815
Ho: mean(diff) = 0 degrees of freedom = 29

Ha: mean(diff) < 0 Ha: mean(diff) != 0 Ha: mean(diff) > 0
Pr(T < t) = 0.9573 Pr(|T| > |t|) = 0.0853 Pr(T > t) = 0.0427

3 Weeks

Paired t test

Variable	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
Wet	30	28994.33	2717.237	14882.92	23436.96	34551.71
Dry	30	15368.83	2330.025	12762.07	10603.4	20134.27
diff	30	13625.5	3741.577	20493.46	5973.115	21277.89

mean(diff) = mean(Wet - Dry) t = 3.6416
Ho: mean(diff) = 0 degrees of freedom = 29

Ha: mean(diff) < 0 Ha: mean(diff) != 0 Ha: mean(diff) > 0
Pr(T < t) = 0.9995 Pr(|T| > |t|) = 0.0010 Pr(T > t) = 0.0005

Planktonic vs Biofilm

0 Hours vs 3 Wks planktonic

Paired t test

Variable	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
Hours	60	4091.5	104.1923	807.0701	3883.012	4299.988
Weeks	60	7835.667	1821.461	14108.98	4190.931	11480.4
diff	60	-3744.167	1825.755	14142.23	-7397.493	-90.84034

mean(diff) = mean(Hours - Weeks) t = -2.0508
Ho: mean(diff) = 0 degrees of freedom = 59

Ha: mean(diff) < 0 Ha: mean(diff) != 0 Ha: mean(diff) > 0
Pr(T < t) = 0.0224 Pr(|T| > |t|) = 0.0447 Pr(T > t) = 0.9776

0 Hours vs 3 Wks Biofilm

Paired t test

Variable	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
Hours	60	4300.333	102.3564	792.8493	4095.519	4505.148
Weeks	60	24605.25	1954.057	15136.06	20695.19	28515.31
diff	60	-20304.92	1943.398	15053.5	-24193.65	-16416.19

mean(diff) = mean(Hours - Weeks) t = -10.4482
Ho: mean(diff) = 0 degrees of freedom = 59

Ha: mean(diff) < 0 Ha: mean(diff) != 0 Ha: mean(diff) > 0
Pr(T < t) = 0.0000 Pr(|T| > |t|) = 0.0000 Pr(T > t) = 1.0000

1 Week

Paired t test

Variable	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
Plankt~c	60	8039.167	1227.538	9508.465	5582.87	10495.46
Biofilm	60	18554.5	1199.791	9293.539	16153.72	20955.28
diff	60	-10515.33	976.2511	7562.008	-12468.81	-8561.859

mean(diff) = mean(Planktonic - Biofilm) t = -10.7711
Ho: mean(diff) = 0 degrees of freedom = 59

Ha: mean(diff) < 0 Ha: mean(diff) != 0 Ha: mean(diff) > 0
Pr(T < t) = 0.0000 Pr(|T| > |t|) = 0.0000 Pr(T > t) = 1.0000

2weeks

Paired t test

Variable	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
Plankt~c	60	8131.5	1514.816	11733.72	5100.359	11162.64
Biofilm	60	23216	1535.545	11894.28	20143.38	26288.62
diff	60	-15084.5	1367.476	10592.43	-17820.81	-12348.19

mean(diff) = mean(Planktonic - Biofilm) t = -11.0309
Ho: mean(diff) = 0 degrees of freedom = 59

Ha: mean(diff) < 0 Ha: mean(diff) != 0 Ha: mean(diff) > 0
Pr(T < t) = 0.0000 Pr(|T| > |t|) = 0.0000 Pr(T > t) = 1.0000

3 Weeks

```
. ttest Planktonic= Biofilm

Paired t test
```

Variable	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
Plankt~c	60	7835.667	1821.461	14108.98	4190.931	11480.4
Biofilm	60	24605.25	1954.057	15136.06	20695.19	28515.31
diff	60	-16769.58	1604.766	12430.46	-19980.71	-13558.45

```
    mean(diff) = mean(Planktonic - Biofilm)                   t = -10.4499
Ho: mean(diff) = 0                             degrees of freedom =       59

 Ha: mean(diff) < 0           Ha: mean(diff) != 0           Ha: mean(diff) > 0
 Pr(T < t) = 0.0000         Pr(|T| > |t|) = 0.0000          Pr(T > t) = 1.0000
```

7.4. Appendix 4 Turn it in report

a0042389:Final_Writeup_December_2021_PATEL.docx

ORIGINALITY REPORT

18%	17%	12%	%
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