Effect of probiotic cell free extracts on antifungal drug resistance and virulence expression in *Candida albicans*

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Declaration

I, Yvonne Dube, 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 the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

I declare that this thesis has the approval of the Ethics Committee for Research on Human Subjects (Medical) with an Ethical clearance number W-CJ-170517-1.

…… (Signature of candidate)

……...day of ……2019

Dedication

In memory of my dad, Mr S. Dube Mutasa.

Publications and Presentations

Publications

Yvonne Dube, Aijaz Ahmad. *Lactobacillus rhamnosus* cell free extract: A potential antifungal against virulence traits and reversal of antifungal drug resistance in *Candida albicans*. Submitted. (Refer to **Appendix 6** for abstract).

Presentations

Poster presentation: **Yvonne Dube**, Aijaz Ahmad. Effect of probiotic cell free extract on virulence expression in *Candida albicans*. African International Conference on Immunity (AICI), Victoria Falls, Zimbabwe, 2018.

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Abstract

Introduction

Candidiasis caused by multidrug resistant *Candida* species continues to be difficult to eradicate and as such it results in life-threatening clinical failures. Moreover, existing antifungal drugs are toxic, inefficient and their misappropriation promotes drug resistance. The use of whole live probiotic bacteria has gained a lot of interest in the treatment of candidiasis; however, it is often associated with high risk of sepsis. Therefore, there is an urgent need of developing new strategies and safer methods for the development of novel antifungal drugs using these beneficial probiotic strains. One such strategy is to use probiotic cell free extracts (CFEs) to target virulence traits which play an important role in pathogenesis of opportunistic microorganisms. The other strategy to combat drug resistance is to inhibit the drug efflux pumps, which are major sources of drug resistance in *Candida albicans*. With this background, we studied the antifungal effects of probiotic CFEs from three probiotic strains against fluconazole susceptible and resistant *C. albicans*. The most active CFE was then assessed against different virulence traits and reversal of drug resistance in *C. albicans*.

Method and Materials

In this study, one laboratory strain along with ten clinical isolates of *C. albicans* were used. All these isolates were stored at -80 ºC in the Department of Clinical Microbiology and Infectious Diseases, University of the Witwatersrand, South Africa. The probiotic cultures were obtained from the culture collection of the Department of Biotechnology and Food Technology, Tshwane University of Technology, South Africa. Identification of *C. albicans* was carried out using germ tube assay and the API 20C AUX kit. To prepare CFEs, probiotic cultures (*L. rhamnosus*, *L. acidophilus* V and *B. animalis* subspecies *lactis*) were extracted with ethyl acetate. Minimum Inhibitory Concentrations (MIC) and Minimum Fungicidal

Concentrations (MFC) of all the CFEs were determined using the microtiter plate dilution assay following CLSI recommended guidelines. The effect of the most active CFE (*L. rhamnosus*) on yeast to hyphae transition, production of proteinases and phospholipases were studied using standard techniques. The effect of *L. rhamnosus* CFE on drug efflux pumps was also investigated using a standard technique. Real time PCR was used to determine the effect of *L. rhamnosus* CFE on expression of virulence and drug efflux pump genes in *C. albicans*. The results were analyzed using Student's two sample t-test and one-way ANOVA.

Result

All the isolates were positive for germ tube assays and API profile confirmed identity as *C. albicans*. The MIC of the three probiotic CFEs ranged from 84.5 to 338 mg/ml. On preliminary screening of the three CFEs, *L. rhamnosus* showed lowest MIC of 84.5 mg/ml. MFC was not determined as growth was observed on all agar plates. *L. rhamnosus* CFE reduced formation of hyphae in *C. albicans* by 87 % at a concentration of 84.5 mg/ml. The length of the hyphae was subsequently reduced in CFE treated cells compared to untreated cells. *L. rhamnosus* CFE exhibited an inhibitory effect on production of proteinases and phospholipases at concentrations of 84.5 and 42.25 mg/ml. *L. rhamnosus* CFE also showed inhibitory effect on drug efflux pumps. Gene expression analysis in the presence of *L. rhamnosus* CFE showed down regulation of virulence and drug efflux pump genes at a concentration of 84.5 mg/ml with varying fold changes.

Conclusion

L. rhamnosus CFE portrayed moderate anticandidal activity against different *C. albicans* isolates. At inhibitory and subinhibitory concentrations, *L. rhamnosus* CFE significantly inhibited virulence traits and drug efflux pump in both susceptible and resistant *C. albicans* isolates. The study also demonstrated that the effect on virulence traits and efflux pumps is at the molecular level by observing the downregulation of virulence and drug efflux pump

genes after *C. albicans* were treated with *L. rhamnosus* CFE. These findings advocate that *L. rhamnosus* CFE has a potential to be used for therapeutic purposes to treat *Candida* infections and to overcome drug resistance. This study also laid a foundation for further research in this area to test more probiotic strains as antifungal and anti-virulent agents in different fungal pathogens.

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Finally, I would like to thank the Almighty God for blessings, favour and opening doors for me. My life is under construction and for this I am grateful.

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List of abbreviations and acronyms

ABC	ATP-binding cassette
AIDS	Acquired immunodeficiency syndrome
ANOVA	Analysis of Variance
ATP	Adenosine triphosphate
API	Analytical Profile Index
BSA	Bovine serum albumin
BSI	Blood stream infections
bp	base pair
cDNA	complementary Deoxyribonucleic acid
CDR1	*Candida* drug resistance 1
CDR2	*Candida* drug resistance 2
CFE	Cell free extract
CNS	Central nervous system
CLSI	Clinical and Laboratory Standards Institute
Cq	Threshold value
DAS	Diallyl sulphides
DADS	Diallyl disulphides
EFG1	Enhanced filamentous growth protein 1
FAO	Food and Agricultural Organization

FBS	Foetal bovine serum
5FC	5 Fluorouracil
FLC	Fluconazole
g	grams
'GRAS'	Generally regarded as safe
HSP90	Heat Shock protein 90
h	hours
HIV	Human immunodeficiency virus
HWP1	Hyphal wall protein 1
IL-8	Interleukin 8
Kb	kilo base
LAB	Lactic acid bacteria
M	Molar
MALDI-TOF MS	Matrix assisted laser desorption ionisation time-of-flight mass spectrometry
MDR 1	Multidrug resistance 1
MFC	Minimum fungicidal concentration
MFS	Multi facilitator superfamily
mg	milligrams
mg/ml	milligrams per millilitre
MIC	Minimum Inhibitory Concentration

min	Minute
ml	millilitres
mM	millimolar
mRNA	messenger ribonucleic acid
MRS	de Man Rogosa and Sharpe
NCBI	National Center for Biotechnology Information
NF-$_k$B	Nuclear factor kappa B
nm	nanometre
OD_{527}	Optical density at a wavelength of 527 nm
PBS	Phosphate buffered saline
PFGE	Pulse field gel electrophoresis
PLB 1	Phospholipase B
R6G	Rhodamine 6 G
RNA	Ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
RVVC	Recurrent vulvovaginal candidiasis
qPCR	quantitative real time Polymerase chain reaction
SAP	Secreted aspartic proteinase
SD	Sabouraud Dextrose

spp	species
TLR2	Toll-like receptor 2
TNF α	Tumour necrosis factor α
UV	Ultraviolet
VVC	Vulvovaginal Candidiasis
WHO	World Health Organization
w/v	weight per volume
µg	micrograms
µl	microlitres
µm	micrometres
%	percent
°C	degrees celsius

CHAPTER 1: INTRODUCTION

With the increase in emerging multidrug resistance forms and species, fungal infections especially candidiasis continue to be life threatening and difficult to eradicate. Bloodstream infections (BSI) known as systemic candidiasis triggered by different *Candida* species account for about 40 % mortality rate among infected patients around the globe (Ahmad *et al*., 2016). Despite an immense improvement in healthcare systems globally, the number of immunocompromised and elderly patients infected by *Candida* species has increased drastically (Kabir *et al*., 2012). The Centers for Diseases Control and Prevention and the National Healthcare Safety Network in the United States of America have reported *Candida* species as the fifth most common hospital-acquired pathogens and the fourth among bloodstream infections (Yapar, 2014). Although other *Candida* species such as *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis*, *Candida krusei* and *Candida auris* have been recovered from disease infected patients (Badenhorst *et al*., 1991; Falagas *et al*., 2010; Kreusch and Karstaedt, 2013; Jeffery-Smith *et al*., 2018), *Candida albicans* still remains the most prevalent infectious fungal agent (Pfaller and Diekema, 2007; Zarrin and Mahmoudabadi, 2009). Thus *C. albicans* has gained importance as a potential human pathogen which has prompted researchers in Africa and around the globe to study the organism in depth for a better understanding of its pathophysiology (Vandeputte *et al*., 2012).

Despite the availability of several classes of antifungal drugs to treat candidiasis, only a few remain in clinics. Among these classes, the azoles (especially fluconazole) are the most widely used antifungal drugs for treating *Candida* infections. The fungistatic nature of fluconazole and its long-term utilisation has resulted in frequent fluconazole drug resistance (Ahmad *et al*., 2013), leaving behind no or very limited choice to treat *Candida* infections. Therefore, there is an urgent need to develop new and safer antifungal drugs along with new strategies to control drug resistance.

C. albicans, being the commensal opportunistic pathogen, has the capability to affect the host at various niches. The transition from commensal to pathogenic form has been attributed to its different virulence traits. Among these virulence traits, adherence to host cells, morphological transition from yeast to hyphae forms and secretion of hydrolytic enzymes are the prerequisite for *Candida* infections (Sardi *et al*., 2013). The yeast cells adhere to the host cell surface and the contact to the host cells induces the transition from yeast to hyphae. The secretion of the hydrolytic enzymes together with the adhesins and physical forces then facilitate the perforation into host cells by breaking down the barriers (Mayer *et al*., 2013). It has been reported that these virulence traits have the potential to be utilised as drug targets to develop new antifungal drugs (Ahmad *et al*., 2016). Utilisation of these emerging drug targets leads to the development of novel drugs, which can bypass drug resistance as these drugs do not impose survival pressure on the pathogens.

There are several drug mechanisms by which *C. albicans* have developed to withstand exposure to drugs. Among them, the most important drug resistance mechanism is over expression of drug efflux pumps (Ahmad *et al*., 2013). Therefore, besides targeting the virulence traits as emerging drug targets, new drugs that inhibit drug efflux pumps can also be used to prevent drug resistance. Besides being antifungal, these drugs may also have a potential to be used as chemosensitising agents and can be used in combination with azoles and other known drugs.

Use of probiotics for treatment of various diseases is gaining increased attention (Sarah *et al*., 2016). There are studies which have reported use of probiotics as whole live cells against *Candida* (Strus *et al*., 2005; Coman *et al*., 2014; Verdenelli *et al*., 2014). However, the chances that the severe risk of sepsis may develop are high especially in immunocompromised patients (Boyle *et al*., 2006). There are several studies reporting cases of sepsis caused by probiotics in infants and elderly patients (Mackay *et al*., 1999; Lestin *et al*., 2003; Kunz *et al*., 2004).

In a bid to prevent sepsis, new measures should be investigated to use the probiotics without the associated risk. One important measure to implement without the chances of causing sepsis is to use probiotic cell free extracts (CFEs) instead of whole live cells. There are few studies that have reported the use of probiotic CFEs against *C. albicans* (Krasowska *et al*., 2009; Murzyn *et al*., 2010; Nyanzi *et al*., 2014; Wang *et al*., 2017a). Most of these research studies involved use of extracts from the culture filtrates (Krasowska *et al*., 2009; Murzyn *et al*., 2010; Wang *et al*., 2017a). In one of the recent studies, anticandidal activity and characterisation of the chemical constituents of probiotic CFEs from *Lactobacilli* strains was investigated (Nyanzi *et al*., 2014). In the study, it was reported that the anticandidal activity of CFEs was due to the presence of some protein-like substances and organic acids (Nyanzi *et al*., 2014). On the other hand, use of other probiotics from *Saccharomyces* species such as *Saccharomyces boulardii* as therapeutic agents are slowly gaining interest among the researchers (Krasowska *et al*., 2009; Murzyn et al., 2010). In one study, the probiotic extract and its constituent capric acid from *Saccharomyces boulardii* were tested against virulence factors and their genes in the standard strain of *C. albicans* SC5314. The CFE and the constituent were found to be effective in inhibiting essential virulence factors such as morphology and adherence (Murzyn *et al*., 2010). As mentioned earlier, most of the research on probiotics has been done on the use of whole live cells, even in the prevention of virulence factors such a morphogenesis and secreted aspartic proteinase genes such as *SAP2* and *SAP6* (Pericolini *et al*., 2017; Wang *et al*., 2017a; Gabrielli *et al*., 2018). Therefore, the challenge of side effects associated with the use of live probiotic microorganisms are still a concern.

With this background, the aim of this study was to unravel the in vitro antifungal effects of probiotic CFEs from three different probiotic strains against fluconazole (FLC) susceptible and resistant *C. albicans*. In this study, we also intended to investigate the effect of the most active probiotic CFE on virulence factors, reversal of antifungal drug resistance and their genes in different *C. albicans* isolates.

1 Literature Review

1.1 Characteristics and taxonomy of *Candida albicans*

Candida albicans is a eukaryotic, opportunistic fungal pathogen and can be found in different population types (neonates, children and adults). On agar culture media, they commonly exist as oval cream-colored colonies with a characteristic sour beer aroma when grown under aerobic conditions at optimum temperature of 37 °C. Growth is usually detected after 24-48 h under these conditions. *C. albicans* belong to the genus *Candida* which is comprised of more than 150 species (Moris *et al*., 2008). *C. albicans* is still the most prevalent, predominant and most studied *Candida* species causing candidiasis worldwide; even among other pathogenic fungi too. *C. albicans*, being a dimorphic fungus, reproduces similar cells by budding to form pseudohyphae. However, under environmental conditions such as body temperature, pH and the presence of serum, the hyphal form may be developed (Mayer *et al*., 2013). Pseudohyphae and hyphae can be differentiated based on their size and shape. True hyphae are narrower with parallel walls that have no constrictions whereas cells with pseudohyphae are wider and form constrictions between elongated buds (Shapiro *et al*., 2011). Even though yeast and true hyphae are often observed during infection, the role of pseudohyphae and switching *in vivo* is rather unclear. Nevertheless, in this report we will make mention of the three common forms of fungal morphology namely yeast, pseudohyphae and true hyphae which occur due to changes in the cell wall. During this morphological transition, cell wall plays a major role and therefore understanding the makeup composition of cell wall is important.

The cell wall comprises of about 80-90 % carbohydrates and is well established as being essential to every aspect of biology and pathogenicity of *C. albicans* (Hall and Gow, 2013). According to Ghannoum (2001), the cell wall of most pathogenic fungi including *C. albicans* is a multi-layered structure. The outer layer is composed of mannan, mannoproteins and 1, 6-

β-glucan and the inner layer is mainly composed of 1, 3-β-glucan, chitin and some mannoproteins as shown in **Figure 1.1.** The cell wall components such as β glucans and chitin (1, 4 linked polymers of N-acetyl-D-glucosamine) have been reported as structural polysaccharides which give strength and shape to cell wall (Gowe and Hube, 2012). In the matrix, there are mannoproteins that are glycosylated with mannose containing less organised polysaccharides called mannans. These polymers form hydrogen bonds between adjacent polysaccharide chains to generate a complex three-dimensional network of micro fibrils (Shapiro *et al*., 2011). The mannan layer does not affect the shape of the cell, however due to its low permeability and porosity it influences the permeability of antifungals through the cell wall. In addition, as cell wall is the outermost layer of the cell, it represents the point of contact between *C. albicans* and the host endothelium. The proteins and carbohydrates at the outer layer have several functions which include the ability to act as adhesion molecules mostly facilitated by mannans. Cell wall is the major difference between fungal and human cells and therefore it provides a potential target for antifungal drugs (Shapiro *et al*., 2011).

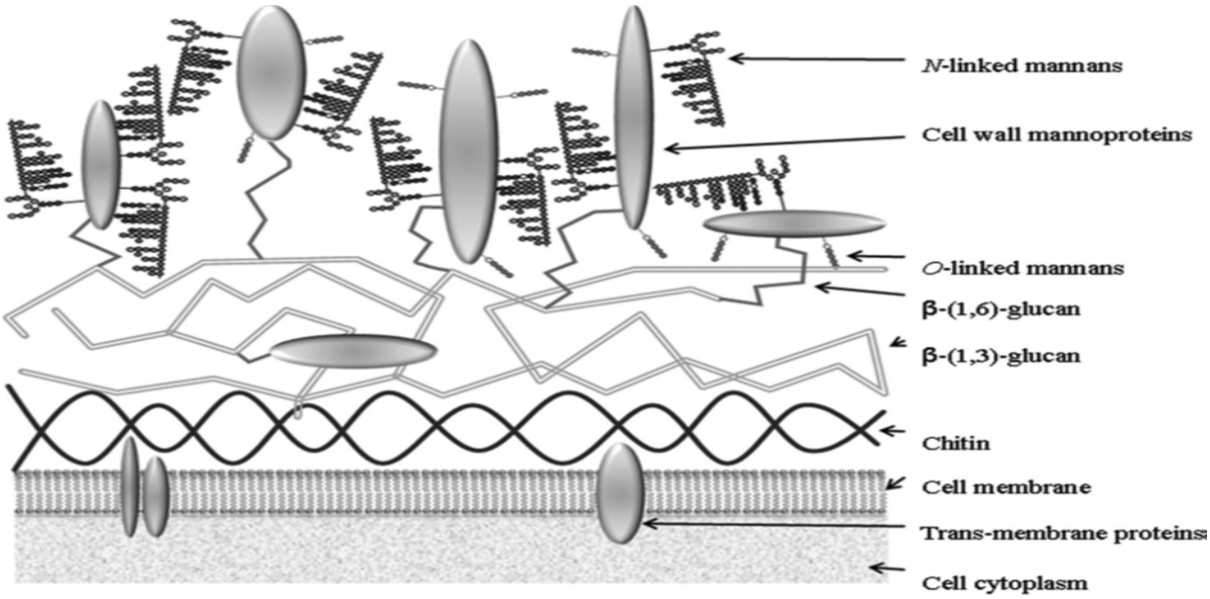

Figure 1.1: Cell wall structure of *C. albicans* showing arrangement of major components that are involved in immune activation and suppression thus making it an ideal drug target (Grubb *et al*., 2008).

1.2 Diseases and Clinical features

C. albicans usually exist as a harmless commensal in the gastrointestinal tract and genitourinary tract in about 70 % of humans, without causing any disease. However, it becomes an opportunistic pathogen in immunocompromised patients, causing a wide variety of diseases ranging from localized membrane infections to life-threatening disseminated diseases (da Silva Dantas *et al*., 2016). Infections due to *C. albicans* are divided into various stages which include colonisation, superficial infections, deep-seated infections and systemic infections. During colonisation, *C. albicans* live as a commensal, with the normal microbial flora on mucosal surfaces without causing any harm to the host. However, superficial infections arise when there is an imbalance in the microbial flora or when the immune system of the host is compromised or suppressed.

Oral infections are very common in immunocompromised patients especially in HIV/AIDS patients. In contrast, vaginal infections often occur mostly in individuals who have no clear signs of immune defects. On the other hand, hospitalized patients are prone to life-threatening systemic candidiasis, identified by dissemination through the bloodstream and infection of internal organs (Jacobsen *et al*., 2012). Based on the severity of the infection, candidiasis is categorised into two main types, detailed below:

1.2.1 Superficial candidiasis

Superficial candidiasis, also called thrush, is a mild type of infection and is sub-categorised into oral and vaginal candidiasis. Oropharyngeal candidiasis arises because of local factors such as the administration of broad-spectrum antimicrobials, inhaled corticosteroids or radiation of the head and neck. It is characterized by white plaques on the buccal mucosa, palate, oropharynx, or tongue (Matsubara *et al*., 2016a). Vulvovaginal candidiasis (VVC) is common among women of child-bearing age. It is the most predominant mucosal expression of *Candida* infection. The risk factors may include increased oestrogen levels due to use of oral contraceptives and conditions such as diabetes mellitus, therapy with corticosteroids or

broad-spectrum antimicrobials as well as HIV (Matsubara, *et al*., 2016a). Symptoms mostly include vaginal discomfort, discharge, and vulvar pruritus. About 75 % of women population have been found to suffer from VVC at least once in a lifetime with around 20 % recurrent vulvovaginal candidiasis (RVVC), where experience of frequent infections is very common (Kabir and Ahmad, 2012; Zeng *et al*., 2018).

1.2.2 Systemic or Invasive Candidiasis

These infections are life-threatening and common in individuals whose immune system is paralyzed such as HIV/AIDS patients, individuals undergoing chemotherapy, organ transplants and radiotherapy treatments for cancer patients (Kabir and Ahmad, 2012; Kabir *et al*., 2012). This type of infection occurs when *C. albicans* cells cross the mucosal barriers and enter the bloodstream from where it is often transported to the vital organs. On this basis, systemic candidiasis is categorised into candidemia (infection in blood) and disseminated candidiasis (infections of organs). Infections caused by *Candida* species are generally nosocomial and are the fourth most common cause of candidemia globally (Guery *et al*., 2009; Picazo *et al*., 2008). Disseminated candidiasis is either an infection of a single organ or may involve several organs.

1.3 Prevalence of *Candida* species in South Africa

There are increasing rates of studies on distribution of *Candida* species and infections, however these studies are limited in South Africa (Kreusch and Karstaedt, 2013; Makhado *et al*., 2014). A recent study suggested that there is limited documentation and surveillance programmes in South Africa and Africa due to treatment of patients without the knowledge of which species they harbour, lack of funding to carry out surveillance programmes and limited number of research collaborations on fungi and fungal infections (Africa and dos Santos Abrantes, 2016).

Badenhorst and co-workers investigated the incidence of hospital fungal infections from blood cultures received over a one-year period in Bloemfontein academic hospitals (Free State province) (Badenhorst *et al.,* 1991). They reported *C. albicans* (42 %) as the most common species that was isolated followed by *C. tropicalis* (26 %), *C. parapsilosis* (20 %) and *C. glabrata* (7 %). In Chris Hani Baragwanath hospital, Soweto, Gauteng province, a surveillance study on the epidemiology of candidemia amongst adults during the period 1990 to 2007 was conducted and it was observed that *C. albicans* and *C. tropicalis* (23 %) were the dominant species in 1990. However, from 2005 to 2007 the common species were *C. albicans* (44-46 %), *C. parapsilosis* (25-28 %) and *C. glabrata* (22-23 %). Other *Candida* species that were reported though in low incidences included *C. tropicalis*, *C. krusei*, *C. guilliermondii* and *C. lusitania*e (Kreusch and Karstaedt, 2012). At Mandela Academic Complex, Mthatha, Eastern Cape, *C. albicans* (45.5 %) was the most frequent species that was isolated; however, a different pattern from the other surveillance studies was observed where the *Candida* species distribution was *C. glabrata* (31.1%), *C. tropicalis* and *C. dubliniensis* (11%) respectively (Mnge *et al*., 2017). A new multidrug resistant non albicans *Candida* species, *C. auris* was recently identified in South Africa. The first case was reported in 2009 but was misidentified as *C. haemulonii*. In 2014, it was confirmed as *C. auris*. In a surveillance study conducted during 2012 to 2016 in South Africa by Govender *et al* (2018), it was observed that *C. auris* was more prevalent in Gauteng province as most cases were reported in this province; however, the causes of the rapid spread were not well established. Based on these surveillance reports, it can be observed that *C. albicans* is still the most frequent *Candida* species isolated in South Africa. In addition, the distribution of non albicans *Candida* species varied amongst the few provinces in South Africa where the studies were carried out and these variations depended on geographical area and hospital units. The species distribution was mostly among immunocompromised adults who were suffering from

HIV, as well as in paediatric and neonatal patient populations (Africa and dos Santos Abrantes, 2016; Kreusch and Karstaedt, 2013).

1.4 Diagnosis

With the ever-increasing number of multidrug resistant *Candida* species and forms, early and appropriate diagnosis becomes significantly important. However, diagnosis of *Candida* infections has proved to be difficult, because some of the etiologic agents are also commonly observed in healthy people. Mucosal candidiasis is easily diagnosed by scraping the lesions and carrying out a Gram stain to determine yeast cells undergoing budding as well as pseudohyphae (Cannon *et al*., 2009). However, the diagnosis of invasive candidiasis is very challenging. Health care providers diagnose invasive candidiasis based on the patient's medical history, symptoms, physical examinations and laboratory tests. The most common and easy method is taking blood samples, culturing them in appropriate media to determine if *Candida* will grow. However, this has been found to take a few days before results are released of which the delay may become detrimental to a seriously ill patient. Another diagnostic method involves microscopic examination of clinical specimens. Both blood culture and microscopic methods are time-consuming and are often associated with false results (Kozel and Wickes, 2014). Commercial kits such as the analytical profile kits (API 20C AUX) are available for more clinically relevant yeasts and fungi including *Candida* species. They are mainly based on biochemical tests and turbidity interpretation often recovering a large range of species. To identify the species, incubation of 24-72 h is required. The advantage of using the API is that the physiological characteristics of *Candida* species can be identified rapidly (Moris *et al*., 2008). The Vitek 2 system is an automated instrument used to accurately identify clinical isolates which include *C. albicans*, *C. parapsilosis*, *C. tropicalis* and *C. glabrata*. The Vitek system identifies 27 species of medically important yeasts. It is used with a yeast biochemical card which includes a programmed computer, a reader incubator unit, a filing module, a sealer module and a printer. The advantage of this

system is that it requires minimal set up, with automated inoculation of the substrates with a suspension of each isolate, and 26 biochemical tests can be performed simultaneously from the same inoculum (Moris *et al*., 2008; Sudhan *et al*., 2016). To every diagnostic technique there are limitations; the Vitek system requires a large inoculum, there is no manual back up, the storage space for the disposable injector tubes is not available and the yeast biochemical card has a short shelf life of 2 months.

Molecular techniques have been employed to facilitate early diagnosis of candidiasis (Cannon *et al*., 2009). This involves the application of techniques such as real time polymerase chain reaction (qPCR) and matrix assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS). The normal conventional PCR and real time PCR are used to measure the amount of fungal DNA present. Though the conventional PCR has been widely used with satisfactory results obtained from employing the technique, the real time polymerase chain reaction (qPCR) has been regarded as the technique of choice as it has been shown to be rapid, highly specific, and sensitive. The technique allows observation of increase in amount of DNA as it is amplified, and post PCR manipulation is not required because the fluorescent signals are measured directly as they pass out of the reaction vessels (El-Naggar *et al*., 2010). In addition, resistant *C. albicans* isolates can be detected rapidly by using primers that are specific for mutations that give rise to antifungal resistance, (Kabir and Ahmad, 2012). The application of MALDI-TOF MS to *Candida* identification is a new approach that has become one of the important and widely used techniques in clinical microbiology laboratories. The technique allows ionisation and vaporisation of large biomolecules such as intact proteins which include structural proteins, DNA, RNA, binding proteins and ribosome modulation factors that produce spectral fingerprints specific to a genus, species and subspecies. The technique distinguishes bacteria and yeasts within 20-35 min; however, it requires single colonies incubated for 18-48 h. Another limitation is that MALDI-TOF MS can give inconclusive and false results due to ribosomal protein spectra

that do not differ adequately in their ribosomal protein. These techniques have been greatly used for identification of *Candida* species, however, their use is limited, and some species cannot be identified and differentiated; due to these shortcomings, molecular methods such as PCR based techniques can be employed instead of conventional methods as more accurate results are obtained within a short period of time (Alam *et al*., 2014).

1.5 Treatment

Development of antifungal therapeutics has become a major problem due to the high resemblance of fungal cells to that of eukaryotic host cells. This resemblance leaves only few targets that can be used to produce antifungal drugs. Based on these targets, current treatment for candidiasis involves antifungal drugs from five major classes which include azoles, echinocandins, allylamines, pyrimidine analogues, antimetabolites, thiocarbamates and morpholines (Sanglard *et al*., 2009).

1.5.1 Azoles

Azoles which include imidazoles and triazoles are the most widely used antifungal drugs in treating both mucosal and systemic fungal infections in clinical settings. Among azoles, fluconazole is the first drug of choice for clinicians to treat any fungal infection including candidiasis. Fluconazole targets the ergosterol biosynthesis pathway, which leads to the reduction of ergosterol and build-up of by-products of the pathway (Bondaryk *et al*., 2013).

1.5.2 Polyenes

The most commonly used polyene is amphotericin B, which exerts its fungicidal effect by interacting with the membrane sterol resulting in the production of pores. This in turn lead to alteration of cell wall permeability, leakage of important cell components such as potassium and sodium ions and eventually death of the microorganism (Ghannoum, 2001). Despite amphotericin B being used in clinics for a long time, its use has declined due to associated

side effects, which promoted researchers to modify these drugs leading to the formulation of liposomal amphotericin B (Voncik *et al*., 2016).

1.5.3 Echinocandins

The recent class of antifungals is echinocandins with micafungin, caspofungin, and anidulafungin as approved drugs to treat fungal infections. This amphiphilic class of lipopeptides target the 1, 3-β-D-glucan synthesis by targeting the 1, 3-β-D-glucan synthases (Sanglard *et al*., 2009; Sanguinetti *et al*., 2015). Despite resistance to this class of antifungal being uncommon, there are studies reporting drug resistance of *C. glabrata* against echinocandins.

1.5.4 Pyrimidine analogues

This class includes flucytosine (5 fluorocytosin, 5FC) which upon conversion to 5-flourouracil in the target cell becomes an antifungal agent. 5-flourouracil acts by interfering with the metabolism of pyrimidines which make up RNA and DNA and protein synthesis in fungal cells (Vandeputte *et al*., 2012). 5-flourouracil can be incorporated into mRNA and thereby inhibit protein synthesis or it can inhibit DNA synthesis by inhibiting thymidylate synthase.

1.5.5 Allylamines, thiocarbamates and morpholines

This class of antifungal drugs is rare and used mostly as topical agents to treat dermatophyte infections. They inhibit the biosynthesis of ergosterol at different stages. Sanglard *et al* (2009) reported that allylamines and thiocarbamates inhibited squalene epoxidase which plays an important role in the early stage of ergosterol biosynthesis.

1.6 Virulence traits and their scope in drug development

Pathogenesis is defined as the progression of any disease in the host (Khan *et al*., 2010). To successfully trigger an infection in a patient, *C. albicans* needs to actively adapt to different niches at various anatomic sites of the host to withstand the lytic activity of the host's

defences. Virulence has been shown to depend on host recognition, interaction, multiplication and damage to host organs. *C. albicans* attaches to the host epithelial cells and when it acquires adequate nutrition, it then penetrates the epithelial cells causing superficial infections. In cases where the host immune system is suppressed, the fungus continues to penetrate further into the epithelial tissue causing deep-seated infections as illustrated in **Figure 1.2**. The fungus may also penetrate other tissues during the process of invasion resulting in disseminated infection which may be fatal to the host (Naglik *et al*., 2003). Expression of various genes, gene products, cell surface proteins and virulence factors have been shown to function as virulence traits and contribute to the ability of *C. albicans* to cause infections (Khan *et al*., 2010).

Virulence traits have recently been reported as emerging drug targets. Researchers have considered these virulence traits as very attractive options that can be used to develop new antifungal drugs for specifically controlling *C. albicans* infections. Targeting virulence traits for therapeutic purposes could be advantageous as targeting these factors will not put survival pressure on the pathogens and thereby minimising chances for drug resistance. This strategy also aids in keeping track of opportunistic pathogens. However, on the other hand, virulence factors are specific and vary from species to species and therefore targeting virulence factors will be limited in acting against any multiple pathogen.

The common primary virulence traits in *C. albicans* include adhesion, morphogenesis and secretion of hydrolytic enzymes. The first indicator of pathogenesis by *Candida* is host recognition which is then followed by adherence and morphology (Mayer *et al*., 2013; Ahmad *et al*., 2016).

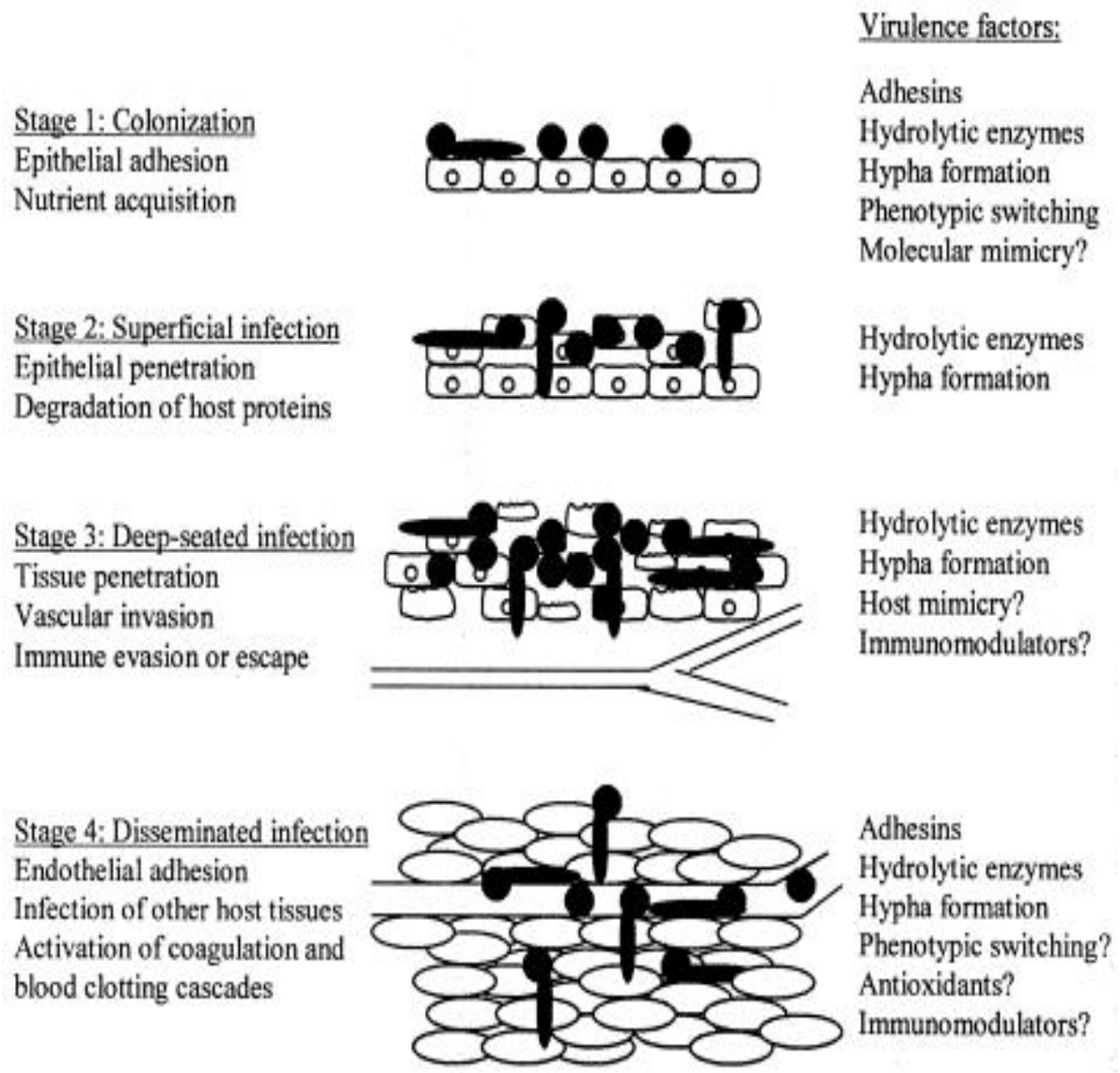

Figure 1.2: Virulence factors that give rise to pathogenicity in *C. albicans* (Naglik *et al*., 2003).

1.6.1 Morphogenesis: transition from yeast to hyphae

Morphogenesis is one of the virulence factors that has been the focus of most of the research done on the human pathogenic fungus *C. albicans* (Gow *et al*., 2002; Mayer *et al*., 2013). It is one of the reversible steps in pathogenesis characterised by formation of germ tubes, which are the projections observed in the initial formation of pseudohyphae or hyphae in the host as illustrated in **Figure 1.3**. Researchers in most clinical laboratories have used the germ tube test to provide a definitive identification of the organism within three hours (Moris *et al*.,

2008). In germ tube test, the test organism is incubated in the presence of foetal calf serum to induce germ tube formation which is then detected microscopically. A total of approximately 75 % of yeasts have been recovered using this method. As a result, it has become one of the most economical methods of identifying yeasts. Studies have shown the germ tube test to be mostly restricted to a few *Candida* species that include *C. dubliniensis* and *C. albicans* and is regulated and facilitated by several external factors such as presence of serum, neutral pH, increased carbon dioxide concentration and physiological temperatures. Serum and elevated temperatures of 37 °C promote the transition from yeast to hyphae (Shapiro *et al*., 2011; Mayer *et al*., 2013).

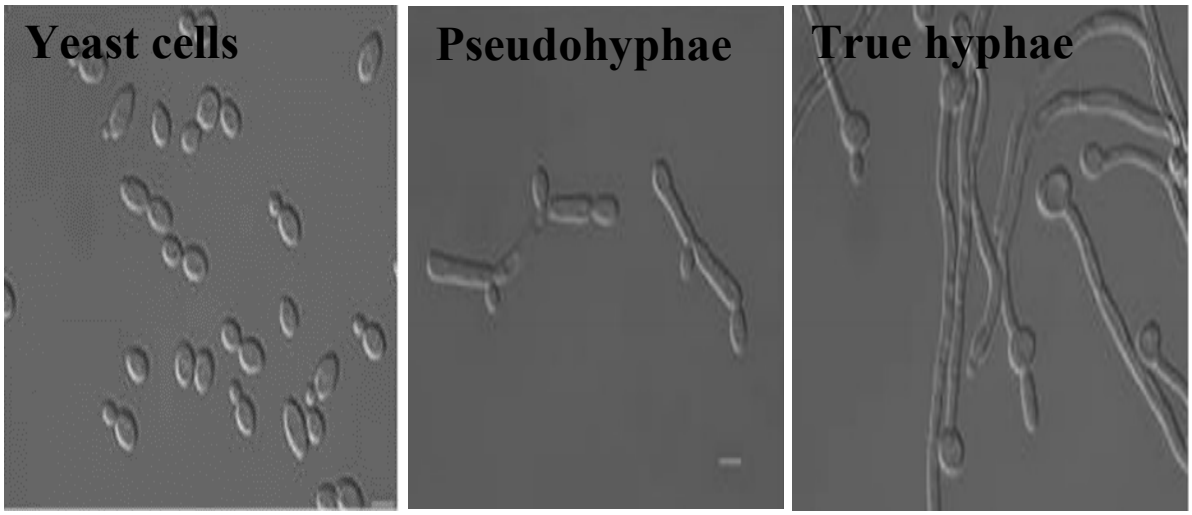

Figure 1.3: Common morphologies in *C. albicans* (de Castro *et al*., 2013).

Both the yeast and hyphae forms have been reported to be essential for virulence and have different functions during the development of a disease (Gow *et al*., 2002; Vila *et al*., 2017). In addition, research studies showed that hyphae formation depends upon adhesion of the yeast cells (Jacobsen *et al*., 2012). When *Candida* cells encounter the host cells, hyphae formation and hyphal associated adhesins are induced simultaneously thereby enhancing the ability of the fungi to adhere to surfaces. In *C. albicans*, the transition from yeast to hyphae also involves a complex network of signalling pathways.

Furthermore, formation of hyphae and the expression of hyphal-associated genes have also been shown to mediate distinct virulence roles such as adhesion. Different morphology associated genes such as *EFG1*, *HWP1*, *TUP1* and *HSP90* have been reported to regulate the yeast to hyphae transition at molecular level. In environmental conditions of high temperature or pH, *EFG1* gene is activated. This in turn induces expression of the *HWP1* gene, which encodes cell surface proteins essential for hyphae formation as well as adhesion to host cells. On the other hand, under normal environmental conditions, morphogenesis is inhibited by suppressor proteins encoded by *TUP1* and *HSP90* (Sharkey *et al*., 1999). Inhibition of the morphological transition would prevent adhesion, invasion and damage of epithelial and endothelial cells, as well as prevent escape from macrophages. It may also aid in modulating the immune response thereby avoiding excessive inflammation during superficial infections.

There are reports of modulating yeast to hyphae transition by several small molecules such as farnesol, fatty acids, rapamycin and geldanamycin. However, many of these small molecules have also been reported to affect fungal viability and growth and contribute to cytotoxicity (Shareck and Belhumeur, 2011). Therefore, further research is required to be done to search for more natural, semi synthetic or synthetic compounds which can target morphological transition at lower concentrations. Researchers have noted that, targeting yeast to hyphae transition is an attractive option to control infections caused by *C. albicans* both prophylactically and as treatment (Mayer *et al*., 2013). This shows that morphogenesis is an important pathogenic trait and can be an emerging drug target to develop new antifungal drugs (Jacobsen *et al*., 2012; Ahmad *et al*., 2016).

1.6.2 Hydrolytic enzymes: Secreted aspartic proteinases

After morphological transition, *C. albicans* secretes hydrolytic enzymes which have been suggested to contribute to pathogenicity by facilitating active penetration of *Candida* cells into the host cells. They are also suggested to increase efficiency of extracellular nutrient acquisition. The hyphal tip has been reported to be the site where hydrolytic enzymes are

secreted (Pawar *et al*., 2014). The major role of these enzymes is to break down host proteins, lipids and other cellular components thereby facilitating the infiltration of the yeast cells into the host cells (Gow *et al*., 2002). The two most common classes of hydrolytic enzymes that have been widely reported are the secreted aspartic proteinases (SAPs) and the phospholipases (Pawar *et al*., 2014).

SAPs are the extracellular hydrolytic enzymes secreted by most *Candida* species including *C. albicans*. *In vitro*, these enzymes are secreted when the yeast cells are cultured in the presence of exogenous protein as a source of nitrogen. SAPs catalyse the hydrolysis of peptide bonds (CO-NH) in human proteins such as albumin, haemoglobin, keratin and secretory immunoglobulin A. The measure of virulence is related to the level of proteinase production which depends on the type of strain and infection, environmental conditions and stage of infection. They have been shown to contribute to pathogenesis by promoting adhesion, through the proteolysis of host surface proteins, damaging of endothelial cells, thereby increasing fungal nitrogen resources and subsequently resulting in host tissue damage (Schaller *et al*., 2005). SAPs also play a complex role in the pathogenicity of *C. albicans* by contributing in hyphae formation and phenotypic switching (Naglik *et al*., 2003; Khan *et al*., 2010; Mayer *et al*., 2013; Pawar *et al*., 2014). In addition, SAPs have also been suggested to be involved in counteracting the host immune response because of their ability to resist phagocytosis and intracellular killing (da Silva Dantas *et al*., 2016).

Several studies have identified SAPs which are encoded by ten genes *SAP1-SAP10*. The genes encode enzymes having similar functions and characteristics but different molecular properties and have been shown to play various roles during the different phases of infection (Pawar *et al*., 2014). *C. albicans* have been shown to express *SAP1-SAP3* in the yeast form whereas *SAP4-SAP6* are expressed in the hyphal form. On the other hand, *SAP9* and *SAP10* have been shown to be expressed in both forms although; they remain bound to the cell surface whilst the roles of *SAP7* and *SAP8* are yet to be fully understood. Several studies on

gene expression have confirmed expression of *SAP1-SAP3* in cutaneous candidiasis based on reconstituted human epidermis (Naglik *et al*., 2003; Schaller *et al*., 1999). However, Stanisvewska *et al* concluded that *SAP1-SAP3* are the main proteinases contributing to the early stages of mucosal infections (Stanisvewska *et al*., 2012).

It is reported that the production of these enzymes was intensified in multidrug resistant *Candida* species when exposed to fluconazole (Wu *et al*., 2000). In addition, it is also reported that SAP activity is comparatively higher in denture wearers (Koga-Ito *et al*., 2006). *In vitro*, SAPs can be detected by culturing *C. albicans* on media supplemented with bovine serum albumin as the only source of protein. The production of proteinases is observed as clear zones around the colonies showing degradation of the proteins by the enzyme (Naglik *et al*., 2003).

1.6.3 Phospholipases

C. albicans have been found to be capable of secreting large amounts of phospholipase enzymes. The secretion of the extracellular phospholipases from *C. albicans* was first identified by growing the cells on solid media supplemented with egg yolk and analysing the products which are produced due to lipid breakdown (Price *et al*., 1982; Yang, 2003). Phospholipases are a group of enzymes that hydrolyse specific ester linkages in glycophospholipids. They are known to attack phospholipids which are the major components of cell membranes in the host cell resulting in damage to cell membrane thereby allowing yeast cells to invade the host tissues with ease (Ahmad *et al*., 2016; Ellepola *et al*., 2016). Phospholipases have been reported to be associated with host cell penetration, adhesion to epithelial cells, invasion of epithelial cells and interaction with host signal transduction pathways (Schaller *et al*., 1999). Activity of phospholipases is increased during damage of the host cell membrane because the enzymes hydrolyse one or more ester linkages of glycerophospholipids. Various studies have shown that increased phospholipase activity is

higher in clinical and blood isolates than in commensal *C. albicans* (Leidich *et al*., 1998; Khan *et al*., 2010).

Four classes (phospholipase A-D) have been identified; each specific towards individual ester bonds in glycerophospholipids; nevertheless, class B has been shown to likely contribute to pathogenesis by damaging and transversing host cell membranes. They possess a wide substrate specificity and have both hydrolase and lysophospholipase-transyclase activities (Ghannoum, 2000). Phospholipase B (PLB) breaks down acyl ester bonds and catalyses the lysophospholipase-transyclase reaction to produce fatty acids, phospholipids and lysophospholipids as illustrated in **Figure 1.4**.

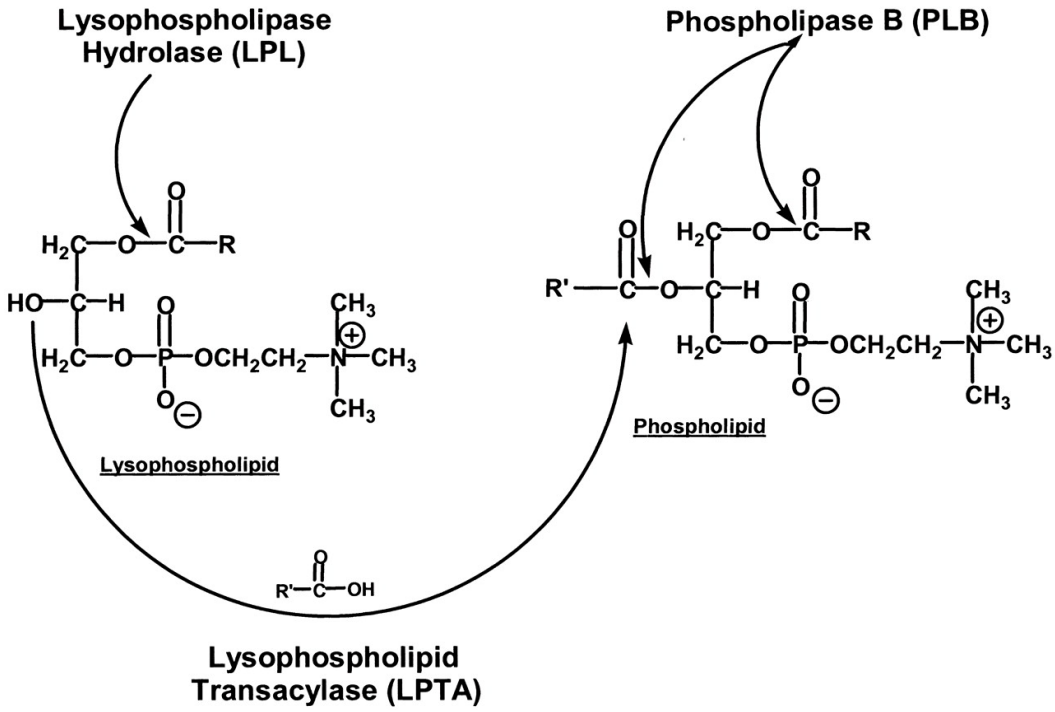

Figure 1.4: Sites of action of phospholipases on phospholipids in *C. albicans* (Ghannoum, 2000).

Production of the phospholipases is also genetically expressed. Five genes (*PLB1-PLB5*) expressed by the class B have been shown to exist and signal their secretion with *PLB1* being the common gene associated with pathogenicity.

1.7 Antifungal Drug Resistance

Drug resistance to the available antibiotics is a global challenge. The rapid emergence of *Candida* species and forms is occurring worldwide thereby endangering millions of lives. Studies have already reported drug resistance to the limited available antifungals (Sanglard *et al*., 2009; Sanguinetti *et al*., 2015; Campoy and Adrio, 2017). Azoles are the most widely used antifungals to treat *C. albicans* infections and therefore progression of azole resistance in *Candida* species is well characterised. Due to the limited numbers of antifungal drugs as compared to the antibacterial drugs; there are limited options to treat these fungal infections as a result of drug resistance against these antifungals. Several factors have been shown to contribute to drug resistance and these include delay in diagnosis, over use and abuse of drugs, improper low dosages or treatment courses that do not comply with the treatment plan (Campoy and Adrio, 2017).

The progression of azole resistance is well characterised in *C. albicans*. Resistance to azoles especially fluconazole involves several mechanisms; which include modification of genes involved in ergosterol biosynthesis pathway, loss of heterozygosity and overexpression of drug efflux pumps (Ahmad *et al*., 2013; Berkow and Lockhart, 2017). The most common mechanism of azole resistance in *C. albicans* is pumping out of drugs by the efflux pumps as shown in **Figure 1.5**. The removal of antifungal drugs through these transporter proteins (efflux pumps) is widespread in fungi and this results in the inability of the drugs to accumulate inside the cells leading to drug resistance (Ahmad *et al*., 2016). The major facilitator superfamily class (MFS) and the ATP-binding cassette family (ABC) transporters are the two major classes of efflux proteins that exist in *Candida* species. These transmembrane proteins translocate substrates across the fungal cell membrane using two different energy sources and thus are capable of mediating resistance to FLC. ABC transporter proteins have broad substrate specificity and depend on hydrolysis of ATP as the source of energy (Pinjon, 2005; Ahmad *et al*., 2016). In *C. albicans*, two of these namely

Candida drug resistance 1(Cdr1p) and *Candida* drug resistance 2 (Cdr2p) have been well characterised and have also been shown to be linked to FLC resistance. Studies have shown that at a molecular level, the *CDR1* gene is highly expressed at detectable levels in susceptible *C. albicans* isolates, however, *CDR2* gene is not usually expressed and its deletion has been shown to have an impact on susceptibility in a drug resistant isolate (Berkow and Lockhart, 2017). On the other hand, FLC resistant *C. albicans* overexpress *CDR1* and *CDR2* and resistance has been reported to be reduced if both genes are deleted (Campoy and Adrio, 2017).

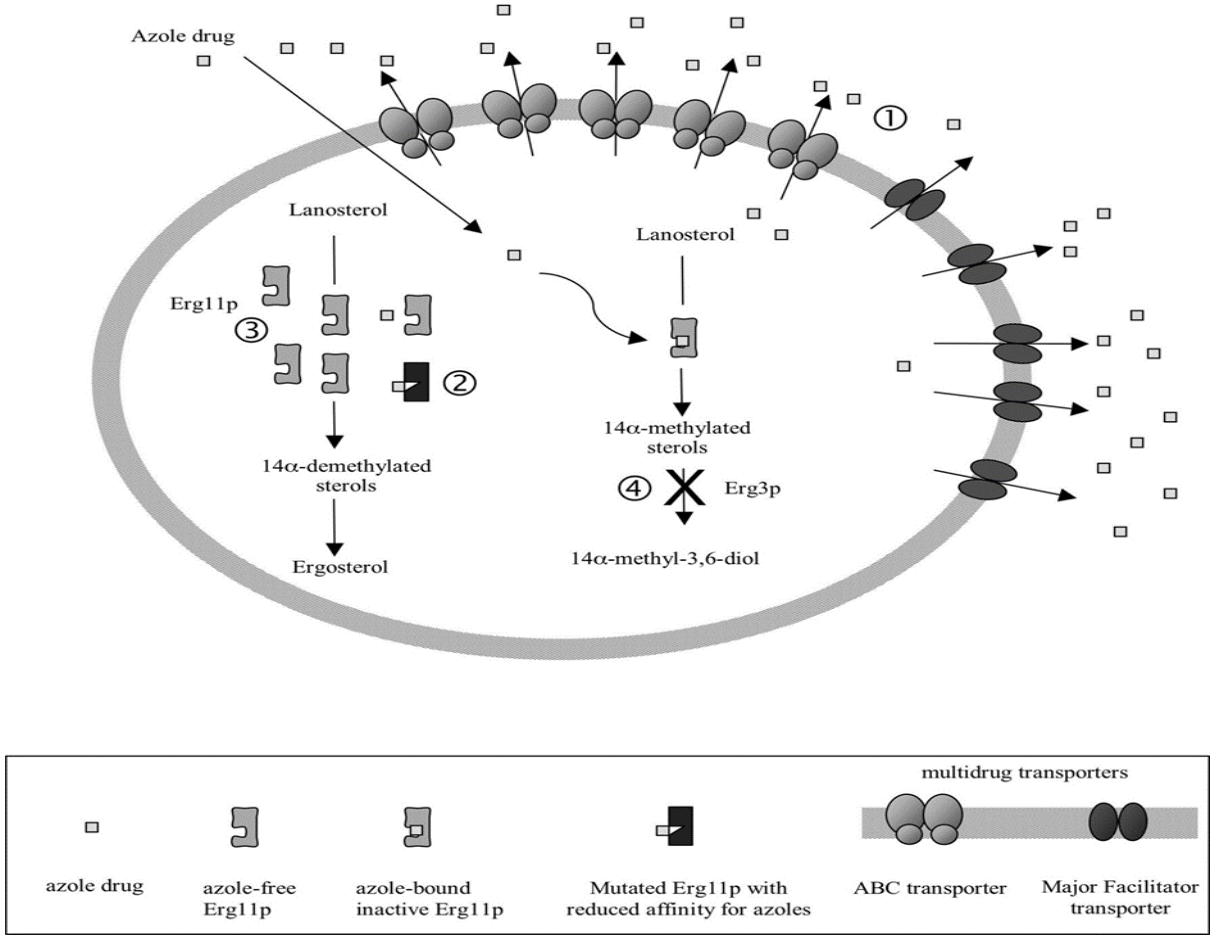

Figure 1.5: Diagrammatic representation of azole antifungal drugs in *C. albicans* and the mechanism by which resistance occurs (Pinjon *et al*., 2005). (1)- Reduced build-up of drug due to up-regulation of ABC (ATP-binding cassette) and major facilitator multidrug transporter genes. (2)- Reduced affinity to azoles of the target enzyme Erg11p. (3)- Elevated cellular content of Erg11p. (4)- Modification of the ergosterol biosynthetic pathway by inactivation of Erg3p.

Compared to the ABC transporter proteins, the MFS transporter proteins have a much narrower scope of substrate specificity and are driven by electrochemical proton-motive force. In the *C. albicans* genome, about 95 MFS transport proteins have been predicted but only one multidrug resistance 1 protein (Mdr1p) has been found to be linked to FLC resistance. In addition, overexpression of *MDR1* gene has been observed mostly in resistant *C. albicans* clinical isolates and its deletion from overexpressing isolates has been proven to reduce resistance (Berkow and Lockhart, 2017). Researchers have also attempted to investigate and understand the drug efflux mechanism of drug resistant strains of *C. albicans* using Rhodamine 6 G (R6G) (Maesaki, 1999; Ahmad *et al*., 2013). It is a fluorescent dye that is used to quantitate mostly the ABC transporter activity of Cdr1p and Cdr2p in *C. albicans*. It is known to be conveyed into or out of the cells in many pathogens that maintain MDR (Maesaki, 1999).

It can therefore be hypothesised, that the inhibition of drug efflux pumps using probiotic cell-free extracts (CFEs) may be considered as a feasible method of overcoming clinical antifungal drug resistance in resistant *C. albicans* isolates.

1.8 Probiotics

The concept of using natural products such as probiotics against microbial infections has been shown to be a feasible therapeutic technique against *Candida* infections. The interest in using probiotics against these opportunistic fungal pathogens is due to limitations of the currently available antifungal drugs (Matsubara *et al*., 2016a).

Probiotics, according to the Food Agricultural Organization and the World Health Organization, are live microorganisms which when administered in adequate amounts confer a health benefit to the host (FAO/WHO, 2002). These can be used in different types of products such as foods, drugs and as dietary supplements. Increased research has been done to explain the molecular mechanisms of the protective action of probiotics against *Candida*

infections, however, their exact role in preventing invasive or systemic candidiasis are still not clearly understood (Fassio, 2016). Probiotics are known to regulate immunity, lower cholesterol, treat rheumatoid arthritis, prevent cancer, improve lactose intolerance, and reduce constipation, candidiasis and urinary tract infections (Nagpal *et al*., 2012; Matsubara *et al*., 2016a). A recent study suggested that for a probiotic to be deemed good, it should be a microorganism of human origin, one which can exert its beneficial effect on the host organism (Moni *et al*., 2017). In addition, it should be neither pathogenic nor toxic and must be able to survive in gastrointestinal tracts. The foodstuff acting as a vehicle for the probiotic should also have a pleasant taste and should ideally have a prolonged shelf life (Matsubara *et al*., 2016a).

1.8.1 Genera of microorganisms used as probiotics

Microorganisms from various genera are being used as probiotics. Common probiotics belong to the heterogenous group of lactic acid bacteria (LAB) which include bacterial genera such as *Enterococci*, *Bifidobacterium* and *Lactobacillus*. Although the term probiotics includes mostly lactic acid bacteria such as *Lactobacillus* and *Bifidobacterium*, it can also be extended to other microorganisms which have not been fully explored but have been found to possess the characteristics of probiotics (Soccol *et al*., 2010). *Bacillus* and fungi such as *Saccharomyces* are some of the species that have also been reported and regarded as probiotics. These probiotics have been well characterised and recommended for their therapeutic and prophylactic potential use against infections (Fijan, 2014). They have also been conferred a 'generally regarded as safe' ('GRAS') status due to their long history of safe use and proven health benefits except for enterococci and streptococci species.

1.8.1.1 *Bifidobacteria* genus

Bifidobacterium species were first isolated and described in 1899-1900 by Tissler who described them as rod-shaped anaerobic microorganisms found in faeces of breast-fed infants. They are characterised as Gram-positive, non-motile, non-spore forming and catalase

negative anaerobic bacteria. They inhabit mostly the gut and vagina of animals as well as humans. In infants, the *Bifidobacterium* genus constitutes about 91 % of the microflora and decrease with increasing age to about 25 % in humans (Soccol *et al*., 2010). The optimal growth conditions for *Bifidobacterium* are in the range 37-40 °C. Probiotics from this genus are known for their resistance mechanism to bile salts. Several *Bifidobacterium* species that are considered as probiotics include *Bifidobacterium animalis* subspecies *lactis*, *B*. *longum*, *B*. *infantis*, *B*. *breve*, *B*. *animalis* subspecies *animalis* and *Bifidobacterium bifidum* (Fijan, 2014).

1.8.1.2 *Lactobacillus* genus

Lactobacillus species are mostly found in the intestinal tract and vagina where they prevent intestinal and vaginal infections. They form the major part of the lactic acid bacterial group. They are characterised by the production of lactic acid as the main product of carbohydrate metabolism (Coman *et al*., 2014). The acidic environment which is produced inhibits growth of pathogenic bacteria and fungi. They are generally identified as Gram-positive rods which do not form flagella or spores. They are either aerotolerant that is they can tolerate conditions with low oxygen or anaerobic, acidoduric, or acidophilic and strictly fermentative. *Lactobacillus* species have received a lot of attention due to their health promoting factors in the gastrointestinal and genitourinary tracts. Probiotic strains of *Lactobacillus* are currently used in a variety of clinical practices due to their ability for high tolerance of bile and acid, ability to adhere to intestinal surfaces and withstanding low pH (Soccol *et al*., 2010; Fijan, 2014). The optimal growth for lactobacilli is in the range 35-40 °C. The common *Lactobacillus* species that have been used as probiotics include *Lactobacillus acidophilus*, *L*. *rhamnosus*, *L*. *helveticus*, *L*. *plantarum*, *L*. *paracasei* and *L*. *casei*. To date, several of these probiotic strains have been used to assess the antifungal activity of probiotic bacteria against human *C*. *albicans* from the oral cavity, gastrointestinal tract and genitourinary tract. Strus *et al* (2005), demonstrated the anticandidal activity of *L*. *rhamnosus*, *L*. *acidophilus*, *L*.

plantarum against *C. albicans* and *C. pseudotropicalis* and noted that the anticandidal activity of the probiotic strains was slight and mostly due to production of hydrogen peroxide. *L. rhamnosus* and *L. paracasei* have been reported to have strong inhibitory activity against *C. albicans* and both Gram-positive and Gram-negative bacteria (Coman *et al*., 2014). Verdenelli *et al* (2014) reported that the inhibitory effect of *L. paracasei* subspecies *casei*, *L. rhamnosus*, *L. plantarum* and *L. fermentum* varied with the clinical isolates of *Candida* species involved.

1.8.1.3 *Saccharomyces* genus

The genus includes *Saccharomyces cerevisiae* and *Saccharomyces* cerevisiae var *boulardii* which have been reported to possess biotherapeutic agents. They tolerate a wide range of pH and temperatures ranging from 30-35 °C. The probable probiotic effect of *Saccharomyces cerevisiae* and *Saccharomyces cerevisiae* var *boulardii* has been shown since they are able to withstand low pH, bile and protect against bacterial infections through the reduction of the intestinal pro-inflammatory response. *S. cerevisiae* var *boulardii* is often marketed as a probiotic in lyophilised form to treat diarrhoea following or during antibiotic treatment of the host, since the yeasts are not susceptible to antibiotics. They are also known to be effective against bacteria-induced gastrointestinal infection, however; there are only a few studies on their effect on *C. albicans* (Krasowska *et al*., 2009). The genus *Saccharomyces* is one such probiotic yeast that has been mostly used in stock feeds.

1.8.2 Beneficial effects and mode of action of probiotics

As there are different genera and species of probiotics, beneficial effects and mechanisms of their action vary depending on the strain. Very few have significant research to back-up the claims. Studies have also shown that probiotics provide these beneficial effects with the aid of two mechanisms;

a) Through the direct effect as live cells (Hardy *et al*., 2013).

b) Through indirect effects by producing various metabolites (Saadatzadeh *et al*., 2013).

1.8.2.1 Competition for adhesion sites

Probiotic bacteria compete with invading pathogens to adhere to epithelial cells and the mucus layer. The competition may occur for nutrient acquisition and mucosal binding sites. Probiotic bacteria modulate the environment to make it unfavourable for pathogens to establish themselves (Tuomola *et al*., 1999). The modulation of the environment is achieved by secretory antimicrobial compounds. *L. acidophilus* LA 1 was reported to inhibit attachment of pathogenic bacteria by binding to the intestinal cell lines of humans (Bernet *et al*., 1994).

1.8.2.2 Modulation of immunity

The immune system is the primary defence against microbial infections. Though the mechanism is not well understood, probiotic bacteria modulate cells which are involved in the immune responses and these include epithelial cells, dendritic cells, monocytes, B cells and T cells. Previous studies have demonstrated that probiotics can mediate antinflammatory effects in ulcerative colitis by inhibiting the tumour necrosis factor α (TNF- α) that is an induced product of interleukin-8 (IL-8) in Caco-2 cells by suppressing the activation of nuclear factor kappa B (NF-$_k$B) (Wang *et al*., 2011). In another study probiotic bacteria exerted immunomodulatory properties via a mechanism that involves synthesis of proinflammatory mediators such as cytokines and participation in signalling pathways such as the toll-like receptor (TLR2) pathway (Rocha-Ramirez *et al*., 2017).

1.8.2.3 Interfering with quorum sensing signal

Quorum sensing is a phenomenon by which bacteria communicate with each other and the surrounding environment through signalling molecules known as auto inducers. This may also extend to fungi as well. Cell to cell signalling enhances colonisation of bacteria and fungi leading to infection in the host tissue. Studies have shown that *L. acidophilus* releases

an active compound that inhibits the quorum sensing signal by interacting with the *Escherichia coli* O157 bacterial transcription gene (Gogineni *et al*., 2013).

1.8.2.4 Release of antimicrobial agents

The beneficial effects of probiotics are based on the understanding that the intestinal flora protects humans against infections and disturbance of the flora increases possibilities of infection. Probiotic bacteria enhance resistance against intestinal pathogens through antimicrobial mechanisms. Antimicrobial substances such as hydrogen peroxide, bacteriocins and organic acids released by probiotics have been proposed as one of the mechanisms of action (Nagpal *et al*., 2012). These include production of primary metabolites such as hydrogen peroxide, antimicrobial peptides such as bacteriocins and organic acids such as lactic and acetic acids. Hydrogen peroxide suppresses pathogens associated with bacterial and fungal vaginosis. The mechanism by which this is achieved is still not clear. Bacteriocins inhibit growth of closely related bacteria by pore formation in the cell membrane surrounding the bacteria (Upadhyay and Moudgal, 2012). In yeast, the mechanism is also not fully understood even though it has been found to have some effect on *Candida* species. Organic acids produced by probiotics have been shown to have a strong inhibitory effect (Nyanzi *et al*., 2014). They permeabilise the outer membrane of pathogens, thereby allowing the organic acid to penetrate the bacteria and fungi leading to cell death due to lowered intracellular pH.

In as much as probiotics have been used for several years, using live cells is the common practice. However, the biggest drawback with using live cells is that sepsis has become an issue affecting their safe use in reducing microbial infections.

1.8.3 Cases of probiotic sepsis

The risk of sepsis due to probiotic use is a major cause for concern. The uses of whole cell forms of probiotics in treatment of candidiasis have been reported to have a high risk of sepsis especially in immunocompromised patients (Kochan *et al*., 2011). Sepsis is the most

severe clinical complication related to administration of probiotics according to Boyle *et al*., (2006). Although sepsis due to probiotic use is rare, an increased number of sepsis cases have been reported across all age populations as shown in **Table 1.1**. Most of the reported cases were from immunocompromised patients who were suffering from conditions such as diabetes, endocarditis, liver abscess and had undergone surgery; some were from premature infants suffering from short gut syndrome. The common probiotics that were administered to these patients to treat the conditions included *L. rhamnosus* GG, *S. boulardii* and *B. longum* subspecies *infantis*. However, the patient's conditions worsened after administering these probiotics and methods of identification to find out the root cause of the worsened conditions were carried out using methods such as pulse field gel electrophoresis (PFGE), API 50 CHL kits and rRNA sequencing. It was noted from these cases studies that the form of sepsis was due to the probiotics and it was either a bacteraemia or fungaemia depending on the probiotic species administered. Several attempts have been made to try and overcome these problems by applying different microencapsulation strategies for live cells and various dried live cells. Use of probiotic CFEs has also gained a lot of consideration as an alternative method.

Table 1.1: Case studies of fungal and bacterial sepsis associated with probiotic use as whole cells.

Study	**Age**	**Risk Factors**	**Probiotics**	**Method of identification**	**Form of Sepsis**
Rautio *et al*., (1999)	74 yrs.	Diabetes, Liver abscess	*L. rhamnosus* GG	PFGE	bacteraemia
Mackay *et al*., (1999)	67 yrs.	Endocarditis,	*L. rhamnosus* GG	API 50 CHL pyrolysis MS	bacteraemia
Lestin *et al*., (2003)	48 yrs.	Diabetes,	*S. boulardii*	API 32C	fatal fungaemia

Kunz *et al*., (2004)	10 weeks	Prematurity, short gut syndrome	*L. rhamnosus GG*	PFGE of DNA restriction enzymes	bacteraemia
de Groot *et al*., (2005)	11 months	short gut syndrome	*L. rhamnosus GG*	rRNA sequencing	bacteraemia
Kochan *et al*., (2011)	24 yrs.	Aortic valve replacement, Endocarditis	*L. rhamnosus*	API 50 CHL PFGE	bacteraemia
Bertelli *et al*., (2014)	26 weeks	Prematurity,	*B. longum* subsp. *infantis*	rRNA sequencing	bacteraemia
	28 weeks	Necrotising enterocolitis	*B. longum* subsp. *infantis*	rRNA sequencing	bacteraemia
Dani *et al*., (2016)	39 weeks	Trisomy 18, triple X syndrome	*L. rhamnosus* GG	PFGE	bacteraemia
	23 weeks	Prematurity, patent ductus arteriosus (PDA)	*L. rhamnosus* GG	PFGE	bacteraemia

1.8.4 Probiotic cell free extracts

In the bid to avoid sepsis, probiotic cell free extracts (CFEs) can be used instead of whole live cells. Recent studies have shown that anti-virulence activity exists in metabolites produced by probiotics. There are also studies showing that lactic acid bacteria have an antioxidative potential which can aid in allowing lactobacilli to protect the intestinal mucosa against oxidative stress (Wang *et al*., 2017b). Few studies reporting the use of probiotic CFEs of *Lactobacillus* species against *C. albicans* have been documented (Nyanzi *et al*., 2014). In these studies, the antifungal activity of the constituents of the CFEs from whole live cells was mostly attributed to protein-like substances and organic acids such as 3-phenyl-L-lactic acid

and caproic acid among others (Schnürer and Magnusson, 2005; Nyanzi *et al*., 2014). In addition, most of the probiotic species have been shown to produce antimicrobial peptides called bacteriocins which specifically target the invading pathogens (Coman *et al*., 2014; Mokoena, 2017). In a study carried out by Matsubara *et al* (2016b), they showed that planktonic probiotic supernatants (*Lactobacillus rhamnosus* LR32, *Lactobacillus casei* L324 and *Lactobacillus acidophilus* NCFM) were effective in inhibiting the early stages of development of biofilm by reducing cell adhesion as well as filamentation. Bacteriocin was found to be the most common extracellular compound released into the broth during the exponential phase (12-16 or 16-18 h of growth of lactic acid bacteria). Kohler *et al* (2012) observed that the lactic acid produced by the culture supernatants from *L. rhamnosus* and *L. johnsonii* inhibited formation of biofilm by *C. albicans* at low pH. Vilela *et al* (2015) reported that the culture filtrate from *L. acidophilus* ATCC 4356 produced substances which inhibited formation of biofilms and hyphae in *C. albicans*. Murzyn *et al* (2010) reported that capric acid; an active compound of *Saccharomyces boulardii* inhibited the *Candida* virulence traits which included hyphae formation, cell adhesion and biofilm formation. The *S. boulardii* CFE and the capric acid further reduced the expression of the *HWP1*, *INO1* and *CSH1* in *C. albicans*.

One important factor that affects the use of probiotic CFEs is the choice of solvents for extraction. The type of solvent used in the preparation and extraction procedure highly affects the antimicrobial activity of the resultant probiotic CFEs. Some important characteristics of solvents which should be considered before their use are that the solvent should have low toxicity, evaporate easily at low heat, promote rapid physiologic absorption of the extract and do not cause the extract to complex or dissociate. In addition, the solvent should not interfere with the bioassay since at the end of extraction the product may contain traces of residual solvent (Ncube *et al*., 2008). The process of extraction is also affected by several parameters such as volume of solvent, extraction time and temperature (Eloff, 1998).

The ratio of the extractant to the sample to be extracted is important. Eloff (1998) reported that it was better to extract repeatedly with a smaller volume instead of once with a larger volume to increase recovery of the extract. Another parameter that should be considered is extraction temperature; Masoko *et al* (2007) reported that room temperature was recommended as the optimum temperature to extract different compounds since at high temperatures the analytes are solubilised by solvents. Moreover, choice of solvents may depend on the targeted compounds to be extracted. In this study, ethyl acetate extraction was used in the initial screening for possible antifungal activities in the probiotics as studies have shown that relatively high concentrations have been obtained from supernatants compared to extracts obtained using acetone (Murzyn *et al*., 2010). Methanol extracts from whole cells have also been reported (Nyanzi *et al*., 2014).

Even though a lot has been done in this area, there is no study information from South Africa reporting the use of probiotic CFEs on the virulence factors of *Candida* and on the reversal of antifungal drug resistance. Globally, there are limited studies on the use of probiotic CFEs against the pathogenic characteristics of *C. albicans*. In a single study of its type, probiotic extract and components of the extract which included caproic acid, caprylic acid, 2 phenylethanol and capric acid from *Saccharomyces boulardii* were tested against the gene expression of virulence factors in the standard strain of *C. albicans* SC5314 and capric acid was found to be effective in inhibiting essential virulence factors such as morphology and adherence (Murzyn *et al*., 2010).

1.9 Aim

The aim of this study was to unravel the *in vitro* antifungal effects of probiotic CFEs from three different probiotic strains against fluconazole (FLC) susceptible and resistant *C. albicans* and to study the effect of the most active probiotic CFE on virulence traits and on the reversal of drug resistance in FLC resistant *C. albicans* isolates.

1.10 Objectives

- To screen the antifungal activity of CFEs of three probiotic strains against FLC susceptible and resistant *C. albicans*.
- To evaluate the effect of the most active probiotic CFE on virulence traits (morphogenesis and secretion of hydrolytic enzymes) of *C. albicans*.
- To study the effect of the most active probiotic CFE on the reversal of drug resistance in *C. albicans*.
- To study the effect of the selected probiotic CFE on the expression of major genes involved in virulence and antifungal drug resistance in *C. albicans*.

CHAPTER 2: MATERIALS AND METHODS

This research study was conducted in the Department of Clinical Microbiology and Infectious Diseases, University of the Witwatersrand, Johannesburg, South Africa.

2.1 Strains and culture conditions

In this study, the laboratory strain *Candida albicans* SC5314 along with seven susceptible and three resistant *C. albicans* clinical strains stored in glycerol stocks at -80 ºC in the Department of Clinical Microbiology and Infectious Diseases, University of the Witwatersrand were used. All clinical strains were collected under the ethical clearance number M000402, obtained from the Human Research Ethics Committee, University of the Witwatersrand. The probiotic cultures used in this study were obtained from the culture collection of the Department of Biotechnology and Food Technology, Tshwane University of Technology, Pretoria, South Africa. To use the clinical *C. albicans* isolates and the probiotic cultures in this study, approval was granted by the Human Research Ethics Committee of the University of the Witwatersrand under ethical waiver reference number: W-CJ-170517-1 **(Appendix 7.1 and 7.2)**.

Before each experiment, the yeast cells were reactivated on Sabouraud dextrose (SD) agar and the probiotic bacteria on de Man Rogosa and Sharpe (MRS) agar. For each experiment fresh cultures were used. All media and chemical reagents used in this study were of high analytical grade.

2.2 Identification of *Candida* strains using conventional methods

Rapid tests for presumptive identification of *Candida albicans* from clinical isolates was carried out using the germ tube assay and the API® 20 C AUX test kit (bioMérieux, France).

2.2.1 Germ tube assay

This is a screening test used to differentiate *C. albicans* from other species. The technique was carried out as described by Mackenzie (1962), with modifications. In 2 ml eppendorf

tubes, 0.5 ml of foetal bovine serum (FBS-Sigma Aldrich), which is a source of proteins, was pipetted. Using a wire loop, a single colony from an overnight fresh plate culture of each strain was then picked and gently emulsified into the FBS. The tubes were incubated at 37 °C for 2-4 h and after incubation a drop of the culture was transferred on to a microscope glass slide (Thermo Scientific) using sterile glass capillary tubes. The drop was smeared and carefully covered with a coverslip to avoid formation of air bubbles. The cells were then examined microscopically using a light microscope (OLYMPUS BX43) under 10X and 40X objective lens to visualize germ tube formation. *Candida albicans* SC5314 (germ tube positive) was included as control.

2.2.2 API 20 C AUX test

The API® 20 C AUX test kit (bioMérieux, France) was used according to the manufacturer's instructions. In this study, cultures from each clinical isolate were subcultured on SD agar and then suspended in a 2 ml sterile distilled water to form a suspension equivalent to a 0.5 McFarland turbidity standard. An aliquot of 100 µl was aseptically transferred to an ampule of API 20C minimal medium provided with the kit and then emulsified. Each cupule of the API 20 C strip was inoculated with the suspension. The strips were carefully placed on an incubation tray, covered loosely with a lid provided by the manufacturer and incubated at 30 °C for 48-72 h. The reactions were visually analysed and determined as positive or negative based on the presence or absence of turbidity in the carbohydrate cupules. A seven-digit code was generated based on the observations by allocating a weighted score to positive reactions. The codes were compared to those listed in the API 20C Analytical Profile Index. The interpretation of the results was done using the apiweb™ identification software (bioMérieux). Only isolates that were more than 96 % identical to *C. albicans* were selected in this study.

2.3 Preparation and screening of probiotic CFEs for antimicrobial activity

2.3.1 Preparation of probiotic CFEs

The procedure was carried out as described by Murzyn *et al* (2010), with modifications. About 80 ml (4 %) overnight MRS broth culture of *Lactobacillus rhamnosus*, *Lactobacillus acidophilus* V and *Bifidobacterium animalis* subsp. *lactis* were inoculated into fresh 2 L MRS broth. The bottles were incubated at 37 °C for 16-20 h anaerobically. The anaerobic conditions were achieved by using anaerobe jars with a lit candle to absorb all the oxygen and supply carbon dioxide required for growth of lactic acid bacteria. The broth cultures were aliquoted into 50 ml falcon tubes and centrifuged at 3000 rpm for 10 min. The supernatants were decanted, and extraction was carried out with one fifth volume (one fifth of the initial volume of culture-400 ml) of ethyl acetate on a Labcon shaker set at 200 rpm. Fresh ethyl acetate was used every time at thirty-minute intervals of the extraction for 3 h. Multiple extractions with ethyl acetate were carried out to achieve maximum recovery of the active compounds. The top layer of ethyl acetate and the middle layer with extract were carefully transferred into pre-weighed beakers. The ethyl acetate was completely removed using a rotary evaporator and the resultant residue was stored in dry form at room temperature until further use. The extracts were weighed and reconstituted by diluting in methanol (1 %) to give a final concentration of 1.35 g/ml in each sample. The concentration of methanol in each sample was always kept at 1 %.

2.3.2 Determination of minimum inhibitory concentrations (MIC) of the probiotic CFEs

Minimum inhibitory concentration of the probiotic CFE against eleven tested *C. albicans* isolates was determined using a broth microdilution technique following CLSI guidelines with modifications (CLSI, 2008). Briefly, 100 µl of SD broth media was added to each of 96 U-shaped wells in a microtitre plate. To the first column of the plate, 100 µl of CFEs from the three probiotic strains reconstituted in methanol was added with an initial concentration of 338 mg/ml. Following a proper mixing by pipetting up and down, serial dilutions were done

by transferring 100 µl to the next column up to and including the last well. Separately, single colonies of clinical *C. albicans* isolates were emulsified in 0.85 % saline solution and the turbidity was adjusted to match that of a 0.5 McFarland standard to achieve colony counts of approximately 1-5 × 10^8 CFU/ml. From this suspension, 100 µl was added to all the wells except for the media control. Addition of the inoculum to the wells further diluted the CFEs by half. The microtitre plates were sealed and incubated at 37 °C for 24 h. In all experiments, methanol (1 %) was used as negative control. Fluconazole, an antifungal agent used to treat fungal infections, was used as a positive control. In addition, media and culture controls were included to confirm sterility of media and culture viability, respectively. After incubation, the MICs were determined as the lowest concentrations of the probiotic CFEs inhibiting the *C. albicans* growth compared with the growth of the control. All the experiments were carried out three times in triplicate.

2.3.3 Determination of minimum fungicidal concentration (MFC) of the probiotic CFEs

After determining the MIC values, MFC values were calculated by taking an aliquot of 10 µl from the wells of MIC, two above MIC and two below MIC wells. The aliquots were spread on SD agar plates and incubated at 37 ºC for 48 h to detect viable *Candida* cells. MFC was determined as the lowest concentration of CFE with no viable colony on agar plate.

After calculating the MIC and MFC values of all the probiotic CFEs, the most active was selected and used for further studies.

2.4 Effect of *L. rhamnosus* CFE on virulence factors

Virulence factors have been recognised to play an important role in the transformation of commensal yeast into pathogenic forms. Among the virulence factors in *C. albicans*, morphological transition and secretion of hydrolytic enzymes have gained of lot of interest and have also been recognised as emerging drug targets (Ahmad *et al.,* 2016). Therefore, we studied the effect of the most active CFE on these virulence factors.

2.4.1 Effect on morphology transition

The effect of most active *L. rhamnosus* CFE on yeast to hyphae transition was determined using a method described by Yousuf *et al* (2011) with modifications. In this experiment, the standard strain SC5314, plus one susceptible (S7) and one resistant (R3) *C. albicans* strains were used. To initiate the growth for experimental purposes, single colonies from 24 h *C. albicans* cultures were inoculated into 150 ml of SD broth and incubated for 12-16 h (late log phase) at 37 °C. Aliquots of grown cells were then transferred into 50 ml fresh SD broth and further incubated for 48 h (stationary phase) at 30 °C to attain a synchronised cell population. To induce hyphal formation, 50 µl of synchronised cell population was transferred into 5 ml of fresh SD broth supplemented with 1 ml of 10 % foetal bovine serum (FBS) at pH 6.5. The effect on hyphae formation was determined by adding different concentrations of CFE (84.5, 42.25 and 21.12 mg/ml) into the media. The external pH was adjusted every 30 min using 0.1 M of sodium hydroxide. All the flasks were incubated at 37 °C and aliquots of 100 µl were taken after every 30 min and placed on a microscope glass slide. The samples were covered using cover slips and viewed under a light microscope at a magnification of 40x (Leica DM 500). Images were taken using a built-in digital camera (Leica DM 500). On each glass slide, thirty cells were randomly selected and the number of cells with hyphae was recorded. The glass slides were viewed twice on different occasions and mean of the two readings was taken as the result. The effect of *L. rhamnosus* CFE on yeast to hyphae transition in each *C. albicans* strain was determined by comparing the treated samples to the control samples. The experiment was carried out using three different concentrations of *L. rhamnosus* CFE.

2.4.2 Effect of *L. rhamnosus* CFE on secretion of hydrolytic enzymes

Effect of the most effective CFE on secreted aspartic proteinases (SAPs) and phospholipases in *C. albicans* strains was determined using the plate assay method. The plate assay for proteinases was supplemented with bovine serum albumin fraction V (BSA) and for the

phospholipase assay, egg yolk emulsion was added as described by Yousuf *et al* (2011) with modifications. Seven susceptible and three resistant *C. albicans* strains including the standard strain *C. albicans* SC5314 were subcultured into flasks containing 5 ml SD broth and incubated at 37 °C for 18 h. The cells were centrifuged at 3000 rpm for 5 min and resuspended in fresh media. The *C. albicans* strains were exposed to different concentrations of the test extract (84.5, 42.25 and 21.12 mg/ml) for 1 h. Cells without any treatment were used as controls. The assays were carried out in duplicate and repeated three times on separate occasions for each yeast isolate.

2.4.2.1 Proteinase assay

From the above prepared treated and untreated suspensions **(section 2.4.2**), aliquots of 2 µl were placed at equidistant points on proteinase agar plates (agar powder, 20 g; bovine serum albumin (BSA) fraction V, 2 g; yeast nitrogen base-without amino acids; ammonium sulphate, 1.45 g; glucose, 20 g; distilled water added to 990 ml) as described by Yousuf *et al* (2011) with modifications. The plates were incubated at 37 °C for 7 days and the effect on proteinase synthesis was determined by the formation of a transparent halo around the yeast colonies. An index (Pz value) was used to determine the effect of CFE on proteinase activity of *C. albicans*. Proteinase activity in CFE treated and untreated *C. albicans* strains were expressed using the formula:

$$Pz = \frac{diameter\ of\ colony}{diameter\ of\ colony\ +\ zone\ of\ \ clearance}$$

2.4.2.2 Phospholipase assay

Aliquots of 2 µl from the above prepared treated and untreated suspensions **(section 2.4.2)**, were placed at equidistant points on phospholipase agar plates (agar powder, 20 g; peptone, 10 g; glucose, 30 g; NaCl, 57.3 g; $CaCl_2$, 0.55 g; distilled water added to 900 ml and 50 % egg yolk emulsion 100 ml). The plates were incubated at 37 °C for 4 days as described by

Price *et al* (1982), with minor modifications. The secretion of phospholipase was determined by the formation of opaque zones due to precipitation around the yeast colonies. The precipitation zones around the yeast colonies were measured to determine phospholipase activity and calculated as follows:

$$Pz = \frac{diameter\ of\ colony}{diameter\ of\ colony\ +\ zone\ of\ \ precipitation}$$

2.5 Effect of *L. rhamnosus* CFE on antifungal drug resistance

The reversal of antifungal drug resistance was determined using the Rhodamine 6 G (R6G) dye as described by Ahmad *et al* (2013). One susceptible (S7) and one resistant (R3) *C. albicans* strain as well as the standard strain *C. albicans* SC5314 were subcultured on SD agar. A single colony from each strain was transferred into each flask with 150 ml of fresh SD broth. The flasks were incubated for 8 h (mid log phase) at 37 °C with shaking at 200 rpm. The cells were then harvested in 50 ml falcon tubes and centrifuged at 3000 rpm for 5 min. The supernatant was discarded, and the cells were washed twice with 25 ml of phosphate buffered saline (PBS; Sigma-Aldrich). The cells were then resuspended in 25 ml PBS and 12.5 ml of 2-deoxy-D-glucose (10 mM) and 12.5 ml of 2, 4-dinitrophenol (10 mM) were added. The suspension was incubated for 45 min at 37 °C with shaking at 200 rpm to deenergise the cells. Following incubation, cells were harvested by spinning at 3000 rpm for 5 min. The cells were washed twice in PBS and resuspended in 25 ml PBS. To determine the effect of *L. rhamnosus* CFE, cell suspensions were exposed to subinhibitory concentrations of CFE for 5 min. Following exposure to CFE, cells were centrifuged at 3000 rpm for 5 min and washed once with PBS. Simultaneously, a stock solution of R6G was prepared by dissolving the dye in water at a concentration of 10 mM. About 25 µl of 10 mM R6G dye was added to the cell suspensions to reach a final concentration of 10 µM. Cell suspensions were then incubated at 37 °C for 40 min with shaking at 200 rpm. The cell suspensions were washed and resuspended in 25 ml PBS. Aliquots of 1.5 ml were withdrawn at specific times (5, 10,

15, 20, 25 min) and centrifuged at 9000 rpm for 2 min. The supernatants were carefully collected, and absorbance was measured at a wavelength of 527 nm using a Shimadzu-UV-VIS double beam spectrophotometer at room temperature.

To assess the effect of glucose, energy dependent efflux was measured after 25 min by adding 1.5 ml of 0.5 M glucose into cells resuspended in PBS (13.5 ml) to reach a final concentration of 0.1 M glucose. Aliquots of 1.5 ml were withdrawn at 5 min intervals (30, 35, 40, 45, 50, 55, 60 min) and centrifuged at 9000 rpm for 2 min. The supernatants were carefully collected and absorbances were measured at 527 nm. In every set of experiments, glucose free controls were also included. The experiment was carried out three times on separate occasions and for each occasion, absorbance was measured three times.

2.6 Effect of *L. rhamnosus* CFE on gene expression

To study the effect of *L. rhamnosus* CFE on the important genes **(Table 2.1)** involved in the virulence and drug efflux pumps of *C. albicans*, gene expression in untreated (control) and CFE treated *C. albicans* cells was measured by real time polymerase chain reaction (qPCR).

2.6.1 Preparation of sample for total RNA extraction

The method was carried out as described by Murzyn *et al* (2010) with minor modifications. Single colonies from *C. albicans* SD agar plates of the standard strain SC5314, susceptible strain (S7) and resistant strain (R3) were inoculated into 10 ml of SD broth to give a final concentration of 5 x 10^6 cells/ml which is equivalent to a 1.5 McFarland turbidity standard. For treatment, a concentration of 84.5 mg/ml of *L. rhamnosus* CFE was added into each flask and incubated at 30 °C for 18-24 h. Flasks which had no CFE were used as controls.

2.6.2. Total RNA extraction

Total RNA was isolated using the Roche High pure RNA Isolation Kit following the manufacturer's instructions. Extraction was carried out in both CFE treated and untreated

(control) cells. About 200 µl of the cell suspension from each strain was aliquoted into 1.5 ml eppendorf tubes and centrifuged (Eppendorf centrifuge 5417C) at 3000 rpm for 10 min. The supernatant was discarded and 200 µl of PBS (pH 7.0) was added to resuspend the resultant pellet. An aliquot of 400 µl lysis/binding buffer was added (Roche). The mixture was vortexed (Velp® Scientifica) for two mins and 520 µl was transferred to a Roche column. After pipette mixing, the samples were centrifuged at 8000 rpm for one minute and flow through was discarded. Genomic DNA was digested using 10 µl DNase mixed with 90 µl of 1 × DNA buffer directly into the Roche column and incubated at room temperature for 15 min. An aliquot of 500 µl of wash buffer 1 (5 M guanidine hydrochloride and 20 mM Tris-hydrochloride; pH 6.6) was added into the column matrix and centrifuged at 8000 rpm for 1 min at room temperature and the flow through was discarded. This was followed by addition of an aliquot of 500 µl of wash buffer II (20 mM NaCl and 2 mM Tris-HCl; pH 7.5) into the mixture. The samples were centrifuged at 8000 rpm for 1 minute and the flow through was discarded. Another 200 µl aliquot of wash buffer II (20 mM NaCl and 2 mM Tris-HCl; pH 7.5) was added and centrifuged at 13 000 rpm for 2 min at room temperature and the flow through was discarded. The collecting tubes were replaced with sterile 1.5 ml eppendorf tubes to collect the purified total RNA. The total RNA was eluted by adding 50 µl of elution buffer (PCR grade water) and this was centrifuged at 8000 rpm for 1 minute at room temperature. The purified total RNA was then collected in 1.5 ml eppendorf tubes and stored at -75 °C until further use.

2.6.3 Determination of total RNA concentration and purity

Total RNA concentration was quantified using the Thermo Scientific Nanodrop 2000 spectrophotometer. Purified total RNA was then converted to cDNA.

2.6.4 cDNA synthesis by reverse transcription of RNA

The Biorad iScript™ cDNA synthesis kit was used to convert purified total RNA to cDNA, following manufacturer's instruction. For each sample, 12 µl comprising of 0.20 µg of total

RNA was added to 4 µl of 5 × iScript reaction mixes. To the reaction mixture, 1 µl of the enzyme reverse transcriptase was added and the mixture was made up to 20 µl with nuclease free water. Reverse transcription (from 5 × reaction mixture) was carried out in a thermal cycler using the following conditions: priming at 25 ºC for 5 min, reverse transcription at 46 ºC for 20 min and reverse transcription inactivation at 95 ºC for 1 min. PCR amplification was performed using 2 × PCR Master Mix (Fermentas, MA, USA), 10 pmol of each fungal primer (Integrated DNA Technologies, IA, USA) and 2 µl cDNA template (100 ng/ µl). The primer sequences used to target and amplify the *HWP1*, *SAP1*, *SAP2*, *SAP3*, *PLB1*, *CDR1*, *CDR2* and *MDR1* gene regions of *C. albicans* are shown in Table 2.1. All amplifications were conducted using the T100^{TM} Thermal Cycler (Bio-Rad, CA, USA) and the following cycling conditions were used to determine the optimum annealing temperature by gradient PCR: one cycle of 95°C for 3 minutes (initial denaturation), 35 cycles of denaturation at 95°C for 30 seconds, annealing ranged from 50-54°C for 30 seconds and extension at 72°C for 1 minute. Final extension step was performed at 72°C for 10 min. Amplified PCR products were electrophoresed on a 2 % agarose gel at 100 volts for 45 min and visualised under UV transillumination (Universal Hood II Gel Doc System, Bio-Rad, CA, USA). The cDNA samples were stored at -20 ºC for further use.

2.6.5 Electrophoresis

This is a technique commonly used to separate charged molecules such as total RNA and DNA based on their sizes.

2.6.5.1 Total RNA agarose gel electrophoresis

Agarose gel electrophoresis was used to assess total RNA integrity based on separation of the 18S and 28S rRNA. A 2 % (w/v) horizontal slab agarose gel was used. About 3 g of agarose gel powder (Seakem® LE) was added to 150 ml of 1 × Tris- Boric Acid EDTA (TBE) buffer

and microwave heated until the agarose boiled. Ethidium bromide (2 µl of a stock of 1% sodium in water; Merck) was added and mixed gently to avoid formation of bubbles.

The gel was gently poured into a casting tray and left to set for 30 min. The gel was then placed in an RNase free treated electrophoresis tank and submerged in 1× TBE buffer. Each sample (10 µl) was mixed with 5 µl 6 × DNA loading dye (Promega). Since 10 µl of sample was used, a 5 µl of molecular mass standard (Fermentas O'Generuler- Thermo Scientific) was loaded in adjacent wells. Electrophoresis was performed at 75 V for approximately one hour. The gel was exposed to UV light (Gel doc; Biorad) to visualise RNA fragments.

2.6.5.2 DNA agarose gel electrophoresis

Agarose gel was used to separate DNA fragments of various sizes. A 2% agarose gel was prepared as described above in section 2.5.5.1. An aliquot of 5 µl of the conventional PCR sample was mixed with 5 µl of 6 × DNA loading dye and 5 µl of molecular mass standard was loaded in adjacent wells. Electrophoresis was performed at 100 V for one hour. The gel was exposed to UV light (Gel doc; Biorad) to visualise the DNA fragments.

2.6.6 Designing primers

Quantitative polymerase chain reaction (qPCR) primers of selected genes were constructed to amplify at most 200 base pairs of each open reading frame of *Candida albicans*. A total of twenty-two primer pairs were used and were designed using NCBI/Primer 3-Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The nucleotide sequence of the virulence factors for *C. albicans* specific primers are listed in **Table 2.1**.

Table 2.1: List of primer sequences

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
HWP1	TCTACTGCTCCAGCCACTGA	GTGGAATGGAAGCTTCTGGA
SAP1	TTTTGACAACAACGCCAAGA	GCAGCAATGTTTGAAGCAGA
SAP2	GGATACCGTTGGATTTGGTG	ATCAACCCCACCGAAAATTA
SAP3	CATGTCAAGCTGGTCAAGGA	AACATCGGCAAATTGTTGCT

PLB1	CAACGAAGCGGTGTTGTCTA	TTGGAATGTTTTGGCCATCT
CDR1	CTTCGAAATTGGGAATTGGA	CAATGTGGAACAACCAGCAC
CDR2	CATGGTCAAGCCATTTTGTG	GCAGCACCAACGACTTGTAA
MDR1	TGGTTTTCAGTCCGATGTCA	CGGCTAACCCAACTGGTAAA
RPP2B	TGGTAACACCTCTCCATCAGC	TTCTTCAGCTTCAGCAGCAC
ACT1	GACAAATGGGTAGGGTGGGAAAAC	TGTGACAGTAACATCCCAAACGAG
PMA1	TTGCTTATGATAATGCTCCATACGA	TACCCCACAATCTTGGCAAGT

2.6.7 Quantitative polymerase chain reaction (qPCR)

Quantitative polymerase chain reactions were performed in eight tube capped strips using the Light cycler® Nano Real Time PCR System (Roche, Basel, Switzerland). Double strand-specific intercalating dye (SYBR Green 1) was used to monitor dsDNA amplification and quantify reaction product at each cycle. Real Time PCR reactions were carried out in 20 µl volumes in 0.2 ml Roche tubes comprising of 10 µl Fast start master mix 2 × with SYBR Green 1, 0.5 µl of 10 mM primer mixes (forward and reverse) and 9 µl of nuclease free water. The tubes were tightly capped to prevent evaporation, spill over and sample contamination. The amplification conditions were as follows: Uracil-DNA glycosylase activation at 50 ºC for 2 min, initial denaturation at 95 ºC for 2 min, followed by 40 cycles of denaturation at 95 ºC for 15 sec, annealing at 50 ºC for 15 sec, elongation at 72 ºC for a minute. Three housekeeping genes (*ACT1, PMA1* and *RPP2B*) were used as reference controls. The relative expressions of the genes for morphogenesis (*HWP1*), proteinases (*SAP1*, *SAP2* and *SAP3*), phospholipases (*PLB1*) and efflux pumps *(CDR1*, *CDR2* and *MDR1*) were determined. Primer sequences for different genes are shown in **Table 2.1**. Results were analysed by Light Cycler Software. Each assay was repeated three times for separately isolated RNA and for reproducibility of results. The relative fold change of each of the virulence and drug efflux pump genes was determined by comparing the gene expression levels of the treated and control samples.

CHAPTER 3: RESULTS

3.1 Identification of clinical isolates of *C. albicans*

A total of eleven *C. albicans* strains were collected from the Department of Clinical Microbiology and Infectious Diseases (University of the Witwatersrand, Johannesburg, South Africa). These included ten clinical isolates and one laboratory strain *C. albicans* SC5314. All the clinical isolates were identified and confirmed as *C. albicans* using an API 20 C AUX kit. Out of the 10 clinical isolates of *C. albicans,* seven *C. albicans* including the standard strain SC5314 were susceptible to fluconazole while the other three were resistant to fluconazole. All the strains were positive for germ tube formation. The API profile confirmed the identity as *C. albicans* with a percentage similarity ranging from 97-99 % **(Table 3.1)**. These *C. albicans* isolates were used in the subsequent assays.

Table 3.1: Germ tube and API test in *C. albicans* strains

Resistant / Susceptible	***C. albicans* strain**	**Fluconazole MIC concentration (µg/ml)**	**API 20 C AUX Profile %**	**Germ tube formation**	**Identification**
Susceptible	SC 5314	0.98	97	+	*C. albicans*
	S1	1.95	97.2	+	*C. albicans*
	S2	3.9	97.2	+	*C. albicans*
	S3	3.9	97.2	+	*C. albicans*
	S4	3.9	97.2	+	*C. albicans*
	S5	1.95	97.2	+	*C. albicans*
	S6	0.98	97.2	+	*C. albicans*
	S7	1.95	97.2	+	*C. albicans*
Resistant	R1	62.5	97.2	+	*C. albicans*
	R2	125	97.2	+	*C. albicans*
	R3	250	99	+	*C. albicans*

3.2 Preparation and characteristics of probiotic CFEs

In this study, three probiotic strains were used to prepare the CFEs. For each strain, the CFEs were combined dried and the total resultant mass was recorded (**Table 3.2**). The results showed that approximately 16 g of the CFE was obtained from multiple extractions using

ethyl acetate in all the three strains. These CFEs had a characteristic brown to dark brown colour with sticky consistency and aromatic smell. The average pH of these extracts was 4.17 (in the range of 4.06-4.24) when it was reconstituted in 1 % methanol.

Table 3.2: Mass of cell free extracts from three probiotic strains

Probiotic strain	**Mass of cell free extract (g)**
L. rhamnosus	15.954
L. acidophilus V	15.842
B. animalis subs. *lactis*	16.435

3.3 Minimum inhibitory concentrations of the three probiotic CFEs against *C. albicans*

The minimum inhibitory concentration (MIC) of the probiotic CFEs is the lowest concentration of the extract to inhibit fungal growth and was determined following CLSI recommended microbroth dilution assay. The MIC results of all the three tested extracts against FLC susceptible and resistant *C. albicans* isolates are shown in **Table 3.3**. The MIC values of the tested CFEs against all the *C. albicans* isolates were in the range of 84.5 to 338 mg/ml. *L. acidophilus* V and *B. animalis* subspecies *lactis* recorded the overall median MIC of 169 mg/ml, while the median MIC for *L. rhamnosus* CFE was 84.5 mg/ml. Based on the results obtained in this assay, *L. rhamnosus* CFE was the most potent antifungal extract and was therefore selected and used for subsequent assays.

Table 3.3: Summary of antifungal activity of three probiotic CFEs against *C. albicans*.

***C. albicans* strains**		***Median MIC of CFE (mg/ml) n= 3**		
		L. rhamnosus	***L. acidophilus V***	***B. animalis* subspecies *lactis***
Susceptible	SC5314	84.5	84.5	169
	S1	84.5	84.5	169
	S2	84.5	169	169
	S3	84.5	169	84.5
	S4	84.5	169	169
	S5	169	169	169
	S6	169	169	169
	S7	84.5	338	169
Resistant	R1	84.5	169	169
	R2	169	84.5	84.5
	R3	84.5	169	169
Median		**84.5**	**169**	**169**

*Refer to **Appendix 2** for full results of MIC concentrations and the repeats.

3.4 Effect of *L. rhamnosus* CFE on morphological transition in *C. albicans*

Candida albicans has been reported to have an ability to switch reversibly from the yeast form of growth to the filamentous form. In this study, three strains of *C. albicans* (standard laboratory strain *C. albicans* SC5314, FLC susceptible strain S7 and FLC resistant strain R3) were used in this assay. Results of the effect of *L. rhamnosus* CFE on the transition from yeast to hyphae in *C. albicans* are represented in **Figures 3.1; 3.2; 3.3**. These representative images show length of hyphae and percentage of cells forming hyphae in control and CFE exposed cells at different concentrations after 300 min **(Figures 3.1; 3.2; 3.3)**. Mean length of hyphae in treated and untreated cells was calculated for all the strains **(Table 3.4)**. In addition, percentage number of hyphae formation after exposure to *L. rhamnosus* CFE was also calculated **(Table 3.4)**. From these results, it is evident that *L. rhamnosus* CFE, at all the concentrations, reduced the length and number of hyphae. Moreover, it was observed that effect of *L. rhamnosus* CFE on morphological transition is highly concentration dependent. At concentrations of 84.5 mg/ml (MIC) and 42.25 (½ MIC), there was evident reduction in the number of hyphae and hyphal length when compared to the untreated control cells. However, at a concentration of 21.12 mg/ml (¼ MIC) of CFE, there was no inhibitory effect

on hyphae formation and length. Statistically, there was an overall significant difference between the control and the MICs and ½ MICs of all strains ($p < 0.05$). However, there was no significant difference in hyphal length and number of cells with hyphae in both clinical susceptible and resistant strains of *C. albicans*.

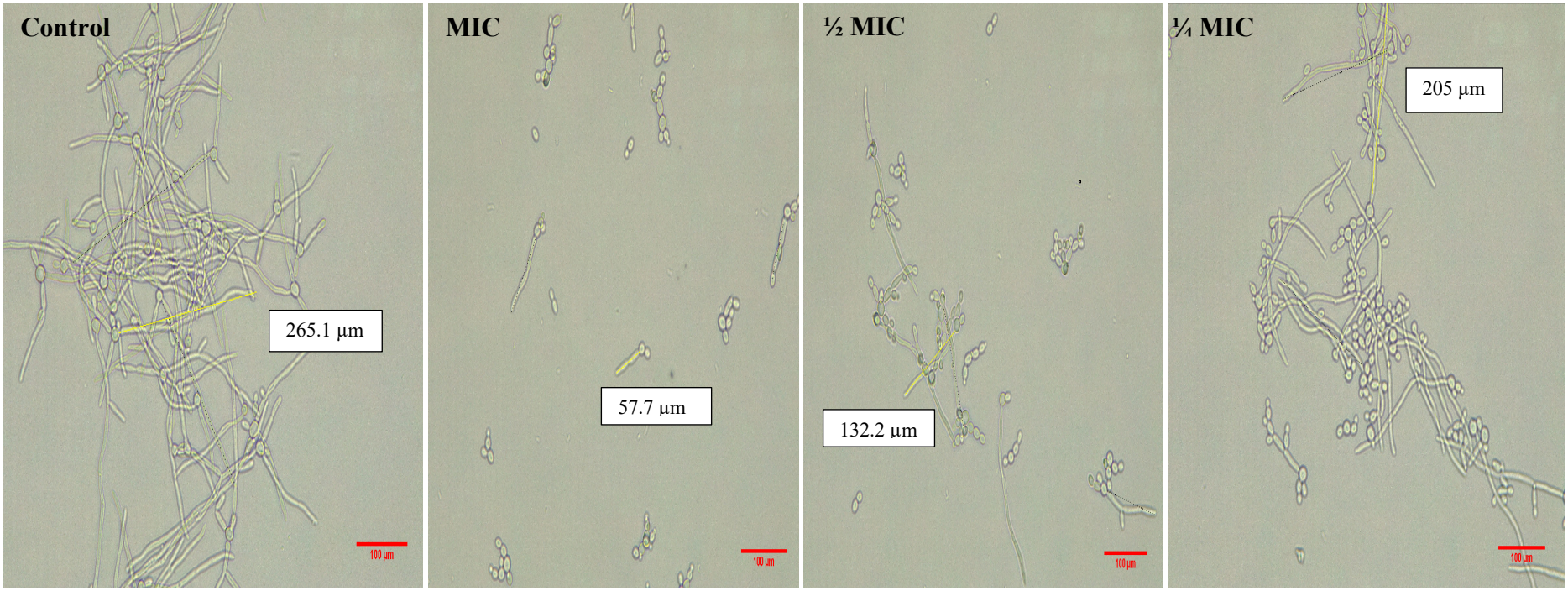

Figure 3.1: Development of hyphae in *C. albicans* SC5314 strain in the absence (control) and presence of *L. rhamnosus* CFE at MIC (84.5 mg/ml), ½ MIC (42.25 mg/ml) and ¼ MIC (21.12 mg/ml).

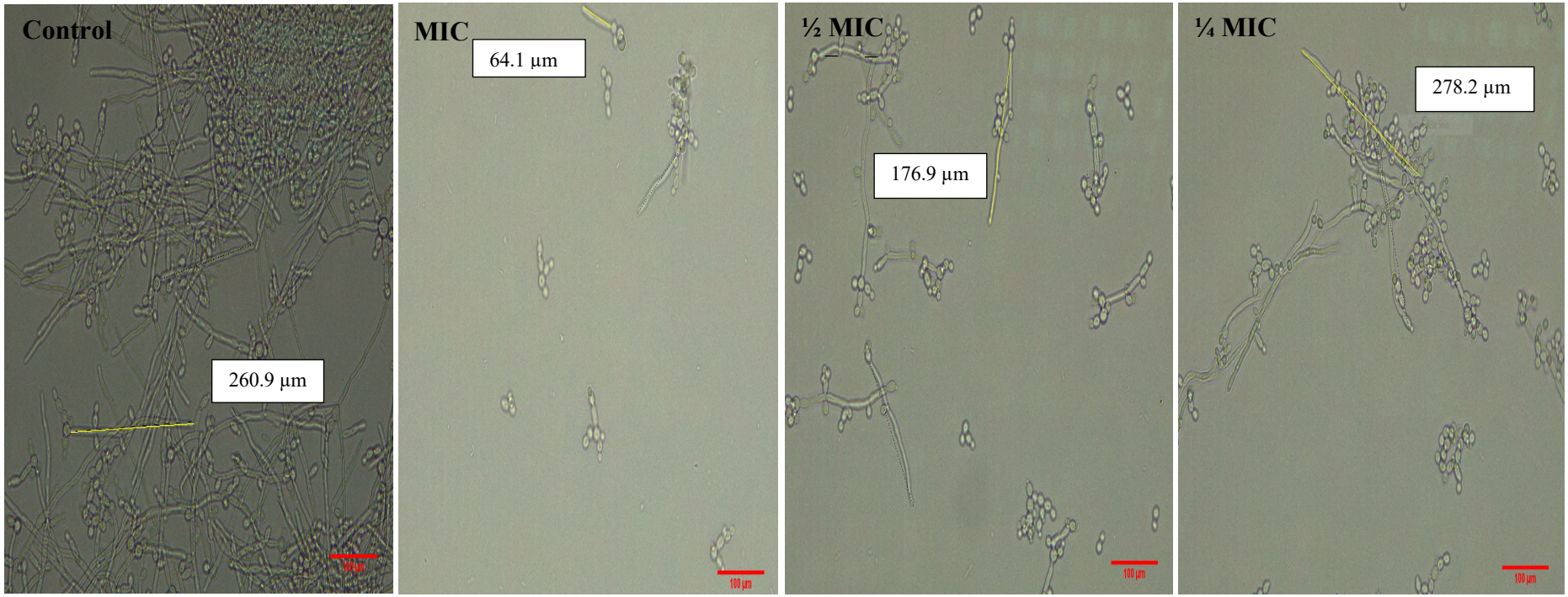

Figure 3.2: Development of hyphae in clinical susceptible *C. albicans* S7 in the absence (control) and presence of *L. rhamnosus* CFE at MIC (84.5 mg/ml), ½ MIC (42.25 mg/ml) and ¼ MIC (21.12 mg/ml).

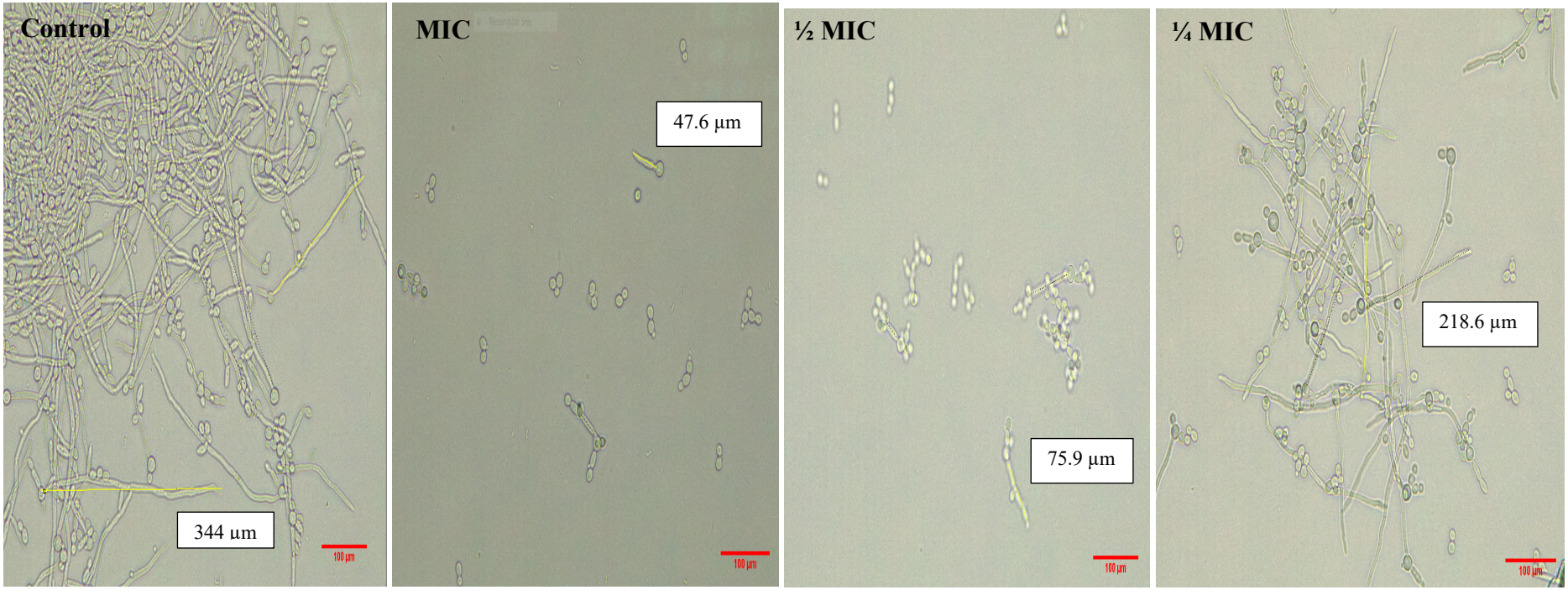

Figure 3.3: Development of hyphae in clinical resistant *C. albicans* R3 in the absence (control) and presence of *L. rhamnosus* CFE at MIC (84.5 mg/ml), ½ MIC (42.25 mg/ml) and ¼ MIC (21.12 mg/ml).

Table 3.4: Yeast to hyphal transition in *C. albicans* cells treated with *L. rhamnosus* CFE.

CFE concentration (mg/ml)	*C. albicans* strain	Length of hyphae (µm) after 300 mins Mean ± SD	No. of cells with hyphae / 30 cells (%)	Test compared to control for the length of hyphae P value
0	SC5314	252.9 ± 17.26	30 (100)	N/A
	S7	227 ± 47.85	30 (100)	
	R3	289.7 ± 76.8	30 (100)	
84.5	SC5314	71.69 ± 19.83	4 (13.3)	0.00
	S7	86.0 ± 30.95	7 (23.3)	
	R3	53.43 ± 8.23	4 (13.3)	
42.25	SC5314	134.88 ± 3.8	14 (46.7)	0.00
	S7	170.12 ± 9.54	16 (53.3)	
	R3	77.21 ± 1.89	9 (30)	
21.12	SC5314	210.39 ± 7.73	25 (83.3)	0.79
	S7	240.90 ± 52.69	21 (70)	
	R3	216.88 ± 2.37	28 (93.3)	

3.5 Effect of *L. rhamnosus* CFE on proteinases production in *C. albicans*

Host tissue damage and invasion is an important step in the pathogenesis of *C. albicans.* To degrade host cell membrane proteins, *C. albicans* secrete proteinases which hydrolyse peptide bonds. In this study, a total of eleven *C. albicans* strains including *C. albicans* SC5314 were tested for proteinase activity in the absence (control) and presence of different concentrations of *L. rhamnosus* CFE. The results of proteinase production at various concentrations of *L. rhamnosus* CFE was determined as zones of clearance (Pz) and are represented in **Table 3.5**. Higher Pz values indicated less enzyme activity and low values represented high enzyme activity. At concentrations of 84.5 mg/ml and 42.25 mg/ml of CFE, there was a significant reduction in proteinase production compared to the control ($P < 0.01$). However, at 21.12 mg/ml of CFE, there was no significant difference when compared to the control ($P > 0.05$). It was observed that after an exposure to MIC value of CFE 28% of proteinase inhibition took place while the respective figure for ½ MIC value was 13%. Interestingly, inhibition of proteinase production was more prominent in FLC resistant (33% inhibition at MIC) than FLC susceptible (26% inhibition at MIC) *C. albicans* isolates.

Table 3.5: Effect of *L. rhamnosus* CFE on proteinase production in *C. albicans*.

***C. albicans* strain**	**Repeats**	**Proteinase production (Pz*) at various concentrations of CFE (mg/ml)**			
		0 (control)	**84.5**	**42.25**	**21.12**
SC5314	1	0.53	0.67	0.62	0.54
	2	0.58	0.67	0.58	0.43
	3	0.39	0.61	0.53	0.4
S1	1	0.39	0.45	0.4	0.4
	2	0.3	0.4	0.35	0.3
	3	0.31	0.4	0.3	0.35
S2	1	0.47	0.56	0.55	0.49
	2	0.49	0.64	0.45	0.4
	3	0.38	0.56	0.37	0.39
S3	1	0.43	0.52	0.5	0.47
	2	0.4	0.55	0.48	0.4
	3	0.3	0.42	0.36	0.35
S4	1	0.45	0.48	0.45	0.38
	2	0.39	0.55	0.48	0.45
	3	0.36	0.47	0.35	0.35
S5	1	0.36	0.55	0.46	0.42
	2	0.45	0.59	0.52	0.5
	3	0.32	0.52	0.43	0.4
S6	1	0.45	0.55	0.49	0.33
	2	0.37	0.64	0.45	0.44
	3	0.3	0.56	0.39	0.36
S7	1	0.39	0.53	0.45	0.43
	2	0.46	0.63	0.53	0.45
	3	0.39	0.53	0.46	0.35
Mean ± SD		0.40 ± 0.07	0.54 ± 0.08	0.45 ± 0.08	0.41 ± 0.06
R1	1	0.41	0.59	0.53	0.47
	2	0.43	0.63	0.53	0.4
	3	0.3	0.51	0.5	0.36
R2	1	0.35	0.47	0.4	0.4
	2	0.39	0.55	0.48	0.5
	3	0.22	0.49	0.4	0.41
R3	1	0.38	0.56	0.36	0.4
	2	0.46	0.61	0.46	0.5
	3	0.38	0.5	0.37	0.4
Mean ± SD		0.37 ± 0.07	0.55 ± 0.06	0.45 ± 0.07	0.43 ± 0.05
Combined Mean ± SD		**0.39 ± 0.07**	**0.54 ± 0.07**	**0.45 ± 0.07**	**0.41 ± 0.06**
Combined P value			**P < 0.01**		
P value			**P < 0.01**	**P < 0.01**	**P > 0.05**

***Pz: ratio of the colony diameter and clear zone therefore lower values indicate greater enzyme activity and higher values indicate less enzyme activity.**

3.6 Effect of *L. rhamnosus* CFE on phospholipases production in *C. albicans*

Phospholipases play an equally important role as that of proteinases in the virulence of *C. albicans.* To degrade host cell membrane lipids, *C. albicans* secrete phospholipases. In this study, a total of eleven *C. albicans* strains including the *C. albicans* SC5314 were tested to evaluate the effect of *L. rhamnosus* CFE on production of phospholipase. The results of the effect of *L. rhamnosus* CFE at various concentrations were determined as zones of precipitation (Pz) and are represented in **Table 3.6**. Means and standard deviations of phospholipase production at different concentrations were calculated for susceptible and resistant strains. *L. rhamnosus* CFE had an overall effect on phospholipase production in *C. albicans* strains. In addition, inhibition of phospholipase production was observed to be dependent on concentration of *L. rhamnosus* CFE. At high concentrations of *L. rhamnosus* CFE (84.5 mg/ml), there was a significant reduction in phospholipase production compared to the control ($P = 0.001$). However, there was no significant effect on phospholipase production when the concentration of CFE was reduced to 42.25 ($P = 0.949$) and 21.12 mg/ml ($P = 1$). Unlike an effect on proteinase production, there was no difference in the effect of CFE on phospholipase production in FLC resistant (17% inhibition at MIC) and FLC susceptible (18% inhibition at MIC) *C. albicans* isolates.

Table 3.6: Effect of *L. rhamnosus* CFE on phospholipase production in *C. albicans*.

***C. albicans* strain**	**Repeats**	**Phospholipase production (Pz*) at various concentrations of CFE (mg/ml)**			
		0 (control)	**84.5**	**42.25**	**21.12**
SC5314	1	0.64	0.83	0.75	0.69
	2	0.5	0.75	0.65	0.5
	3	0.69	0.84	0.74	0.59
S1	1	0.4	0.72	0.54	0.52
	2	0.41	0.68	0.43	0.42
	3	0.41	0.67	0.55	0.53
S2	1	0.73	0.95	0.85	0.79
	2	0.64	1	1	0.68
	3	0.71	0.95	0.84	0.79
S3	1	0.5	0.69	0.67	0.61
	2	0.6	0.69	0.64	0.6
	3	0.61	0.65	0.63	0.62
S4	1	0.66	0.74	0.67	0.62
	2	0.69	0.82	0.67	0.56
	3	0.65	0.71	0.67	0.63
S5	1	1	1	1	1
	2	1	1	1	1
	3	1	1	1	1
S6	1	0.65	0.72	0.68	0.68
	2	0.58	0.63	0.61	0.58
	3	0.67	0.71	0.64	0.51
S7	1	0.52	0.73	0.64	0.58
	2	0.68	0.78	0.75	0.73
	3	0.67	0.77	0.65	0.56
Mean ± SD		0.65 ± 0.17	0.79 ± 0.12	0.72 ± 0.16	0.66 ± 0.16
R1	1	0.59	0.67	0.54	0.52
	2	0.75	0.82	0.69	0.65
	3	0.63	0.75	0.58	0.64
R2	1	0.69	0.85	0.73	0.72
	2	0.71	0.77	0.67	0.65
	3	0.73	0.79	0.71	0.73
R3	1	0.81	0.9	0.79	0.74
	2	0.67	0.85	0.71	0.65
	3	0.77	0.91	0.83	0.77
Mean ± SD		0.67 ± 0.07	0.80 ± 0.08	0.71 ± 0.09	0.66 ± 0.08
Combined Mean ± SD		**0.66 ± 0.15**	**0.80 ± 0.11**	**0.71 ± 0.14**	**0.66 ± 0.14**
Combined P value			**0.04**		
P value			**0.001**	**0.949**	**1**

***Pz: ratio of the colony diameter and precipitation zone therefore lower values indicate greater enzyme activity and higher values indicate less enzyme activity.**

3.7 Effect of *L. rhamnosus* CFE on drug efflux pumps

Drug efflux pumps play an important role in azole drug resistance in *C. albicans* and had therefore been studied in detail. To study the effect of *L. rhamnosus* CFE on the efflux pumps of *C. albicans*, we measured the efflux of R6G in FLC susceptible (*C. albicans* SC5314 and *C. albicans* S7) and FLC resistant *C. albicans* R3. R6G is a fluorescent dye and was taken up by the deenergised FLC susceptible and FLC resistant *C. albicans* cells without any significant differences. The uptake of R6G by all the isolates is evident as there was a sharp drop in absorbance in all the strains in the first 5 minutes of incubation. In FLC susceptible isolates, due to inactive efflux pumps, no efflux of R6G was observed as shown by a consistency in relatively low absorbances over a period of 60 min. Even after addition of 1.5 ml of 0.5 M glucose after 25 min there was no significant change in absorbance as shown in **Figures 3.4** and **3.5.** In contrast, a different trend was observed in FLC resistant *C. albicans* R3. After addition of 1.5 ml of 0.5 M glucose, there was a gradual increase in absorbance from 30-60 min, which could be related to active efflux pumps in this isolate. Upon addition of glucose, cells effluxed R6G which was measured in the supernatant at an absorbance of 527 nm. Efflux of R6G was measured by recording the change in absorbance with time **(**Refer to **Appendix 3.1)**. The effect of different concentrations of *L. rhamnosus* CFE on efflux pumps of different *C. albicans* isolates are summarised in **Table 3.7** and represented in **Figures 3.4, 3.5 and 3.6**. As there was no efflux of R6G is FLC susceptible isolates, there was no considerable reduction in absorbance when compared to the control **(Figure 3.4 and 3.5)**. On the other hand, at concentrations of 84.5 and 42.25 mg/ml of *L. rhamnosus* CFE the efflux pumps were inhibited prominently as shown by lower absorbances when compared to the untreated control. However, there is no effect on R6G efflux in comparison to the control when cells were treated with a concentration of 21.12 mg/ml **(Figure 3.6)**. From these results, it is evident that FLC resistant cells have active efflux pumps compared to FLC susceptible

cells which can efflux drugs from the cells. These results also showed that *L. rhamnosus* CFE has a potential to inhibit these efflux pumps and thereby reverse drug resistance.

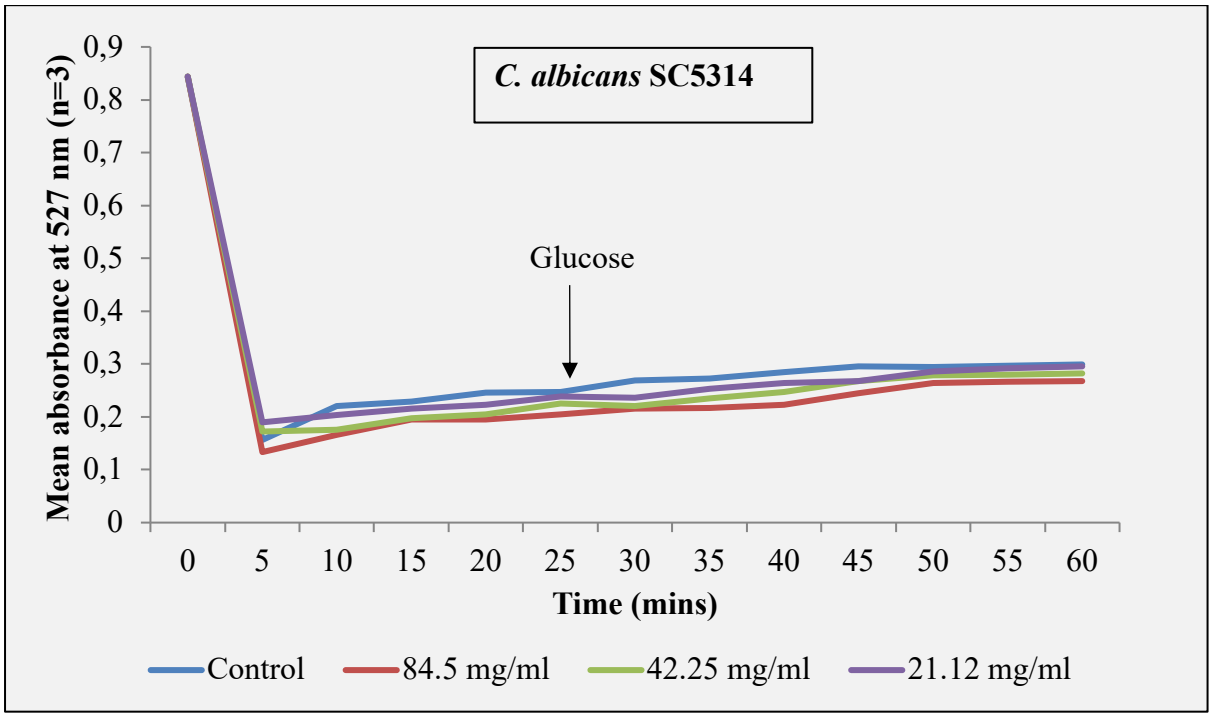

Figure 3.4: Effect of R6G efflux in the presence and absence of various concentrations of *L. rhamnosus* CFE in *C. albicans* SC5314.

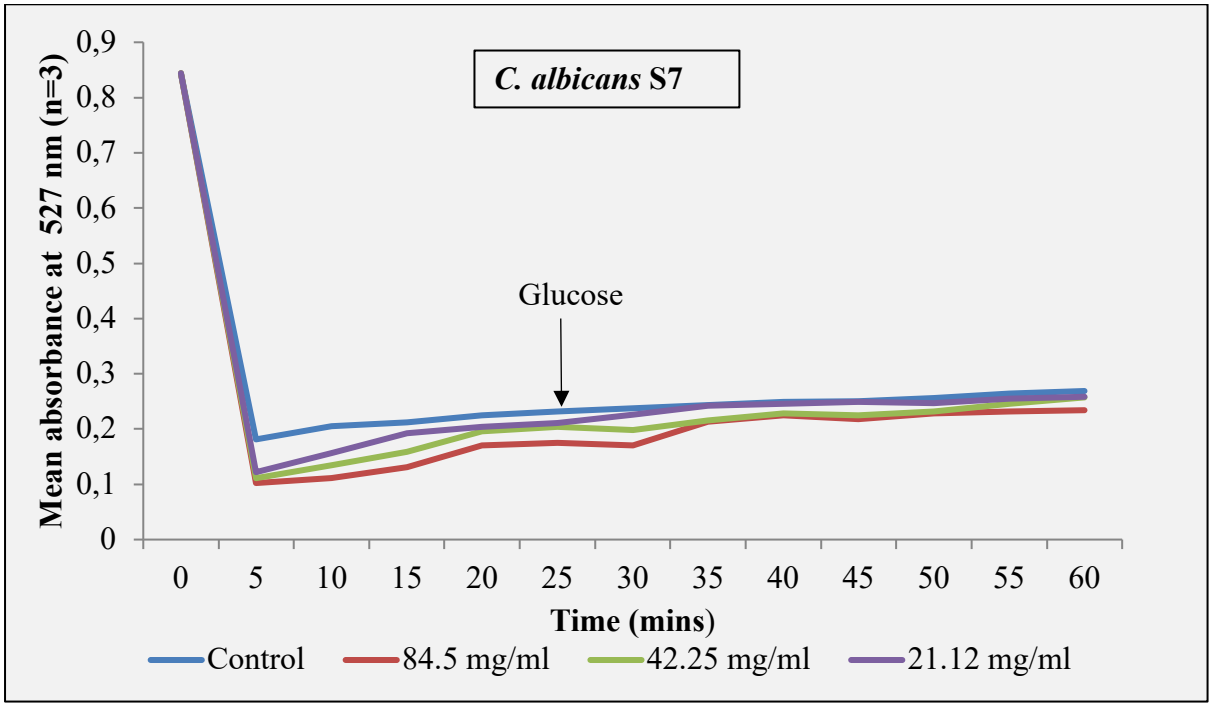

Figure 3.5: Effect of R6G efflux in the presence and absence of various concentrations of *L. rhamnosus* CFE in susceptible *C. albicans* S7 strain.

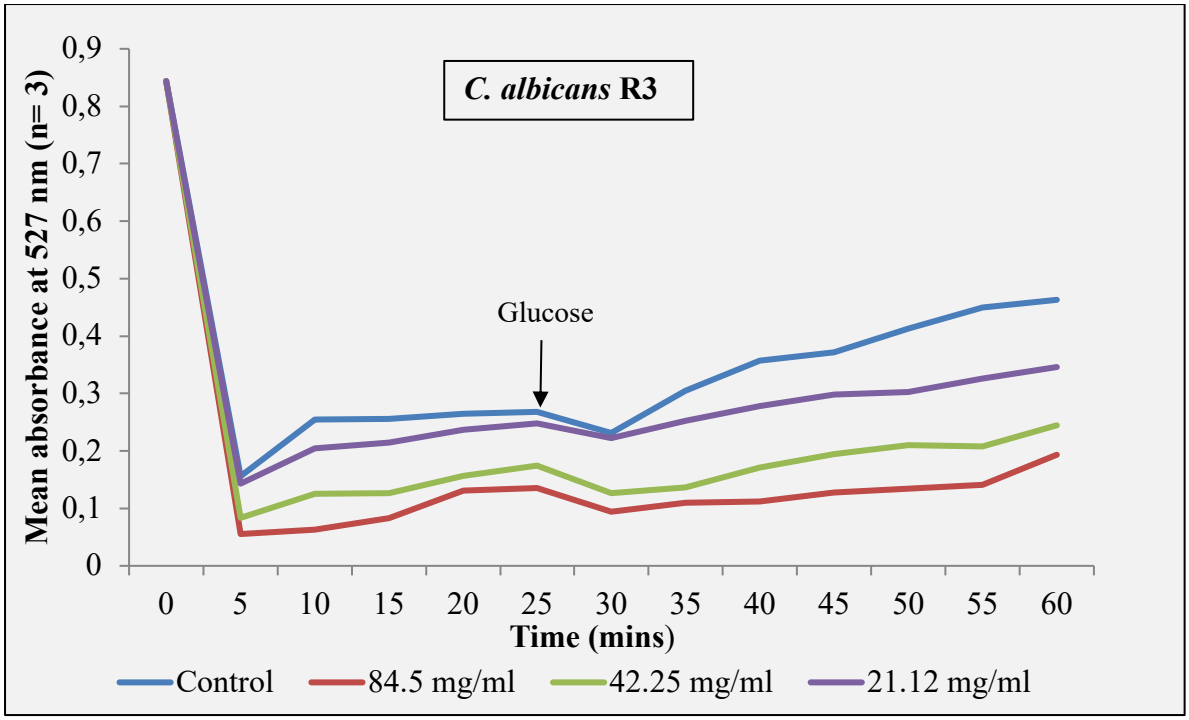

Figure 3.6: Effect of R6G efflux in the presence and absence of various concentrations of *L. rhamnosus* CFE in resistant *C. albicans* strain (R3).

3.8 Regulation of expression of virulence and drug resistant genes in *C. albicans* by *L. rhamnosus* CFE

The effect of *L. rhamnosus* CFE on the expression of virulence genes responsible for yeast to hyphae transition (*HWP1*), proteinases (*SAP1*, *SAP2*, *SAP3*) and phospholipases (*PLB1*) was studied. In addition, the effect on the expression of drug resistant genes (*CDR1*, *CDR2*, and *MDR1*) was also studied. To perform these assays, validation of primers and gene profiling was necessary.

3.8.1 Validation of primers and gene profiling

Initially, for each strain total RNA was extracted and cDNA was synthesised. Untreated cells served as controls. The concentration of *L. rhamnosus* CFE used in this assay was 84.5 mg/ml.

3.8.1.1 Total RNA purity and integrity

Initially, four strains of *C. albicans* (SC5314, S7, R2 and R3) were employed for molecular characterisation. However, only three C. *albicans* strains (SC5314, S7, and R3) were further assayed as they were used in previous experiments. The results for total RNA purity are represented in **Appendix 4.1** as absorbance ratios. An absorbance ratio of A_{260}/A_{280} of 1.8-2.1 indicated highest RNA purity. In this study, the highest absorbance ratio was 2.1 in *C. albicans* S7 (untreated) and the lowest was recorded in *C. albicans* SC5314 and R3 isolates with ranges from 1.51-1.69 **(Figure 3.7).**

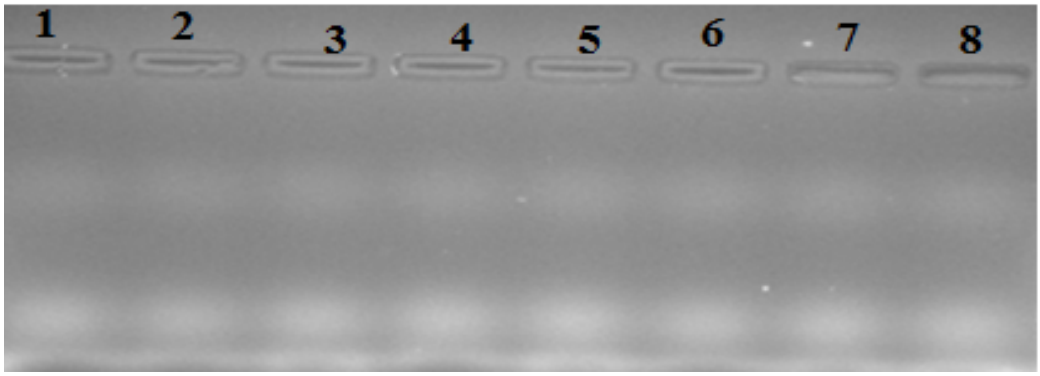

Figure 3.7: A representative 2 % agarose gel profile showing *C. albicans* RNA bands stained with ethidium bromide. (Lane 1-untreated strain R2, Lane 2-treated R2, Lane 3-untreated strain SC5314, Lane 4-treated strain SC5314, Lane 5-untreated strain R3, Lane 6-treated strain R3, Lane 7-untreated strain S7, Lane 8-treated strain S7).

Assessment of total RNA integrity is an important step in assessing nucleic acid purity and stability. As such, the quality and quantity of total RNA was analysed using the Thermo Scientific Nanodrop 2000 spectrophotometer.

Inclusion of the O'Generuler, a DNA molecular weight marker on the gel allowed determination of band sizes besides serving as a control to ensure optimal electrophoresis of nucleic acids. *C. albicans* R2 was not used for further studies as the RNA concentration obtained was lower compared to the RNA concentrations for the other three clinical strains.

3.8.1.2 Validation and specificity of primers

A total of five primer pairs for virulence genes (*HWP1*, *SAP1*, *SAP2*, *SAP3*, *PLB1*) and three for efflux pump genes (*CDR1*, *CDR2*, *MDR1*) were utilised to investigate gene expression profiling. Additionally, three reference genes (*ACT1*, *PMA1* and *RPP2B*) were also included in the study and served as positive controls for constitutive expression. The untreated standard strain *C. albicans* SC5314 was used to optimise PCR amplification conditions. Primer pairs were first validated for their specificity prior to being employed in quantitative real time PCR. The best annealing temperature to use for each primer pair was carried out by determining the annealing temperature which ranged between 50-54 °C. PCR products were characterised on a 2 % agarose gel. The tested possible annealing temperature for each primer

pair and corresponding PCR product is represented in each lane **(Figure 3.8)**. Each lane was comprised of a single band of predicted size and all annealing temperatures produced a similar single band of same size in all annealing temperatures **(Figure 3.8 and Appendix 4.2)**. Therefore, the highest annealing temperature of 54 °C was chosen for gene expression by qPCR analysis.

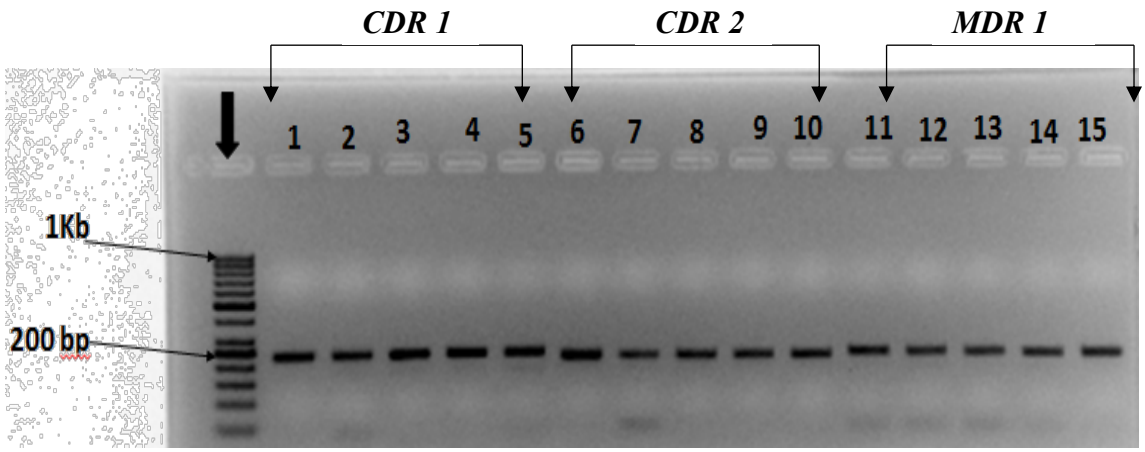

Figure 3.8: PCR amplification of *C. albicans* SC5314 cDNA on 2 % agarose gel. PCR amplicons ranged from 150-200 bp. Note arrow shows the DNA ladder (50 bp) (Refer to **Appendix 4.2** for rest of amplicons on 2 % agarose gel).

3.8.1.3 Screening of reference genes

The effect of *L. rhamnosus* CFE on expression of three reference genes (*ACT1*, *PMA1* and *RPP2B*) was evaluated. The control and treated *C. albicans* SC5314 cDNA were used for this assay. Before commencing with gene expression profiling, three reference genes were tested to confirm constitutive expression in samples derived from susceptible and resistant fungal pathogens. Conventional PCR was conducted to evaluate the optimum annealing temperature for each target and reference gene employed in the current study. There was no significant difference between control and treated genes for *ACT1* and *PMA1* ($P > 0.05$), whereas for *RPP2B* there were significant difference between untreated and treated genes ($P < 0.05$). Therefore, only one housekeeping gene *ACT1* was selected as a reference gene for gene expression studies. Although the band corresponding to the *ACT* reference gene was not

prominent as observed on a 2% agarose gel following conventional PCR, RT-qPCR provides improved sensitivity and specificity relative to the former. Importantly, there was no significant difference in *ACT* gene expression between treated and untreated samples, thus indicating constitutive expression. Statistical analysis is detailed in **Appendix 8.1**.

3.8.2 Gene expression assay

The control and treated cDNA samples from three *C. albicans* strains (SC5314, S7, and R3) were used for this assay. The effect of *L. rhamnosus* CFE on expression of virulence genes (*HWP1*, *SAP1*, *SAP2*, *SAP3* and *PLB1*) and drug resistance genes (*CDR1*, *CDR2* and *MDR1*) was determined. In each strain, the average ratio of the target gene to mRNA expression of *ACT1* in the control and treated samples was calculated using threshold values (Cq) for each gene **(Figures 3.9; 3.10; 3.11; 3.12; 3.13; 3.14; Appendix 5.1 and 5.2)**. The fold change was used to describe the level of gene expression **(Appendix 5.3)**.

3.8.2.1 Virulence gene expression in *C. albicans* SC5314

The results of relative fold change of virulence genes in untreated and treated strains are represented in **Figure 3.9**. *L. rhamnosus* CFE significantly reduced expression of *HWP1* ($P = 0.04$). The expression of *SAP1*, *SAP2*, *SAP3* and *PLB1* genes were significantly reduced in treated microbial strains as shown in Figure 3.9 ($P < 0.01$). The fold change was in the range 0.82 to 0.91 . *PLB1* was highly downregulated with a fold difference of 0.82. The least fold change of 0.91 was recorded in *HWP1*, *SAP1,* and *SAP2*.*SAP3* gene displayed a fold change of 0.88.

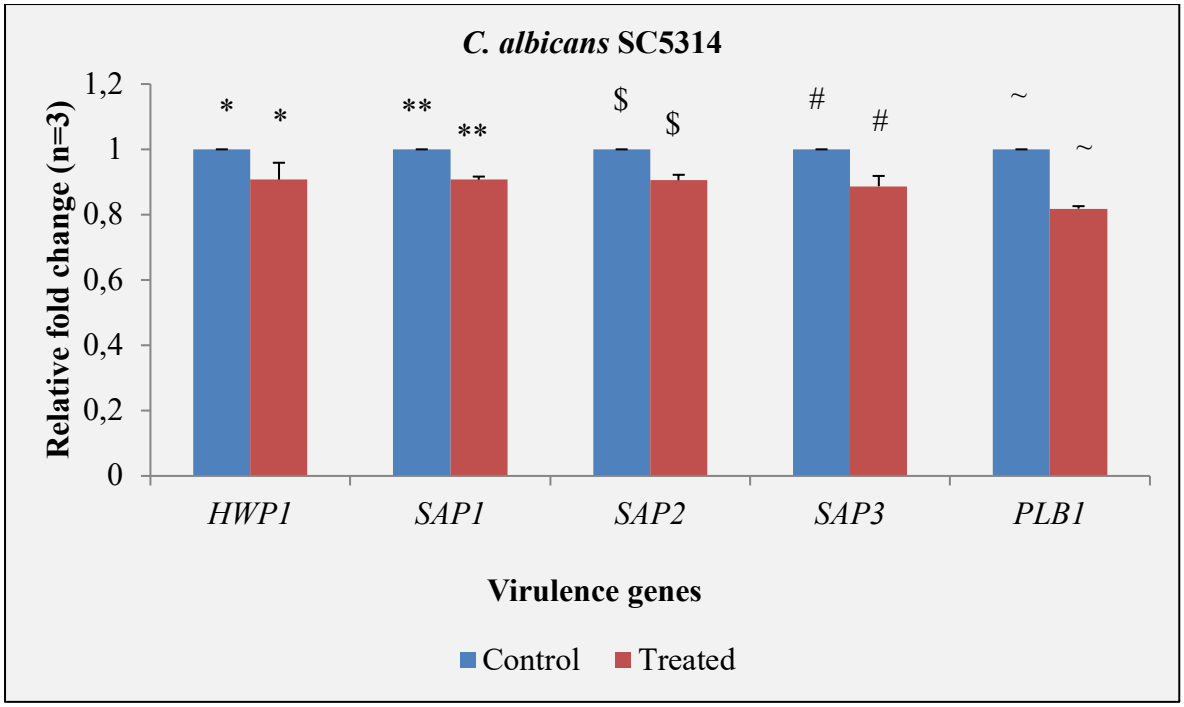

Figure 3.9: Effect of *L. rhamnosus* CFE at MIC on expression of virulence genes in *C. albicans* SC5314. *: P = 0.04; **, $, #, ~: P < 0.01. *, **, $, #, ~: Comparison between control and treated target genes of virulence using Student's two-sample t-test.

3.8.2.2 Virulence gene expression in *C. albicans* S7

The results for relative fold change in the presence or absence of *L. rhamnosus* CFE are represented in **Figure 3.10**. The expression levels of *HWP1*, *SAP1*, *SAP2* and *PLB1* were significantly downregulated (P < 0.01). However, there was no significant difference in *SAP3* expression between treated and untreated samples (P > 0.05). The fold change in the virulence genes was in the range 0.83 to 0.96. Expression of *SAP2* was significantly decreased with a fold change of 0.83, while *SAP3* levels were moderately downregulated with a fold change of 0.96. The levels of expression in *HWP1*, *SAP1* and *PLB1* were moderate with fold changes of 0.89, 0.87, and 0.91respectively.

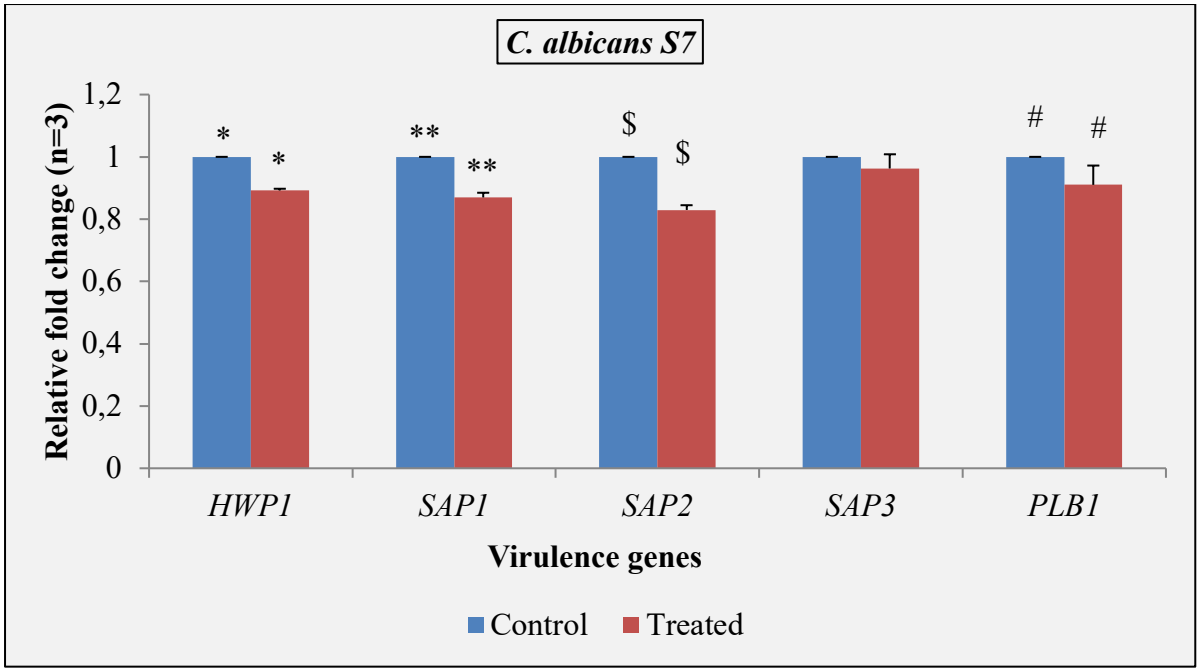

Figure 3.10: Effect of *L. rhamnosus* CFE at MIC on expression of virulence genes in susceptible *C. albicans* S7. *, **, $, #: P < 0.01. *, **, $, #: Comparison between control and treated target genes of virulence using Student's two-sample t-test.

3.8.2.3 Virulence gene expression in *C. albicans* R3

The results of relative fold change in samples treated with *L. rhamnosus* CFE and untreated controls are represented in **Figure 3.11**. There was an overall effect of *L. rhamnosus* CFE on the virulence genes. The expression levels of *HWP1, SAP1*, *SAP2* and *PLB1* were markedly reduced in treated relative to untreated cells (P < 0.01). Interestingly, expression of *SAP3* was significantly reduced (P < 0.01). The fold change in the virulence genes was in the range 0.83 to 0.93. *HWP1* was highly downregulated with a fold change of 0.83. *SAP3* was slightly downregulated with a fold change of 0.93. The levels of expression in *SAP1*, *SAP2* and *PLB1* were also moderate with fold changes of 0.86, 0.83, and 0.91 respectively.

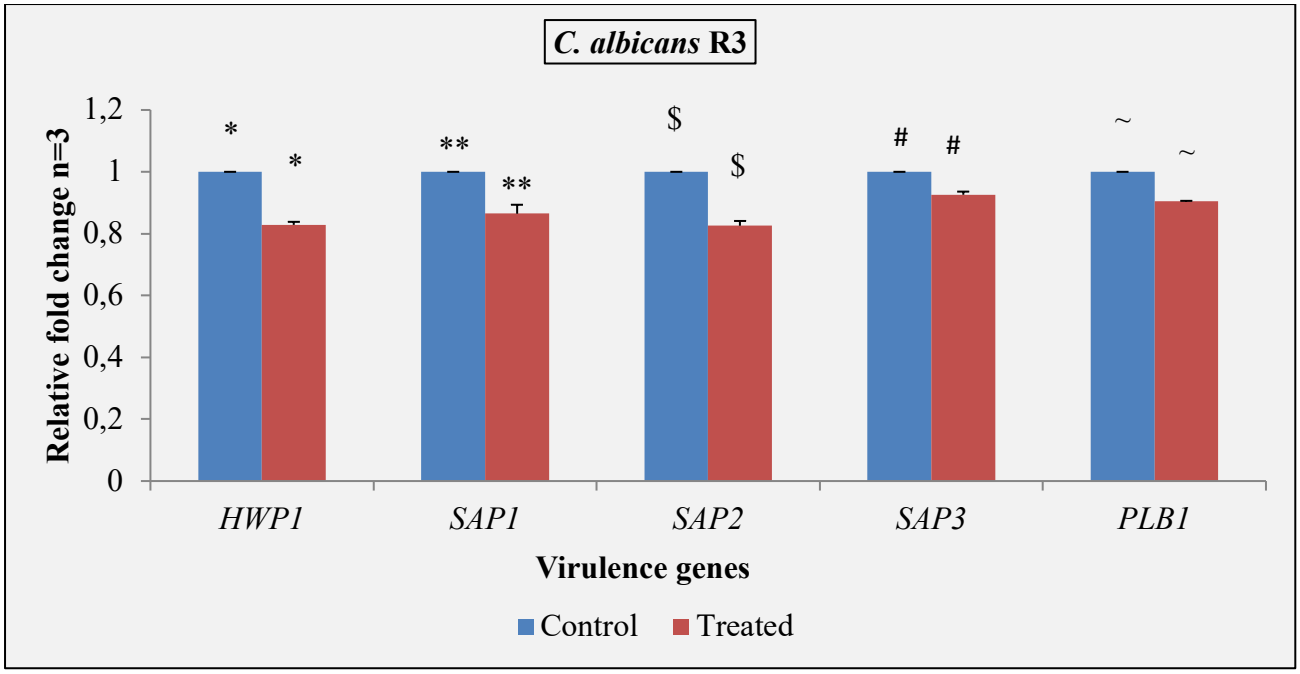

Figure 3.11: Effect of *L. rhamnosus* CFE at MIC on expression of virulence genes in resistant *C. albicans* R3 strain. *, **,$, #, ~: P < 0.01. *, **, $, #, ~: Comparison between control and treated target genes of virulence using Student's two- sample t- test.

3.8.2.4 Summary results of expression of virulence genes in *C. albicans*

The summary of the effect of *L. rhamnosus* CFE on expression of virulence genes in *C. albicans* are represented in **Table 3.7**. For each gene, the overall mean relative fold change in the control and untreated samples was evaluated. *L. rhamnosus* CFE reduced the expression of the virulence genes. The reduction in gene expression was significant in *HWP1*, *SAP1*, *SAP2*, *SAP3* and *PLB1* (P values: < 0.01) respectively. n summary, relative to untreated strains administration of *L. rhamnosus* CFE significantly decreased expression of virulence genes.

Table 3.7: Summary of the antifungal effect of *L. rhamnosus* CFE on expression of virulence genes in *C. albicans*

Target gene	*C. albicans* strain	Mean relative fold change n= 3	
		Untreated (Control)*	**Treated***
HWP1	SC5314	**1**	0.91
	S7	**1**	0.89
	R3	**1**	0.83
P value		**< 0.01**	
SAP1	SC5314	1	0.91
	S7	1	0.87
	R3	1	0.86
P value		**< 0.01**	
SAP2	SC5314	1	0.91
	S7	1	0.83
	R3	1	0.83
P value		**< 0.01**	
SAP3	SC5314	1	0.89
	S7	1	0.96
	R3	1	0.93
P value		**< 0.01**	
PLB1	SC5314	1	0.82
	S7	1	0.91
	R3	1	0.91
P value		**< 0.01**	

3.8.2.5 Expression of drug resistant genes in *C. albicans* SC5314

The results for mean relative fold change of drug resistant genes in the presence or absence of *L. rhamnosus* CFE are represented in **Figure 3.12**. The expression levels of *CDR1*, and *CDR2* were significantly downregulated ($P < 0.01$). *L. rhamnosus* CFE had no effect on the expression of *MDR1* as there was no significant difference between the control and treated samples ($P > 0.05$). The fold change in *CDR1*, *CDR2* and *MDR1* was in the range 0.87 to 0.98.*CDR2* was highly downregulated with a fold change of 0.87. *MDR1* showed considerable expression as noted by the slight fold difference of 0.98while the level of expression in *CDR1* was moderate with fold change of 0.89.

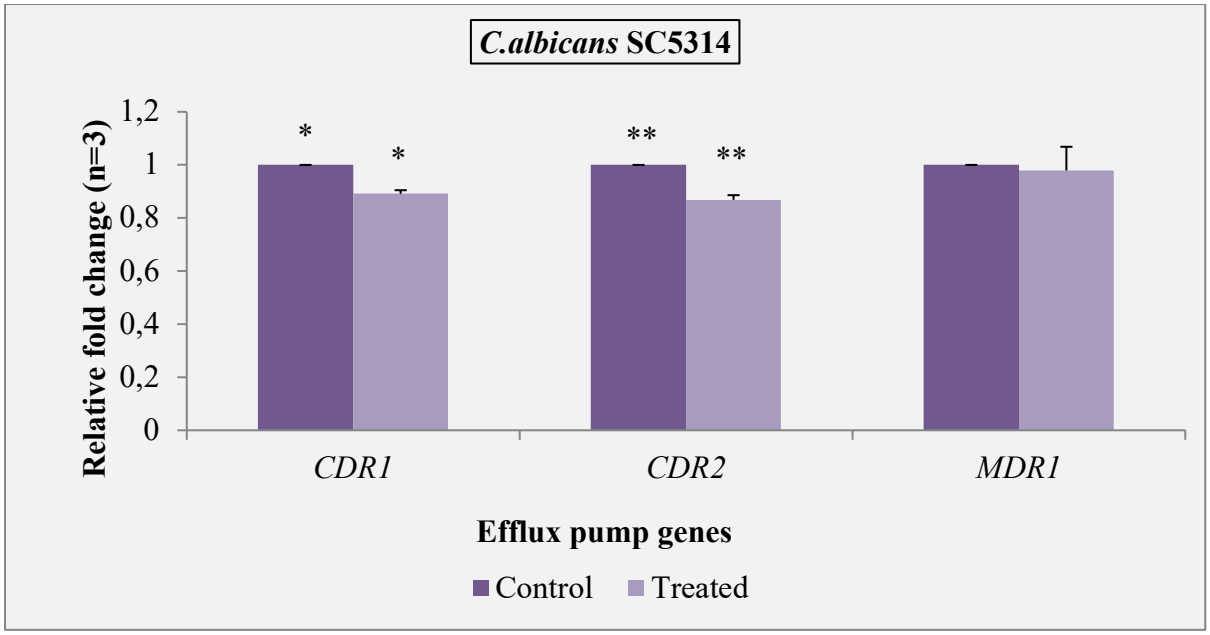

Figure 3.12: Effect of *L. rhamnosus* CFE at MIC on expression of drug efflux pump genes in *C. albicans* SC5314. *, **: P <0.01. *, **: Comparison between control and treated target genes of efflux pumps using Student's two-sample t-test.

3.8.2.6 Expression of drug resistant genes in *C. albicans* S7

Results of the effect of *L. rhamnosus* CFE on expression of drug resistant genes is represented in **Figure 3.13**. The relative fold change of the gene of interest is also represented. A significant reduction in the expression of *CDR1* and *CDR2* was observed P < 0.01) respectively. Interestingly there was a slight upregulation of mRNA expression levels of *MDR1* in the presence *L. rhamnosus* CFE. However, there was no significant difference in expression of *MDR1* in both control and treated samples (P > 0.05). The fold change in *CDR1*, *CDR2* and *MDR1* was in the range 0.88 to 1.01 *CDR2* was highly downregulated with a fold change of 0.88. *MDR1* instead was upregulated and displayed a slight fold change of 1.01. The level of expression in *CDR1* was slightly moderate with a fold change of 0.91.

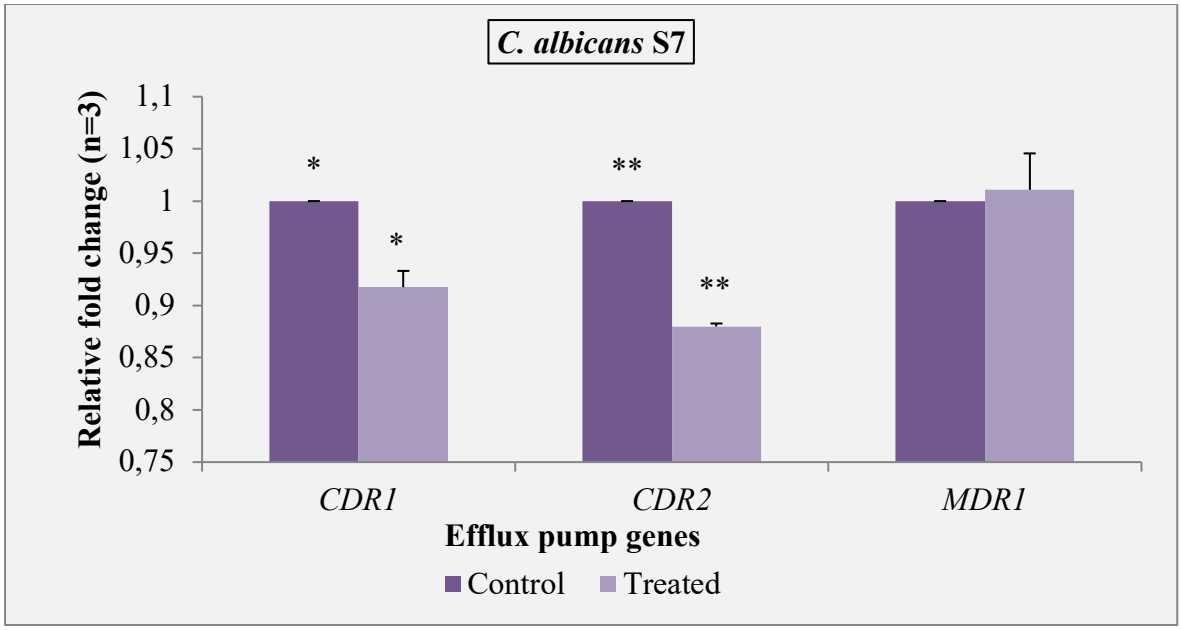

Figure 3.13: Effect of *L. rhamnosus* CFE at MIC on expression of drug efflux pump genes in susceptible *C. albicans* S7. *; **: P <0.01. *, **: Comparison between control and treated target genes of efflux pumps using Student's two-sample t-test.

3.8.2.7 Expression of drug resistant genes in *C. albicans* R3

The results for relative fold change of drug resistant genes in untreated and samples treated with *L. rhamnosus* CFE are represented in **Figure 3.14**. There was a marked reduction in the expression of *CDR1*, and *CDR2* were significantly downregulated ($P < 0.01$ and $P = 0.01$). Conversely, there was no significant difference in the expression of *MDR1* ($P > 0.05$). This means that *L. rhamnosus* CFE had no effect on expression of *MDR1*. The fold change in *CDR1*, *CDR2* and *MDR1* was in the range 0.87 to 0.97. *CDR2* was highly downregulated with a fold change of 0.87. *MDR1* showed minimal expression as noted by the slight fold difference of 0.97. The level of expression in *CDR1* was slightly moderate with fold change of 0.92.

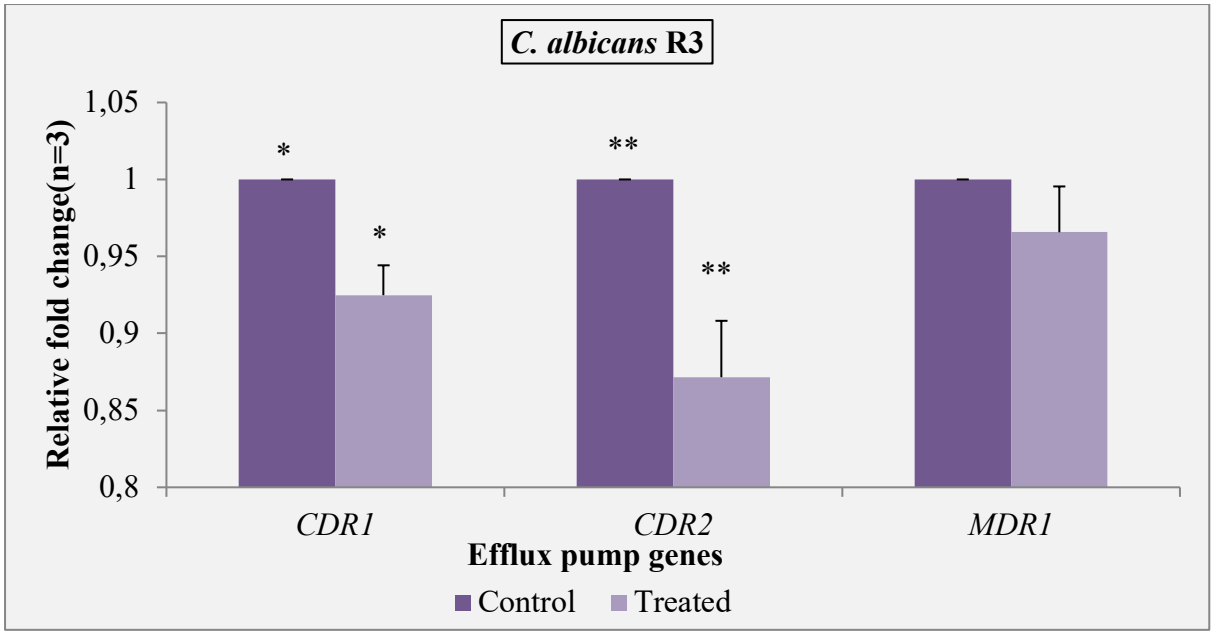

Figure 3.14: Effect of *L. rhamnosus* CFE at MIC on expression of drug efflux pump genes in resistant *C. albicans* R3. *: P < 0.01; ***: P = 0.01. *, ***: Comparison between control and treated target genes of efflux pumps using Student's two-sample t-test.

3.8.2.8 Summary results of drug resistance gene expression assay

Results of the effect of *L. rhamnosus* CFE on expression of drug resistant genes in *C. albicans* are summarised and represented in **Table 3.9**. For each gene, the overall relative fold change in the control and untreated samples was evaluated. The reduction in gene expression was significant in *CDR1* and *CDR2* (P values: < 0.01) respectively. Interestingly, there was no overall significance between the control and treated samples of *MDR1* genes in all strains (P > 0.05). This means that the expression of the drug resistant genes was not affected by administration of *L. rhamnosus* CFE.

Table 3.8: Effect of *L. rhamnosus* CFE at MIC concentration on expression of drug efflux pump genes in *C. albicans*.

Target gene	***C. albicans* strain**	**Mean relative fold change n= 3**	
		Untreated (Control)*	**Treated***
CDR1	SC5314	1	0.89
	S7	1	0.92
	R3	1	0.92
P value		**< 0.01**	
CDR2	SC5314	1	0.87
	S7	1	0.88
	R3	1	0.87
P value		**< 0.01**	
MDR1	SC5314	1	0.98
	S7	1	1.01
	R3	1	0.97
P value		**0.42**	

CHAPTER 4: DISCUSSION

Candidiasis continues to be a serious threat causing mortality rates of 30-50 % globally in immunocompromised patients (Ahmad *et al*., 2016; Irfan *et al*., 2017). In addition, the burden of antifungal drug resistance in immunocompromised patients has become a major concern due to limited options of treatment especially in developing countries. All these factors advocated the need for new drugs along with the novel strategies to combat antifungal drug resistance in *C. albicans*. An attractive alternative is to target virulence factors, which play an important role in the pathogenesis of *C. albicans*. In addition, targeting efflux pumps that are specific to *C. albicans* and are mainly responsible for antifungal drug resistance by effluxing the drug from the cells before they reach their targets. Targeting virulence traits and efflux pumps has emerged as important aspects and promising strategies of dealing with drug resistance. Targeting these factors can bypass drug resistance mechanisms in these pathogens as these approaches inhibit pathogenesis rather than killing or impeding fungal growth (Mane *et al*., 2012; Shareck and Belhumeur, 2011). Therefore, developing new antifungal drugs that can maintain *C. albicans* cells in its harmless state by blocking virulence traits that contribute to its pathogenicity is a prime requirement. One emerging solution is to use the compounds and CFEs of other microorganisms, especially probiotics.

The use of probiotics as whole cells in reducing bacterial and fungal infections is well documented (Coman *et al*., 2014; Verdenelli *et al*., 2014). However, questions on their safety have been raised due to cases of sepsis that have been reported because of use probiotics as whole live cells (Boyle, 2006). Most of the cases of sepsis have been attributed to fungaemia and bacteraemia (Kunz *et al*., 2004; Lestin *et al*., 2003). Due to this side effect, use of probiotics against different infections has declined and therefore, uses of probiotic CFEs as alternatives have emerged. Several reports on the potential use of probiotic CFEs have been documented (Nyanzi *et al*., 2014; Murzyn *et al*., 2010). *Saccharomyces boulardii* CFE and the active compound capric acid have been reported to inhibit filamentation and biofilm

formation in *C. albicans* (Murzyn *et al*., 2010). In the same study, the genes responsible for virulence have also been reported to be downregulated in the presence of both the *S. boulardii* CFE and capric acid (Murzyn *et al*., 2010). In another study, Nyanzi *et al* (2014) characterised methanol cell free extracts (CFE) from *Lactobacilli* species and their constituents and were tested for antifungal activity against *C. albicans.* Wang and co-workers stated that antimicrobial compounds that were produced by *L. crispatus* were able to inhibit the growth and hyphae formation in *C. albicans* (Wang *et al*., 2017a). In the same study, it was highlighted that these antimicrobial compounds also regulate the expression of genes related to virulence. As these studies were only restricted to standard *C. albicans* strains and the effect of CFE was tested on yeast to hyphae transition only, there is need to investigate the effect of probiotic CFE on expression of other virulence traits in clinical *C. albicans* strains.

In this study, the clinical strains used were identified and confirmed as *C. albicans*. The three probiotic CFEs were prepared from *L. rhamnosus*, *L. acidophilus* V and *B. animalis* subspecies *lactis*. Results from preliminary screening indicated that all the three probiotic CFEs had a fungistatic effect against the tested clinical *C. albicans* strains. These results also indicated that the inhibitory effect was the same on both susceptible and resistant *C. albicans* strains. Based on the minimum inhibitory concentrations, *L. rhamnosus* showed the highest antifungal activity against all the tested clinical *C. albicans* strains and therefore was selected for further assays in this study.

Besides antifungal activity, the effect of *L. rhamnosus* CFE at subinhibitory concentrations was tested against the main virulence traits and their genes which are responsible for the initial stages of pathogenesis in *C. albicans* strains. In addition, effect of *L. rhamnosus* CFE was tested on reversal of drug resistance and its genes in *C. albicans* strains.

4.1 *C. albicans identification*

All the clinical isolates of *C. albicans* used in this study were identified and confirmed by germ tube formation and API 20 C AUX test kit (Biomerieux, France) following the manufacturer's instructions. Germ tube formation is a characteristics of *C. albicans* and is the first stage in yeast to hyphae morphological transition. All the clinical *C. albicans* strains used were germ tubes positive (**Table 3.1**). API 20 C AUX test is a rapid, cheap, precise and effective test for identification of most frequently encountered yeasts such as *Candida* and *Saccharomyces* species among others. The principle of API 20 C AUX kit uses strips and each strip consists of 20 cupules each with dehydrated substrates. The cupules are inoculated with a semi-solid minimal medium. Yeasts capable of utilising each substrate as the sole carbon source will grow. The reactions are compared to growth control and identified by referring to the identification software. The germ tube and the API 20C AUX tests can be used together for identification and confirmation of *C. albicans*. It has been reported by Smith *et al* (1999) that the API 20C AUX has an accuracy of 95.6-99 %. This correlates well with the results obtained in this study where the accuracy ranged from 97-99 % for identification of *C. albicans*.

4.2 Preparation of probiotic CFEs

In this study, cell free extracts of *L. rhamnosus*, *L. acidophilus* V and *B. animalis* subspecies *lactis* were successfully prepared using ethyl acetate. Ethyl acetate is categorised as a class III solvent and has medium polarity and low toxicity which has no effect on the test strains. It is one of the preferred solvents as it extracts mainly polar and non-polar biological compounds. This solvent is moderately safe and is preferred over methanol, ethanol, chloroform and hexane for use in pharmaceutical industries for drug development (Masoko *et al*., 2007).

The resultant final mass of 16 g was obtained for all the three-probiotic cell free extracts. A mass of 1.35 g was diluted using 1 % methanol to give a final concentration of 1.35 g/ml, which was prepared fresh each time before an assay. The nature of the CFE and yield could

not be compared with other studies as there are no studies to our knowledge that have reported the nature and yield of the CFE obtained. However, the final concentration obtained in this study was higher than final concentration of CFE reported by Murzyn *et al* (2010). This is attributed to the fact that the same method including the solvent was used to prepare the CFE but the difference between the two studies was the species that were used and subsequently the growth conditions. In our study, the lactic acid bacteria were used and these were cultured in MRS broth and grown in anaerobic conditions for 16-20 h whereas Murzyn and co-workers used *S. boulardii,* which was cultured in SD broth and grown in aerobic conditions for 24 h.

The chemical composition of CFE varies with the differences in the incubation times. In a study by Taheri, it was reported that during the log phase to late log phase (12-20 h), lactic acid bacteria secrete large amounts of bacteriocin into the media (Taheri *et al.*, 2012). However, after 20 h the biomass is reduced probably due to proteolytic degradation during fermentation of lactic acid bacteria. In another study by Nyanzi and co-workers, CFE from different *Lactobacilli* strains were rich in organic acids such as 3-phenyl-L-lactic acid and caproic acid (Nyanzi *et al*., 2014). Furthermore, there are reports of preparing CFE with different methods, however all these studies reported the antimicrobial property of these CFE to varying extents (Taheri *et al.*, 2012; Nyanzi *et al*., 2015; Wang *et al*., 2017a). With this background, the CFE prepared in this study were screened for their anticandidal activity against different clinical FLC susceptible and resistant *C. albicans* isolates.

4.3 Antifungal activity of probiotic CFEs against *C. albicans* cells

The three probiotic CFEs were tested for their antifungal activity by determining their MIC and MFC values following the CLSI recommended guidelines. All the three CFEs showed anticandidal effect on ten tested clinical *C. albicans* strains including the standard strain *C. albicans* SC5314 at varying levels. The median MICs ranged from 84.5-338 mg/ml. Among all the three extracts, *L. rhamnosus* CFE had the lowest median MIC of 84.5 mg/ml and

based on these results, *L. rhamnosus* CFE was selected for further assays. Both *L. acidophilus* V and *B. animalis* subspecies *lactis* CFE showed median MICs of 169 mg/ml.

Comparatively, MIC values of all the tested probiotic CFEs in this study were higher than those reported for probiotic CFEs by other authors. In a study by Nyanzi *et al* (2014), the MICs of CFEs from *L. helveticus* Z, *L. rhamnosus* (M, Y, O D, C) and *L. acidophilus* (U, V and W) were in the range 1.25-5 mg/ml. In another study, Strom (2005) reported MIC values of 20 mg/ml from CFE of *L. plantarum* MiLAB 393 against *A. fumigatus* and *P. roqueforti*. Lavermicocca *et al* (2000) stated that phenyllactic acid, form *L. plantarum* 21 B showed activity against *Eurotium* species and *Aspergillus* species with MICs of 50 mg/ml. On the other hand, the MIC results in the present study closely correlated with results reported by Lavermicocca *et al* (2000). Lavermicocca and co-workers reported MIC of *L. plantarum* 21B CFE as 166 mg/ml against *P. roqueforti* IBT18687 and *P. corylophium* IBT6978. They also reported phenyllactic acid to be the most active compound responsible for the antifungal activity. The discrepancies between the previously reported results and the results from the current study could be due to the nature of the CFE, method of CFE preparation and the use of different fungal species.

After determining the MIC values, we intended to determine the MFC values of *L. rhamnosus*, *L. acidophilus* V and *B. animalis* subspecies *lactis* CFEs. However, the MFC of the test CFEs could not be determined as growth was observed from the wells when plated out on SD agar. This finding is congruent with the previous study by Nyanzi *et al* (2014), where they reported lower MICs of CFEs but could not determine MFCs values either. Therefore, it can be concluded that the CFEs had fungistatic activity against all the tested *C. albicans*.

4.4 *L. rhamnosus* CFE against expression of virulence traits in *C. albicans*

In *C. albicans* and other commensal pathogens, transition from the normal commensal to pathogenic form depends upon several factors known as virulence traits. The expression of these virulence traits varies according to the stage, site of infection and the nature of the host immune system (das Mohan and Ballal, 2008). Fungal virulence traits such as yeast to hyphae transition and production of hydrolytic enzymes are known to play a role in pathogenesis and due to this characteristic; they are attractive potential targets for drug development (Mayer *et al*., 2013). Since these traits play a role in pathogenesis and are not required for cell survival, blocking these traits does not pose survival pressure on pathogens. Targeting virulence traits have emerged as a new antifungal paradigm (Wang *et al*., 2017a), especially against drug resistant pathogens. In this study, the effect of *L. rhamnosus* CFE, a potential antifungal, was evaluated against virulence traits of clinical strains of fluconazole susceptible and resistant *C. albicans* isolates.

4.4.1 Effect on yeast to hyphae transition

The initial stage that enables *C. albicans* to invade the host cells is the morphological transition from normal yeast to pathogenic hyphae form. Successful transition from yeast to hyphae is dependent on environmental pH, physiological temperature, presence of serum and carbon dioxide concentrations (Mayer *et al*., 2013). *C. albicans* invades the host cells using two various mechanisms namely induced endocytosis and active penetration. In induced endocytosis, specialised proteins on the cell surface such as invasins are expressed by fungi to mediate binding to the host thereby triggering the engulfment of the fungal cell wall into the host cell. Reports have shown that this process usually occurs during the early stages of invasion and is passive as viable yeast cells are not required for the process to occur. In contrast, active penetration is a fungal-driven process that occurs later as it requires viable *C. albicans* hyphae to penetrate between or through epithelial cells (da Silva Dantas *et al*., 2016).

In this study, clinical *C. albicans* strains were incubated at 37 °C in the presence of *L. rhamnosus* CFE at various concentrations. The pH was adjusted to 6.5 and hyphae formation was induced with foetal bovine serum. The results showed that *L. rhamnosus* CFE inhibited the transition in a concentration dependent manner **(Figures 3.1; 3.2; 3.3 and Table 3.4)**. At concentrations of 84.5 mg/ml and 42.25 mg/ml, there was a significant inhibition of hyphae which advocates the potential of the CFE to inhibit the initial stage of pathogenesis in *C. albicans*.

However, at low concentrations (21.12 mg/ml), there was no effect of CFE on morphological transition. Not only was the number of hyphae forming cells reduced with CFE treatment, but the hyphal length was also recorded to be lowered in a concentration dependent manner **(Figures 3.1; 3.2; 3.3 and Table 3.4)**. The hyphal length also plays an important role in the ability of *C. albicans* cells to penetrate and invade the host tissue during pathogenesis. These results are in line with previous similar findings where *S. boulardii* extract and capric acid were reported to have inhibitory activity on yeast to hyphae transition in *C. albicans* cells (Murzyn *et al*., 2010), with differences of the concentrations used and the probiotic species from which the extract was prepared. In the present study, *L. rhamnosus* CFE was used at concentrations of 84.5, 42.25 and 21.12 mg/ml, whereas Murzyn *et al* (2010) used *S. boulardii* extract and its constituent capric acid at concentrations of 160 µg/ml and 45.3 µg/ml respectively. In another study, the transition from yeast to hyphae was inhibited by 80 % in the presence of *L. crispatus* cell free supernatant (Wang *et al*., 2017a). Matsubara *et al* (2016b) evaluated the inhibitory effects of the planktonic probiotic *Lactobacilli* species and their supernatants. They observed that *L. rhamnosus* supernatant inhibited the early stages of biofilm development which is hyphal formation by cell to cell interactions and probably secretion of antifungal compounds present in the supernatant.

Besides probiotic CFEs, other antimicrobial compounds have been reported to have a similar effect on transition from yeast to hyphae in *C. albicans*. Mafojane *et al* (2017) investigated

the effect of gentian violet on yeast to hyphae transition and found that 98 % of germ tubes were inhibited. In another study, *Ocimum sanctum* essential oil was reported to inhibit *C. albicans* hyphae development by 92 % at ½ MIC (0.1 µl/ml), (Khan *et al*., 2014). The same group of authors in a separate study investigated the effect of diallyl sulphide (DAS) and diallyl disulphide (DADS) on yeast to hyphae transition in *C. albicans* (Yousuf *et al*., 2011). In this study, they reported 85 % and 95 % inhibition of yeast to hyphae at subinhibitory concentrations of DAS and DADS, respectively after 300 mins. From these results, it can be concluded that the yeast to hyphae transition is an attractive virulence trait that can be targeted for therapeutic purposes. *L. rhamnosus* CFE showed inhibitory activity against formation of hyphae and its effect was further assayed against other virulence traits.

4.4.2 *L. rhamnosus* CFE on proteinase secretion

The other important virulence trait, following hyphae formation, is the production of secreted aspartic proteinases (SAPs). Like hyphae formation, secretion of SAPs also varies according to *Candida* strain, type of infection, environmental conditions and stage of infection (Mayer *et al*., 2013; Pawar *et al*., 2014). The SAPs facilitate the active penetration into the host target cell membranes which are composed mainly of proteins and lipids. In addition, they have been reported to enhance efficiency and acquisition of extracellular nutrients, digestion of cells and molecules of host immune system to resist antimicrobial attack by the host. It is also reported that the presence of increased activity of SAPs leads to increased and rapid development of candidiasis (das Mohan and Ballal, 2008; Naglik *et al*., 2003). Therefore, the absence and/ or reduced expression of SAPs may indicate the less virulent nature of *C. albicans*.

The effect of *L. rhamnosus* CFE on production of SAPs by *C. albicans* was investigated using inhibitory and subinhibitory concentrations of the CFE. Bovine serum albumin fraction V was used as the only source of nitrogen and activity was reported by calculating zones of protein clearing (Pz). The production of SAPs in clinical *C. albicans* strains was significantly

reduced in the presence of *L. rhamnosus* CFE ($P < 0.01$) in a concentration dependent manner. At concentrations of 84.5 mg/ml and 42.25 mg/ml, the inhibition was more significant when compared to the control ($P < 0.01$, **Table 3.5**). Low concentrations of 21.12 mg/ml showed no effect on SAP production. Even though there were significant differences in proteinase production in treated and untreated cells, there was no difference in the index values (Pz) between the susceptible and the resistant *C. albicans* strains.

Despite there being several reports on the effect of antimicrobials on the production of SAPs in *C. albicans*, there is no study so far reporting the effect of *L. rhamnosus* CFE on production of SAPs in *C. albicans*. In a study by Yousuf and co-workers, the effect of DAS and DADS on production of SAPs was investigated; they reported the reduced secretion of SAPs and related it to the ability of these compounds to increase reactive oxygen species and inducing apoptosis in *C. albicans* (Yousuf *et al*., 2011). In another study, *Ocimum sanctum* essential oil had inhibitory effects on SAPs in *C. albicans* (Khan *et al*., 2014). They also suggested that the effect may prevent the hydrolysis of host tissues when they are colonised with *C. albicans*.

4.4.3 *L. rhamnosus* CFE on phospholipase secretion

Phospholipases are a group of heterogenous enzymes capable of hydrolysing one or more ester linkages in glycerophospholipids (Ghannoum, 2000). They are secreted at the periphery of the yeast cell and the tip of the hyphae. Phospholipases lyse host cells to facilitate adhesion and penetration by breaking down the phospholipids of the cell membrane causing cell lysis. The mechanism of phospholipase activity was reported by Price *et al* (1982) where they demonstrated that phospholipases break down phospholipids present in egg-yolk namely phosphatidylcholine and phosphatidylethanolamine resulting in formation of a calcium complex with the released fatty acids causing damage to the cell membrane. Studies have also reported that clinical *C. albicans* strains have high phospholipase activity compared to

commensal *C. albicans* strains (Mahmoudabadi *et al*., 2010), depicting the crucial role of these enzymes in pathogenicity of *C. albicans*.

Therefore, inhibition of phospholipases using antimicrobial agents could have a beneficial effect on the course of the *C. albicans* infections and can be used as an emerging antifungal drug target.

In the current study, effect of *L. rhamnosus* CFE on production of phospholipase in *C. albicans* was investigated. Phospholipase agar was supplemented with egg yolk emulsion which was used as the only source of phospholipids to induce secretion of the phospholipases. Inhibitory and subinhibitory concentrations of *L. rhamnosus* CFE were used and it was observed that high concentrations inhibited phospholipase production in comparison to the untreated control cells. As that of proteinase activity, inhibition of phospholipase production was also concentration dependant with no significant differences between susceptible and resistant *C. albicans* strains. Phospholipase activity was inhibited by CFE in all the tested strains. The inhibitory effect was the same in both susceptible and resistant *C. albicans* strains, except FLC susceptible strain S5, where no inhibition was observed for any of the tested concentrations. These results observed in strain S5 were similar to a study done by Patel *et al* (2009) on the effect of *Dodonaea viscosa var angustifolia* on phospholipase and proteinase production in *C. albicans*. In this study, they reported that *Dodonaea viscosa var angustifolia* had no effect on production of proteinase or phospholipase enzymes. They suggested that the extract had no effect because the *C. albicans* had already colonised the host tissue and hence could not prevent the infection process. The authors also suggested that the low concentrations could have changed the surface characteristics and did not penetrate the yeast cells to cause further changes.

To the best of our literature search, this is the first report on phospholipase inhibitory activity of *L. rhamnosus* CFE, however, there are studies reporting the effect of antimicrobials on

phospholipase activity. In a study by Yousuf *et al* (2011), DAS and DADS which are garlic derived sulphides, have been shown to reduce secretion of phospholipases in *C. albicans*. *Ocimum sanctum* essential oil was reported to inhibit phospholipase activity in *C. albicans* (Khan *et al*., 2014). In a recent report, effect of geraniol on production of phospholipases was studied and it was found that the phospholipase activity was reduced at MIC when compared to the negative control (Singh *et al*., 2018). These studies and the results from our study indicate the potential of natural products and probiotic CFE to inhibit or reduce the phospholipase activity in *C. albicans* and thereby inhibiting their virulence property. These results advocate the use of *L. rhamnosus* CFE at the subinhibitory concentrations to target pathogenicity of *C. albicans*, however, further studies such as use of other *Candida* species and other fungal pathogens are required to support these claims.

4.5 Effect of *L. rhamnosus* CFE on efflux pumps

Various mechanisms drive *Candida* to escape the action of antifungal drugs so that they can survive, leading to drug resistance. Amongst these mechanisms, overexpression of drug efflux pumps is the most dominant strategy for extrusion of drugs (Singh *et al*., 2018). Drug efflux pumps which belong to the adenosine triphosphate (ATP) binding cassette (ABC) and major facilitator superfamily (MFS) transporters have been reported to be the major causes of azole resistance in *C. albicans*. ABC superfamily use energy derived from hydrolysis of ATP to drive efflux of drugs and MFS utilise the electrochemical gradient of protons across the cell membrane to efflux substrates (Manzoor, 2015). Inhibition of these drug efflux pumps could reduce extrusion of the drugs and subsequently restore antifungal susceptibility in azole resistant *C. albicans* strains.

This study determined the effect of *L. rhamnosus* CFE on drug efflux mechanisms in fluconazole resistant *C. albicans* R3 at various concentrations in the presence and absence of glucose. The standard strain *C. albicans* SC5314 and fluconazole susceptible strain S5 were also investigated. Our results were based on the efflux of Rhodamine 6 G (R6G) the

fluorescent dye, which was measured in the supernatant using the spectrophotometer. R6G is a xanthene dye which appears red to violet in solution and is mostly used in biotechnology applications such as flow cytometry, fluorescence microscopy and spectroscopy. Moreover, this drug is known as a substrate of efflux pumps and can be used to identify multidrug resistance in many organisms (Maesaki *et al*., 1999).

The results showed that in all the test strains there was a sharp drop in absorbance at 5 min **(Figures 3.4; 3.5; 3.6)** indicating that the R6G dye was transported from the extracellular to the intracellular compartment probably by passive diffusion. However, there was no considerable difference in absorption from 5-25 min in all tested concentrations indicating that the concentration of the R6G remained high inside the cells compared to outside. This could be related to the deenergisation of cells using 2, 4-dinitrophenol and 2-deoxy-D-glucose. The former is a biochemically active compound that inhibits production of energy mainly ATP by disrupting the proton gradient in cells, whereas the latter competitively inhibits glycolysis through its action on hexokinase enzyme which is the rate limiting step of glycolysis. It is phosphorylated to 2-D-glucose-phosphates which cannot be further metabolised by phosphoglucose isomerase. This leads to accumulation of 2-D-glucose phosphate in the cells and the depletion in cellular ATP. The consistency in the absorbances could also suggest that the efflux of R6G may be dependent on energy. Drug resistant *C. albicans* R3 did not show considerable consistency from 0-25 min, which could be due to incomplete inhibition of the glycolytic pathway resulting in production of ATP and efflux of a small amount of R6G dye.

At 25 minutes, when cells were reenergised by adding glucose, there was no significant efflux of R6G, as shown by the slight differences in absorbances, in fluconazole susceptible *C. albicans* SC5314 and *C. albicans* S7. However, there was a considerable difference in cells exposed to *L. rhamnosus* CFE at a concentration of 84.5 mg/ml compared to the

untreated cells. This could suggest that the efflux pumps may have been present but in very low levels and were not induced to reduce intracellular accumulation of the drug.

In contrast, untreated fluconazole resistant *C. albicans* R3 pumped out higher concentrations of R6G as noted by a steady increase in absorbance after addition of glucose at 25 minutes. However, in *L. rhamnosus* CFE treated cells at inhibitory and subinhibitory concentrations, R6G efflux was inhibited. As R6G dye is a known specific substrate for ABC superfamily transporters namely *Candida* drug resistance protein 1(CaCdr1p) and *Candida* drug resistance protein 2 (CaCdr2p), inhibition in the efflux of R6G in treated resistant cells suggests that this CFE inhibited these energy dependent efflux pumps.

Maesaki *et al* (1999) investigated the mechanism of drug efflux using R6G in *C. albicans* and showed that azole resistant *C. albicans* strains had increased efflux of R6G when induced with glucose. These findings were similar to the results observed in this study. Efflux assays, using R6G and other fluorescent dyes, have been found to be simple and convenient to determine the basis of fluconazole resistance in clinical isolates. The R6G assays have already been reported to possess a strong agreement between high efflux rates and azole drug resistance. It is also confirmed that susceptible strains show less efflux compared to resistant strains (Maesaki *et al*., 1999; Szczepaniak *et al*., 2017) .

There is no study so far reporting the effect of *L. rhamnosus* CFE on drug efflux pumps in *C. albicans*. However, there are several studies on effect of natural compounds on the ABC family transporters. In a study by Ahmad *et al* (2013), two potent antifungal compounds namely thymol and carvacrol, inhibited the efflux pumps from multi drug resistant *C. albicans.* In the same study, they reported that thymol inhibited the efflux pump proteins by 70-75 % whereas carvacrol inhibited by 80-85 %. In another study, curcumin, a natural product from rhizomes of *Curcuma longa*, was shown to have inhibitory effects on drug transporters of yeasts (Sharma *et al*., 2009).

The fungicidal nature of geraniol, a natural monoterpenoid from Palmarosa oil, was reported to inhibit the activity of ABC drug transporter CaCdr1p. They carried out enzyme kinetic studies and identified the type of inhibition as competitive. The researchers further evaluated their results and showed that geraniol exerted the competitive effect by binding to the active site cavity of CaCdr1p (Singh *et al*., 2018).

Farnesol, a quorum sensing molecule and styrylquinolines have been reported to have an effect on efflux activities of ABC drug transporters only (Sharma *et al*., 2011; Szczepaniak *et al*., 2017). In addition, there are several studies reporting the effect of derivatives from jatrophane, palmarumycin P3 and phialocephalarin B on CaMdr1p efflux activities (Xie *et al*., 2016). These studies are in line with the findings of our study reporting the effect of compounds from different sources on efflux pump inhibition. These results suggest that reversal of antifungal drug resistance can be achieved. Our results are basis for further studies to test the effect of CFE from different probiotic microorganisms on the efflux pumps related to drug resistance in *C. albicans* and other fungal pathogens.

4.6 Effect of *L. rhamnosus* CFE on gene expression

One of the criteria to prove that a gene or its product plays an important role in the disease process is to show that it is expressed by the microorganism during the infectious process. As *L. rhamnosus* CFE had a significant effect on the virulence factors and efflux pumps. Its effect on the specific genes was determined.

HWP1 gene codes for cell wall mannon proteins which function as adhesion required for hyphal formation (Murzyn *et al*., 2010; Wang *et al*., 2017a). *SAP1*, *SAP2* and *SAP3* genes encode for secreted aspartic proteinases which are important for pathogenesis of candidiasis (Naglik *et al*., 2003). *PLB1* gene encodes phospholipases which contribute to pathogenesis in *C. albicans* (Ghannoum, 2000). Based on the results from previous assays conducted in this study, a concentration of 84.5 mg/ml was used for this study as the effects at lower

concentrations (42.25 and 21.12 mg/ml) were insignificant. Changes in the expression of virulence genes of *C. albicans* treated with *L. rhamnosus* CFE were analysed by calculating the fold changes of treated and untreated cells. The expression of the virulence genes was shown as the relative fold change. Our results showed the downregulation of all the test genes upon treatment with the CFE. The test genes had high threshold values (Cq). There were significant changes which were observed in all virulence genes except *SAP3* from the susceptible *C. albicans* S7 **(Figures 3.9; 3.10; 3.11)**, where no significant difference between the control and the treated samples ($P > 0.05$) was observed. These results agree with the findings of Khan *et al* (2014) where they reported high Cq values on three genes namely *HWP1*, *SAP1* and *PLB2* after treating *C. albicans* cells with *Ocimum sanctum* essential oil.

4.6.1 *HWP1* gene

The Hwp1p protein is expressed on the surface of germ tubes and hyphae but not in the yeast morphology and plays an important role in filamentation and hence in virulence. Moreover, *HWP1* forms cross links between the *HWP1* and host cell surface proteins mediating stable attachment of hyphae to epithelial cells (Samaranayake *et al*., 2013). Due to importance of this gene, effect of *L. rhamnosus* CFE on expression of *HWP1* was evaluated in three *C. albicans* strains. In all strains, *L. rhamnosus* CFE downregulated the expression of *HWP1*. Downregulation of this gene corresponds well with the physiological changes observed in yeast to hyphae transition of *C. albicans*. A concentration of 84.5 mg/ml of *L. rhamnosus* CFE reduced the *HWP1* transcript with a fold change of 0.91 in *C. albicans* SC5314. These results are in line with the previous similar findings of Murzyn and co-workers where they investigated the effect of *S. boulardii* extract and its constituent capric acid on the expression of *HWP1* gene and observed an 8-fold down regulation in comparison to the untreated *C. albicans* SC5314 cells (Murzyn *et al*., 2010). Wang *et al* (2017a) investigated the effect of *L. crispatus* cell free supernatant on modulation of gene expression in *C. albicans* and reported downregulation of *HWP1* with a fold change of 0.075. Compared to the standard laboratory

strain, expression of *HWP1* gene in fluconazole susceptible *C. albicans* S7 and resistant *C. albicans* R3 was highly reduced with fold changes of 0.89 and 0.83respectively. These results encourage further studies of *L. rhamnosus* CFE on other genes in different *Candida* species.

4.6.2 *SAP* genes

Secreted aspartic proteinases play a key role in yeast colonisation. They have been reported to contribute to virulence by degrading the proteins for further penetration into host cells. The various *SAP* genes are differentially and selectively regulated in yeast and filamentous forms at different stages of infection leading to virulence in *C. albicans* strains (Samaranayake *et al*., 2013). Thus, downregulation of *SAP* genes by antimicrobial compounds may reduce virulence strategy in *C. albicans*. Therefore, in this study the effect of *L. rhamnosus* CFE on expression of *SAP1*, *SAP2* and *SAP3* was evaluated. It was observed that all the genes were down regulated in comparison to untreated control cells **(Figures 3.9; 3.10; 3.11)**. Significant downregulation of all the tested *SAP* genes was observed in all the tested strains of *C. albicans* except for *SAP3* in *C. albicans* S7, where the relative fold change t in the control and treated samples was not significant ($P > 0.05$). In addition, the effect of *L. rhamnosus* CFE on the expression of these genes vary from strain to strain, which could possibly be because *C. albicans* strains could have been in different stages of infections. These results are in line with the previous findings, where they reported differential expression of *SAP* genes in different *C. albicans* strains and related the difference in expression to the different stages of infection (Naglik *et al*., 2003). In the same study, they also reported that *SAP2* was the most dominant gene expressed in *C. albicans* followed by *SAP1*. Our results recorded that *SAP2* genes were highly downregulated by *L. rhamnosus* CFE with fold change of 0.83 in the resistant *C. albicans* R3 and susceptible *C. albicans* S7 strains respectively. The *SAP1* gene was also downregulated with fold changes of 0.86 and 0.87 in resistant and susceptible *C. albicans* strains, respectively. As *SAP1* and *SAP2* are the most highly expressed genes for proteinases, our results recorded both these genes as the most downregulated in the presence

of *L. rhamnosus* CFE. The other tested gene, *SAP3* was found to be the least expressed and hence its regulation with *L. rhamnosus* CFE could have resulted in very low fold change values which ranged between 0.93 and 0.96. Low expression of *SAP3* has also been previously reported and it was found that it was not one of the frequently detected members of the *SAP* gene family (Naglik *et al*., 2003). Downregulation of *SAP* genes by *L. rhamnosus* CFE were in reflection with the low activity of proteinase enzymes when treated with the CFE. These results are highly significant and can be utilised for further studies to test other CFEs against different proteinase producing fungal pathogens.

4.6.3 *PLB1* gene

Phospholipases are one of the proven virulence enzymes in *C. albicans*, which hydrolyse acyl ester bonds and catalyse lysophospholipase-transyclase reaction to produce fatty acids from phospholipids and lysophospholipids (Ghannoum, 2000). *C. albicans* produces different types of phospholipases A, B, C and D. However, of the four types, class B has been reported to contribute to pathogenicity in *C. albicans* (Pawar *et al*., 2014). The secretion of phospholipase is regulated by different genes of which the most dominant and virulent is *PLB1* which is highly expressed in yeast and hyphae forms of this pathogen. As significant effect of *L. rhamnosus* CFE was observed on phospholipase secretion, we evaluated the effect of this extract expression of mRNA transcripts of the *PLB1* gene in *C. albicans*. As expected, *L. rhamnosus* CFE reduced the expression of this gene in all three *C. albicans* strains **(Figures 3.9; 3.10; 3.11)**. There was a significant difference in relative mRNA expression between the untreated and treated cells with fold changes which ranged from 0.82 to 0.91. The results suggest that one possible mechanism of action of *L. rhamnosus* CFE is inactivation of the *PLB1* gene that regulates secretion of phospholipase B. Though there are no studies on inhibitory activity of probiotic CFEs on phospholipases and its regulatory genes, it was hard to support our findings with known literature. However, there are studies reporting the inhibitory effects of antimicrobials from plants and essential oils on

phospholipase and its genes. Khan *et al* (2014) showed that *Ocimum sanctum* essential oil downregulated the *PLB2* gene, which they correlated to contribute to tissue invasion as well.

4.6.4 Drug efflux genes

Drug efflux from the cells has been reported to be one of the components of resistance in *C. albicans* as overexpression of two types of efflux pumps have been associated with antifungal drug resistance. The ABC transporter genes *CDR1* and *CDR2* are ATP driven efflux pumps that are mostly overexpressed in azole resistant *C. albicans* strains whereas the major facilitator superfamily gene *MDR1* encodes an efflux pump that utilises a proton motive force at the membrane to transport drugs and other compounds across the cell membrane. Overexpression of this gene has been reported to be related to drug resistance (White *et al*., 2002). Therefore, inhibition of these drug efflux pump genes may confer sensitivity of the *C. albicans* strains to drugs. Several studies have already confirmed that the role of *CDR1* was more important than *CDR2* in regulation of transporter efflux pumps to confer resistance to drugs (Maesaki *et al*., 1999; Ahmad e*t al*., 2013). However, in this study we tested the effect of *L. rhamnosus* CFE on both *CDR1* as well as *CDR2* and found that the expression of *CDR2* (0.87 to 0.88 fold) was inhibited more than *CDR1* (0.89 to 0.92 fold). Though there are no studies on regulation of gene expression using probiotic CFEs, similar results were obtained by Shao *et al* (2016). In their study, they used kaempferol (an active flavonoid which exists in fruits and plants) alone and in synergy with fluconazole and reported that the inhibition of gene expression of *CDR2* (7.14-11.1 fold) was more than *CDR1* (2.78-8.2 fold). In addition, we also tested the effect of *L. rhamnosus* CFE on expression of *MDR1* gene and found that different strains have different effects. In *C. albicans* SC5314, the expression was downregulated whilst in *C. albicans* S7, the gene was upregulated in a negligible manner after treating the cells with *L. rhamnosus* CFE. In resistant *C. albicans* R3 there was no significant difference between the untreated control and treated cells. These inconclusive results are difficult to interpret; however, we assumed that the upregulation could be due to

oxidative damage induced by *L. rhamnosus* CFE and in response *MDR1* was over expressed as an oxidative defence system to protect the cells and not necessarily as a resistance mechanism. These results provide the basis to understand the role of different genes and the importance of *L. rhamnosus* CFE to regulate these genes and thereby controlling pathogenesis and drug resistance in *C. albicans*.

CHAPTER 5: CONCLUSION, LIMITATIONS TO THE STUDY AND FUTURE RESEARCH

5.1 Conclusion

In the quest of finding new options and strategies to treat *Candida* infections, especially caused by drug resistant *C. albicans*, we tested three different probiotic CFEs against a panel of FLC susceptible and resistant *C. albicans* isolates. All the tested probiotic CFEs showed varying levels of anticandidal activity with *L. rhamnosus* CFE being the most active CFE with the MIC of 84.5 mg/ml. *L. rhamnosus* CFE significantly inhibited virulence factors of C. *albicans*, which played an important role in its pathogenesis. Our findings also reported the reversal of drug efflux pumps when FLC resistant cells were treated at varying concentrations of *L. rhamnosus* CFE. The results also revealed that exposure of *L. rhamnosus* downregulated the important genes involved in the expression of virulence traits and efflux pumps. These results advocated the moderate anticandidal activity in *L. rhamnosus* CFE, which could inhibit the virulence traits and inhibit the drug efflux proteins. Therefore, *L. rhamnosus* CFE has a potential to be used for therapeutic purposes to treat drug resistant *Candida* infections. This study also laid a platform for further in-depth studies to unveil the effect of other probiotic strains against different fungal pathogens. To the best of our literature search, this is the first report on inhibitory activity of *L. rhamnosus* CFE on production of proteinases and phospholipases as well as on reversal of drug resistance in *C. albicans*.

5.2 Limitations of the study

1. This study was limited to a small number of probiotic strains and also tested against a small number of *C. albicans* isolates. For concrete evidences of the claims made, more probiotic strains should be tested against more *C. albicans* and non albicans *Candida* isolates.

2. During the preparation of probiotic CFEs, it is possible that some important constituents were either lost or not extracted properly, which could be reason for the moderate anti-candidal activity of these CFEs.
3. During solvent extractions, some extract was eluted which affected the final obtained yield.
4. The gas liquid chromatography coupled with mass spectrometry (GC-MS) technique to identify the active constituents of the CFEs of *L. rhamnosus*, *L. acidophilus* V and *B. animalis* subspecies *lactis* was not done in the study due to limited study time available to complete the degree.

5.3 Future research

1. Anticandidal activity of *L. rhamnosus* CFE has been revealed in pathogenicity of *C. albicans*. It will be ideal to use other *Candida* species as well as fungal pathogens to advocate the use of the CFE.
2. Drug efflux pumps are emerging drug targets. *L. rhamnosus* CFE has proved to inhibit these efflux pumps. Determining the effect of CFE from different probiotic microorganisms on the efflux pumps related to drug resistance in *C. albicans* and other fungal pathogens would be an important study.
3. The effect of *L. rhamnosus* CFE on other genes in different *Candida* species can also be investigated.
4. Investigating the effect of *L. rhamnosus* CFE on biofilm formation and adherence in *Candida* species that also contribute to pathogenicity would be a step forward.
5. In vivo studies should be preceded by safety studies in cultured mammalian cells to determine the toxicity profile of cell free extracts by incorporating the *L. rhamnosus* CFE orally in the form of capsules, in foods or beverages and to determine its efficacy in preventing *Candida* infections

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APPENDICES

Appendix 1: Composition and preparation of media and reagents

Sabouraud dextrose agar (4%) –Sigma Aldrich

65g	Sabouraud agar
1000ml	distilled water

65 g of Sabouraud agar was dissolved in 1000 ml of distilled water and sterilised by autoclaving at 121 °C for 15 min. After cooling to below 50 °C, the media was poured into petri dishes and allowed to set. The plates were refrigerated.

Sabouraud dextrose broth (2%) - Sigma Aldrich

30g	Sabouraud dextrose broth
1000 ml	distilled water

30 g of Sabouraud dextrose broth was dissolved in 1000 ml distilled water. It was sterilised by autoclaving at 121 °C for 15 min. The Sabouraud broth was refrigerated after cooling to below 50 °C.

de Man Rogosa and Sharpe (MRS) agar

65 g	de Man Rogosa and Sharpe agar (Biolab)
1000 ml	distilled water

65g of MRS agar was suspended in 1000 ml distilled water. The suspension was allowed to stand for 15 minutes and then sterilised by autoclaving at 121 °C for 15 min. The media was cooled to 45-50 °C and poured into petri dishes. The plates were allowed to set and refrigerated.

de Man Rogosa and Sharpe (MRS) broth

50g	de Man Rogosa and Sharpe broth (Biolab)
1000 ml	distilled water

50 g of MRS broth was suspended in 1000 ml of distilled water. The suspension was allowed to stand for 15 mins and autoclaved at 121 °C for 15 min.

0.85 % saline

0.85 g	sodium chloride (Merck)
1000 ml	distilled water

0.85 g was dissolved in 1000 ml of distilled water and autoclaved at 121 °C for 15 mins.

1x Phosphate buffered saline (PBS)

8 g	sodium chloride
0.20 g	potassium chloride
1.44 g	di-sodium hydrogen phosphate
0.24 g	di-hydrogen potassium phosphate

The solids were dissolved in 900 ml of distilled water. pH was adjusted to 7.5 and autoclaved at 121 °C for 15 min.

Proteinase agar

20 g	agar powder
1.45 g	ammonium sulphate
1.45 g	yeast nitrogen base without amino acids
20 g	glucose
10 g	bovine serum albumin (BSA) fraction V
0.22 µm	filter membranes
950 ml	distilled water

Agar powder; ammonium sulphate; yeast nitrogen base without amino acids and glucose were dissolved in 950 ml of distilled water and autoclaved at 121 °C for 15 min. 2 % BSA was prepared by dissolving 10 g in 50 ml autoclaved distilled water. BSA was filter sterilisedusing 0.22 µm filter membranes. After cooling to below 50 °C, 50 ml of sterile BSA was added into the media and mixed gently. The media was poured into petri dishes and allowed to set.

Egg yolk agar

20 g	Agar powder
57.3 g	sodium chloride
10 g	peptone powder
0.55 g	calcium chloride
100 ml	egg yolk emulsion
900 ml	distilled water

The solids were dissolved in 180 ml of distilled water and autoclaved at 121 °C for 15 min. After cooling to below 50 °C, 100 ml of egg yolk emulsion was added into the media and

mixed gently. The media was poured into petri dishes and allowed to set. The plates were then refrigerated.

Rhodamine 6 G dye (Sigma Aldrich)

2.4 g	rhodamine 6 G dye
500 ml	distilled water

2.4 g of rhodamine 6 G dye was dissolved in 500 ml of autoclaved distilled water. The suspension was stored in a dark place.

2, 4 Dinitrophenol (Sigma Aldrich)

0.92 g	2,4 dinitrophenol powder
500 ml	ethyl acetate

0.92 g of 2, 4 dinitrophenol was dissolved in 500 ml of ethyl acetate. The suspension was stored in a brown bottle and wrapped in aluminium foil to avoid exposure to light.

2 Deoxy D glucose (Sigma Aldrich)

0.82 g	2 deoxy d glucose
500 ml	distilled water.

0.82 g was dissolved in 500 ml autoclaved distilled water. The suspension was filter sterilised using 0.45 μm filter membrane. The suspension was then refrigerated.

10x TBE solution

54 g	Tris
27.5g	Boric acid
0.5 M	EDTA
500 ml	distilled water

The solids were suspended in 250 ml distilled water and 20 ml EDTA (pH-8.0) was added. The suspension was made to 500 ml mark with distilled water.

1x TBE solution

100 ml	10x TBE
900 ml	distilled water

100 ml of 10x TBE was diluted in 900 ml of 10x TBE solution.

2% agarose gel

3 g	Seakem ® LE agarose (White Science)
150 ml	1x TBE buffer
6 µl	ethidium bromide (Merck)

3 g of Seakem ® LE agarose was suspended in 150 ml of 1x TBE buffer. The suspension was heated in a microwave until the powder dissolved completely. After cooling to below 55 °C, 6 µl of ethidium bromide was added and gently mixed. The mixture was poured into the electrophoresis tank and allowed to set.

Appendix 2: Results of antifungal susceptibility assay

Table 2.1: The clinical *C. albicans* strains exposed to different probiotic cell free extracts

Resistant / Susceptible	*C. albicans* Strain	Repeats	Median MFC of cell free extract (mg/ml)		
			L. rhamnosus	*L. acidophilus* V	*B. animalis* subspecies *lactis*
Susceptible	SC5314	1	84.5	84.5	338
		2	84.5	84.5	169
		3	84.5	84.5	169
	Median		**84.5**	**84.5**	**169**
	S1	1	338	169	338
		2	84.5	84.5	169
		3	84.5	84.5	169
	Median		**84.5**	**84.5**	**169**
	S2	1	169	169	338
		2	84.5	169	169
		3	84.5	169	169
	Median		**84.5**	**169**	**169**
	S3	1	169	338	169
		2	84.5	169	84.5
		3	84.5	169	84.5
	Median		**84.5**	**169**	**84.5**
	S4	1	169	169	338
		2	84.5	84.5	169
		3	84.5	169	169
	Median		**84.5**	**169**	**169**
	S5	1	338	338	338
		2	169	169	169
		3	169	169	169
	Median		**169**	**169**	**169**
	S6	1	338	338	169
		2	169	169	169
		3	169	169	169
	Median		**169**	**169**	**169**
	S7	1	338	338	338
		2	84.5	338	169
		3	84.5	338	169
	Median		**84.5**	**338**	**169**
Resistant	R1	1	338	338	338
		2	84.5	169	169
		3	84.5	169	169
	Median		**84.5**	**169**	**169**
	R2	1	169	169	338
		2	169	84.5	84.5
		3	169	84.5	84.5
	Median		**169**	**84.5**	**84.5**
	R3	1	338	338	338
		2	84.5	169	169
		3	84.5	169	169
	Median		**84.5**	**169**	**169**

Appendix 3: Results of efflux pump proteins exposed to *L. rhamnosus* cell free extract in *C. albicans*

Table 3.1: Effect of *L. rhamnosus* CFE on drug efflux pumps in *C. albicans*

Concentration of CFE (mg/ml)	*C. albicans* strain	Mean OD_{527} at time intervals (min) n = 3												
		0	5	10	15	20	25	30	35	40	45	50	55	60
0 (control)	SC 5314	0.84	0.16	0.22	0.23	0.25	0.25	0.27	0.27	0.28	0.30	0.29	0.30	0.30
	S7	0.84	0.18	0.21	0.21	0.22	0.23	0.24	0.24	0.25	0.25	0.26	0.26	0.27
	R3	0.84	0.16	0.25	0.26	0.27	0.27	0.23	0.30	0.36	0.37	0.41	0.45	0.46
84.5	SC 5314	0.84	0.13	0.17	0.2	0.19	0.20	0.22	0.22	0.22	0.25	0.26	0.27	0.27
	S7	0.84	0.10	0.11	0.13	0.17	0.18	0.17	0.21	0.22	0.22	0.23	0.23	0.23
	R3	0.84	0.06	0.06	0.08	0.13	0.14	0.1	0.11	0.11	0.13	0.13	0.14	0.19
42.25	SC 5314	0.84	0.17	0.18	0.2	0.2	0.23	0.22	0.24	0.25	0.27	0.28	0.28	0.28
	S7	0.84	0.11	0.13	0.16	0.2	0.2	0.2	0.21	0.23	0.23	0.23	0.25	0.26
	R3	0.84	0.08	0.13	0.13	0.16	0.17	0.13	0.14	0.17	0.2	0.21	0.21	0.24
21.12	SC 5314	0.84	0.19	0.20	0.22	0.22	0.24	0.24	0.25	0.26	0.27	0.29	0.29	0.30
	S7	0.84	0.12	0.16	0.19	0.20	0.21	0.23	0.24	0.25	0.25	0.25	0.26	0.26
	R3	0.84	0.14	0.20	0.21	0.24	0.25	0.22	0.25	0.28	0.30	0.30	0.33	0.35

Appendix 4: Validation results in *C. albicans*

Table 4.1: Purity and Integrity of total RNA and cDNA from *C. albicans* strains (Figures 3.7 and 3.8)

***C. albicans* Strain**	**RNA Purity A260/280**	**RNA concentration (ng/µl)**	**DNA purity A260/280**	**cDNA concentration (ng/µl)**
SC5314 untreated	1.69	70.1	1.8	1139.5
SC5314 treated	1.51	99.4	1.83	1193.2
S7 untreated	2.08	46.6	1.84	1146.8
S7 treated	1.84	28.9	1.83	1222.3
R3 untreated	1.65	90.5	1.81	1211.7
R3 treated	1.58	27.8	1.81	1206

Figure 4.2 Results of cDNA amplicons in *C. albicans*

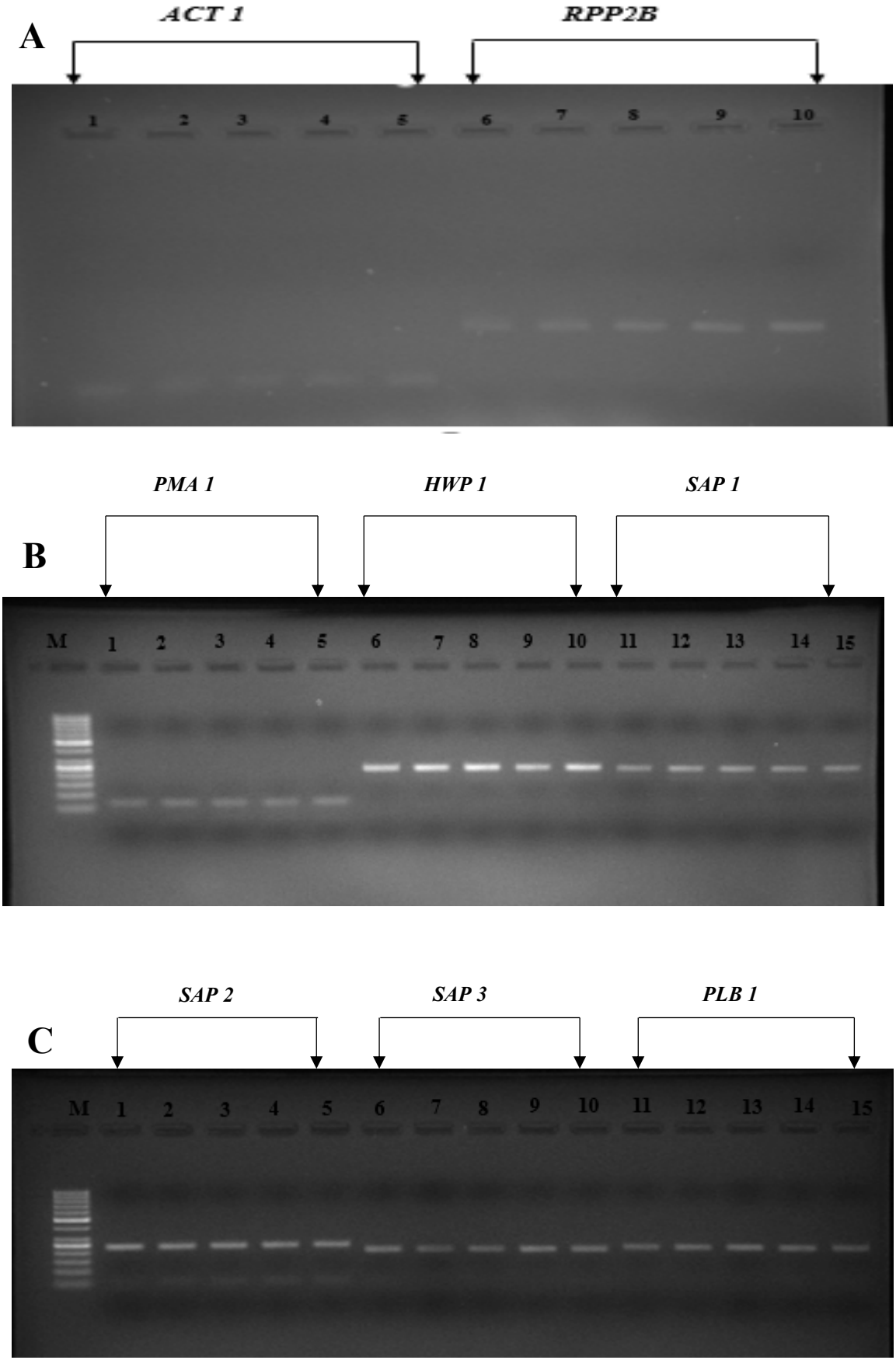

Table 5.1: Threshold values and relative ratios of virulence and drug resistant genes in *C. albicans* (Figures 3.9; 3.10; 3.12 and 3.13)

C. albicans	Target gene	Threshold values (Cq)			Ratio of target gene to *ACT* mRNA (Relativised) $2^{\Delta Cq}$			Mean ΔCq	SD
SC5314	*HWP 1*Control	31.8474	30.31765	32.60532	5.71942455	1.9547851	9.206482153	5.626897262	3.6267339
	HWP 1 Treated	28.32145	29.27293	28.26597	0.496518714	0.9475605	0.454798689	0.632959309	0.27325
	SAP 1 Control	33.47616	34.5564	33.41003	17.68715375	36.905399	16.08183357	23.55812892	11.58691
	SAP 1 Treated	30.49447	30.95601	30.5342	2.239132652	3.0427378	2.190909664	2.490926692	0.4784903
	SAP 2 Control	32.5073	32.6767	32.07563	9.036536962	10.028681	6.377362992	8.480860397	1.8880184
	SAP 2 Treated	29.40916	29.05606	29.58785	1.05528304	0.8153097	1.136958819	1.00251717	0.1671907
	SAP 3 Control	33.09602	31.5688	33.60085	13.59015495	4.6529962	18.35600246	12.19971786	6.9565136
	SAP 3 Treated	29.20608	29.06388	28.83695	0.916718059	0.819741	1.480126005	1.07219501	0.3565907
	PLB 1 Control	34.73133	35.53231	35.21417	42.21840003	72.588545	56.16112771	56.98935769	15.202003
	PLB 1 Treated	28.42772	28.81961	29.09144	0.53447338	0.6920605	0.805954333	0.67749606	0.1363252
	*CDR 1*Control	38.24361	39.01384	40.24779	481.7296791	810.79872	1839.528101	1044.018833	708.30633
	CDR 1 Treated	34.21703	35.21394	35.26926	29.55847636	58.214232	58.34713854	48.70661569	16.582908
	CDR 2 Control	39.02419	37.65239	38.32977	827.5255237	315.55551	410.6313599	517.9041309	272.32134
	CDR 2 Treated	33.32133	33.43392	33.08457	15.88727733	16.950783	12.83400905	15.22402308	2.1370274

	*MDR 1*Control	39.55679	33.43834	32.16594	1197.043612	17.002795	6.789333717	406.9452469	684.26431
	*MDR 1*Treated	34.6929	34.04766	33.51679	41.10864744	25.938443	17.31703464	28.12137517	12.045086
	Target gene	**Threshold values (Cq)**			**Ratio of target gene to *ACT* mRNA (Relativised**			**Mean ΔCq**	**SD**
S7	*HWP 1*Control	41.18455	40.33337	40.10731	1361.8763	1094.8064	999.1379	1151.9402	187.99735
	HWP 1 Treated	36.92789	36.04231	35.53295	71.245155	55.924169	41.937821	56.369048	14.658731
	SAP 1 Control	40.84829	40.63544	40.70344	1078.3277	1349.8001	1510.3529	1312.8269	218.37286
	SAP 1 Treated	35.81881	34.65543	35.81781	33.01615	21.384892	51.092513	35.164518	14.96988
	SAP 2 Control	41.14206	40.31541	41.29033	1322.3515	1081.2617	2268.5583	1557.3905	627.57533
	SAP 2 Treated	33.47155	34.0292	34.29988	6.4907267	13.85457	17.84082	12.728705	5.7581969
	SAP 3 Control	41.45732	41.34742	41.47117	1645.3172	2211.0409	2571.5099	2142.6227	466.87155
	SAP 3 Treated	41.1388	40.75149	37.75149	1319.3668	1462.8641	195.18793	992.47293	694.18686
	PLB 1 Control	41.40328	41.0546	41.35231	1584.8274	1804.8835	2368.1422	1919.2844	403.99405
	PLB 1 Treated	35.74586	40.25005	36.65787	31.399942	1033.3691	91.461964	385.41035	561.95179
	*CDR 1*Control	40.79534	40.50132	40.68414	1039.8574	1229.9722	1490.2824	1253.3707	226.12226
	CDR 1 Treated	37.42897	36.54213	37.96734	100.83132	79.078851	226.68891	135.53303	79.689013
	CDR 2 Control	41.32002	40.30521	39.82758	1495.9539	1073.644	823.03502	1130.8776	340.09076

	CDR 2 Treated	36.3819	35.34682	35.14744	48.79732	34.533149	32.103752	38.478074	9.0189037
	*MDR 1*Control	39.87456	40.60596	38.09897	549.27697	1322.4982	248.34467	706.7066	554.11145
	*MDR 1*Treated	39.71761	40.02855	40.02856	492.65709	886.29306	946.06139	775.00385	246.33886

Table 5.2: Threshold values and relative ratios of virulence and drug resistance genes in *C. albicans* R3 (Figures 3.11 and 3.14)

C. albicans	Target gene	Threshold values (Cq)			Ratio of target gene to *ACT* mRNA (Relativised)			Mean ΔCq	SD
R3	*HWP 1*Control	36.89297	35.82813	36.0328	16.934224	6.5947136	12.52629625	12.01841138	5.1884324
	HWP1 Treated	30.53033	29.34675	30.21888	0.2057879	0.0738085	0.222667051	0.167421134	0.0815091
	SAP 1 Control	41.12184	41.12953	40.97766	317.528	260.06168	385.8082009	321.1326251	62.950712
	SAP 1 Treated	36.71833	34.34521	35.47431	15.003529	2.3593509	8.505464344	8.622781504	6.3229055
	SAP 2 Control	40.61061	40.34557	40.3365	321.68392	151.03614	247.3791329	240.0330638	85.560735
	SAP 2 Treated	33.90845	32.5969	33.64829	3.0894285	0.7022614	2.39889498	2.063528297	1.2284115
	SAP 3 Control	35.31491	35.42926	35.34045	8.1896398	5.0017744	7.751792751	6.981068998	1.728043
	SAP 3 Treated	33.09896	32.68092	32.43692	1.762777	0.7443742	1.035982764	1.181044671	0.5244695
	PLB 1 Control	37.57421	38.48594	37.17223	39.208648	41.617546	27.59452219	36.14023888	7.4981766
	PLB 1 Treated	34.05285	34.80407	33.62369	3.4146554	3.2428224	2.358337179	3.005271656	0.5668111
	*CDR 1*Control	41.17767	40.99802	41.07997	476.57518	237.40393	414.1616039	376.0469034	124.05752
	CDR 1 Treated	37.64952	38.8332	37.50613	41.309738	52.943439	34.78057792	43.01125154	9.2002033
	CDR 2 Control	35.77519	35.50158	36.52948	11.267379	5.2588961	17.67404062	11.40010511	6.2086364
	CDR 2 Treated	32.3987	31.10694	30.4245	1.084921	0.2500208	0.256775596	0.530572465	0.4800918
	*MDR 1*Control	39.81244	37.16402	35.4772	184.9934	16.647112	8.522519528	70.05434421	99.623001
	*MDR 1*Treated	37.8353	37.16384	33.59372	47.084816	16.645035	2.309851334	22.01323426	22.865095

Table 5.3: Fold change values of virulence and drug resistant genes in *C. albicans* (Figures 3.9; 3.10; 3.11; 3.12; 3.13 and 3.14)

C. albicans	**Target gene (n=3)**	**Mean fold changes, n=3**	**SD**
SC5314	*HWP 1* Control	1	0
	HWP1 Treated	0.9072466	0.0517089
	SAP 1 Control	1	0
	SAP 1 Treated	0.9068884	0.0097091
	SAP 2 Control	1	0
	SAP 2 Treated	0.9054441	0.0166337
	SAP 3 Control	1	0
	SAP 3 Treated	0.8871126	0.031474
	PLB 1 Control	1	0
	PLB 1 Treated	0.8185714	0.0075237
	CDR 1 Control	1	0
	CDR 1 Treated	0.8912055	0.0134953
	CDR 2 Control	1	0
	CDR 2 Treated	0.8683274	0.0176281
	MDR 1 Control	1	0
	MDR 1 Treated	0.9790863	0.0891702
S7	***HWP 1* Control**	1	0
	HWP1 Treated	0.892067135	0.005513
	SAP 1 Control	1	0
	SAP 1 Treated	0.869893943	0.014852
	SAP 2 Control	1	0
	SAP 2 Treated	0.829444905	0.015296
	SAP 3 Control	1	0
	SAP 3 Treated	0.962737005	0.04553
	PLB 1 Control	1	0
	PLB 1 Treated	0.910079351	0.061989
	CDR 1 Control	1	0
	CDR 1 Treated	0.917649683	0.015489
	CDR 2 Control	1	0
	CDR 2 Treated	0.879986589	0.00279
	MDR 1 Control	1	0
	MDR 1 Treated	1.010830266	0.034863
R3	*HWP 1* Control	1	0
	HWP1 Treated	0.828428372	0.009806
	SAP 1 Control	1	0
	SAP 1 Treated	0.864554739	0.02895
	SAP 2 Control	1	0
	SAP 2 Treated	0.825699136	0.015383
	SAP 3 Control	1	0
	SAP 3 Treated	0.925840094	0.010145
	PLB 1 Control	1	0
	PLB 1 Treated	0.905050827	0.001072
	CDR 1 Control	1	0
	CDR 1 Treated	0.924839558	0.019373
	CDR 2 Control	1	0
	CDR 2 Treated	0.871569106	0.036594
	MDR 1 Control	1	0
	MDR 1 Treated	0.965747974	0.029708

Mean fold change for control = untreated / untreated =1
Mean fold change for treated = treated/untreated

Appendix 6: Publication Abstract

The increase in multi drug resistant *Candida* species has caused candidiasis, a systemic fungal infection to continue to be life threatening and difficult to eradicate hence resulting in clinical failure. Moreover, existing antifungal drugs are toxic, inefficient and their misappropriation develops drug resistance. Probiotic strains as whole cells have been used in treatment of candidiasis, which leads to high risk of probiotic sepsis. Thus, new and safer methods for combating fungal infections are of high medical priority. One strategy is to target the virulence traits and inhibit the drug efflux pumps using probiotic cell free extracts (CFEs). CFEs prepared from *Lactobacillus acidophilus* V, *Lactobacillus rhamnosus* and *Bifidobacterium animalis* subspecies *lactis* were screened against seven fluconazole susceptible and three resistant clinical *Candida albicans* strains using the antimicrobial assay. *L. rhamnosus* was selected as the most effective CFE and its biological activity was further evaluated on *C. albicans* virulence traits and reversal of antifungal drug resistance. The study demonstrated that *L. rhamnosus* CFE significantly inhibited formation of hyphae thereby subsequently reducing the secretion of secreted aspartic proteinases (SAPs) and phospholipases. Moreover, *L. rhamnosus* inhibited the drug efflux pump proteins in resistant clinical *C. albicans* strain thus reducing resistance in the *C. albicans* strains. The expression profile of selected genes associated with *C. albicans* virulence and antifungal drug resistance by real time quantitative polymerase chain reaction (PCR) showed a reduced expression of *HWP1*, *SAP1*, *SAP2*, *PLB1*, *CDR1*, *CDR2* in *C. albicans* cells treated with *L. rhamnosus* CFE. However, there was no significant difference between the control and treated samples in expression of *SAP3* in S7 and R3 and *MDR1* in all strains ($p > 0.05$). Therefore, this aligns probiotic CFEs especially *L. rhamnosus* CFE as one of the potentially potent natural products against *Candida albicans* virulence traits and antifungal drug resistance.

Appendix 7: Ethics

7.1: Ethical clearance

Human Research Ethics Committee (Medical) 50 years 1966 – 2016

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
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Website: www.wits.ac.za/research/about-our-research/ethics-and-research-integrity/

Ref: W-CJ-170517-1 17/05/2017

TO WHOM IT MAY CONCERN:

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

Investigators: Y Dube (student no 1156158), Dr A Ahmad.

Project title: Effect of probiotic cell free extracts on antifungal drug resistance and virulence expression in *Candida albicans*.

Reason: This is an *in vitro* laboratory study using the following stock cultures: *Candida albicans* (isolated in study M000402) and commercial bacterial and fungal stock cultures from the American Type Culture Center *Candida albicans*, *Lactobacillus acidophilus*, *Bifidobacterium longum* and *Saccharomyces boulardii*. There are no human participants.

Professor Peter Cleaton-Jones

Chair: Human Research Ethics Committee (Medical)

UNIVERSITY OF THE WITWATERSRAND
PROF PE CLEATON-JONES
HREC (MEDICAL)
2017 -05- 17
JOHANNESBURG

Copy – HREC (Medical) Secretariat: Zanele Ndlovu, Rhulani Mkansi, Lebo Moeng.

7.2: Ethical clearance with amendment

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG	HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

7 December 2018

Ms Y Dube
School of Pathology
Dept of Clinical Microbiology & Infectious Diseases
SAIMR

Sent by e-mail to: 1156158@students.wits.ac.za

Dear Ms Dube

Re: **Protocol Ref No:** W-CJ-170717-1
Protocol Title: *Effect of probiotic cell-free extracts on antifungal drug resistance and virulence expression in Candida albicans*
Principal Investigator: Ms Y Dube

Thank you for your letter of 20 November 2018.

I confirm that the proposed amendment to your study has been noted and approved. The ethics waiver of 17 May 2017 remains valid.

For the record, the amendment is:

- the replacement of cultures of *Bifidobacterium longum* and *Saccharomyces boulardii by Bifidobacterium animalis* and *Lactobacillus rhamnosus*

Thank you for keeping us informed.

Yours Sincerely

I. Burns

.....
Mr I Burns
For the Human Research Ethics Committee (Medical)

cc Dr A Ahmad

Appendix 8: Statistics

8.1 Statistics for reference genes

Compare control with CFE at concentration A. Does CFE have any effect on expression of ACT 1 gene?

```
. ttest ACT1, by(Concentration1)

Two-sample t test with equal variances
```

Variable	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
A	3	29.43507	.0925066	.1602261	29.03705	29.83309
Control	3	29.71799	.349118	.6046901	28.21585	31.22012
combined	6	29.57653	.1734654	.4249018	29.13062	30.02244
diff		-.2829167	.361166		-1.285674	.7198408

```
    diff = mean(A) - mean(Control)                                t =  -0.7833
Ho: diff = 0                                     degrees of freedom =        4

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.2386         Pr(|T| > |t|) = 0.4772          Pr(T > t) = 0.7614
```

No significant difference.

Compare control with CFE at concentration A. Does CFE have any effect on expression of PMA 1 gene?

```
. ttest PMA1, by(Concentration2)

Two-sample t test with equal variances
```

Variable	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
A	3	28.20377	.0996178	.1725431	27.77515	28.63239
Control	3	33.37432	2.811138	4.869033	21.27897	45.46967
combined	6	30.78904	1.70857	4.185125	26.39702	35.18106
diff		-5.170547	2.812902		-12.98042	2.639322

```
    diff = mean(A) - mean(Control)                                t =  -1.8382
Ho: diff = 0                                     degrees of freedom =        4

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0699         Pr(|T| > |t|) = 0.1399          Pr(T > t) = 0.9301
```

Not significant.

Compare control with CFE at concentration A. Does CFE affect expression of RPP2B gene?

```
. ttest RPP2B, by(Concentration3)

Two-sample t test with equal variances
```

Variable	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
A	3	27.9896	.0780377	.1351653	27.65383	28.32537
Control	3	29.29717	.1657704	.2871227	28.58392	30.01043
combined	6	28.64339	.303646	.7437778	27.86284	29.42394
diff		-1.30757	.1832204		-1.816271	-.7988687

```
    diff = mean(A) - mean(Control)                                t =  -7.1366
Ho: diff = 0                                     degrees of freedom =        4

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0010         Pr(|T| > |t|) = 0.0020          Pr(T > t) = 0.9990
```

Significant difference in expression of RPP2B gene by CFE.

8.2: Statistics for effect of *L. rhamnosus* CFE on yeast to hyphae transition

Compare control to CFE overall difference at concentrations A, B, C. which concentration of CFE affect length of hyphae?

concentration	Summary of hyphae length Mean	Std. Dev.	Freq.
A	70.3735	22.290096	6
B	127.40617	42.211732	6
C	222.72317	27.840748	6
Control	256.54383	49.889645	6
Total	169.26167	83.342191	24

Analysis of Variance

Source	SS	df	MS	F	Prob > F
Between groups	132042.366	3	44014.122	31.76	0.0000
Within groups	27713.813	20	1385.69065		
Total	159756.179	23	6945.92082		

```
Bartlett's test for equal variances:  chi2(3) =   3.5516  Prob>chi2 = 0.314
```

Comparison of hyphae length by concentration (Bonferroni)

Row Mean-Col Mean	A	B	C
B	57.0327 0.091		
C	152.35 0.000	95.317 0.002	
Control	186.17 0.000	129.138 0.000	33.8207 0.788

Significant reduction in length of hyphae by CFE at concentrations A and B.

8.3: Statistics for effect of *L. rhamnosus* CFE on production of proteinases in *C. albicans*

Compare control to CFE at concentrations A, B or C. which concentration affects production of proteinases?

Concetration	Summary of Proteinaise production		
	Mean	Std. Dev.	Freq.
A	.54424242	.07232869	33
B	.45393939	.07466339	33
C	.41272727	.05580506	33
control	.39333333	.07347052	33
Total	.45106061	.09009967	132

Analysis of Variance

Source	SS	df	MS	F	Prob > F
Between groups	.445269697	3	.148423232	30.73	0.0000
Within groups	.618181818	128	.004829545		
Total	1.06345152	131	.00811795		

Bartlett's test for equal variances: chi2(3) = 3.2888 Prob>chi2 = 0.349

Comparison of Proteinaise production by Concetration
(Bonferroni)

Row Mean-Col Mean	A	B	C
B	-.090303 0.000		
C	-.131515 0.000	-.041212 0.105	
control	-.150909 0.000	-.060606 0.003	-.019394 1.000

Significant difference in proteinase activity between control and treated at concentrations A = 84.5 mg/ml and B = 42.25 mg/ml

Compare control and CFE overall at concentrations A+B+C. Does CFE have any effect on production of proteinases?

Two-sample t test with equal variances

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
A	99	.470303	.0087575	.0871365	.452924	.4876821
control	33	.3933333	.0127896	.0734705	.3672818	.4193848
combined	132	.4510606	.0078422	.0900997	.4355469	.4665743
diff		.0769697	.0168804		.0435738	.1103656

diff = mean(A) - mean(control) t = 4.5597
Ho: diff = 0 degrees of freedom = 130

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

Mean difference in proteinase activity between control and CFE is 0.0769. CFE gave more activity because the mean is higher (0.47).

P=0.000 highly significant difference, CFE effective.

8.4: Statistics for effect of *L. rhamnosus* CFE production of phospholipases in *C. albicans*

Compare control to CFE at concentrations A, B or C. which concentration affects production of phospholipases?

Concentration	Summary of Phospholipase		
	Mean	Std. Dev.	Freq.
A	.79818182	.11234585	33
B	.71272727	.13967088	33
C	.66242424	.13968783	33
Control	.66545455	.14677983	33
Total	.70969697	.14459704	132

Analysis of Variance

Source	SS	df	MS	F	Prob > F
Between groups	.397018182	3	.132339394	7.23	0.0002
Within groups	2.3419697	128	.018296638		
Total	2.73898788	131	.020908304		

Bartlett's test for equal variances: chi2(3) = 2.5101 Prob>chi2 = 0.473

Comparison of Phospholipase by Concentration
(Bonferroni)

Row Mean-Col Mean	A	B	C
B	-.085455 0.069		
C	-.135758 0.000	-.050303 0.800	
Control	-.132727 0.001	-.047273 0.949	.00303 1.000

significant difference in phospholipase activity between control and treated at concentration A = 84.5 mg/ml

Compare control and CFE overall at concentrations A+B+C. Does CFE have any effect on production of phospholipases?

Two-sample t test with equal variances

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
A	99	.7244444	.0142259	.1415456	.6962137	.7526752
Control	33	.6654545	.0255511	.1467798	.6134087	.7175004
combined	132	.709697	.0125856	.144597	.6847997	.7345942
diff		.0589899	.0287143		.0021821	.1157977

diff = mean(A) - mean(Control) t = 2.0544
Ho: diff = 0 degrees of freedom = 130

Ha: diff < 0 Pr(T < t) = 0.9790
Ha: diff != 0 Pr(|T| > |t|) = 0.0419
Ha: diff > 0 Pr(T > t) = 0.0210

Mean difference in phospholipase activity between control and CFE is 0.059. CFE gave more activity because the mean is higher (0.72. P=0.04 significant difference, CFE effective

8.5 Statistics for effect of *L. rhamnosus* CFE on gene expression in *C. albicans*

Compare control with CFE at concentration A. Does CFE have any effect on expression of *HWP1, SAP1,SAP2 and SAP3* genes in *C. albicans* SC5314?

```
. ttest HWP1, by(concentration1)

Two-sample t test with equal variances

   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       A |       3    .9072466    .0298541    .0517089    .7787946    1.035699
 control |       3           1           0           0           1           1
---------+--------------------------------------------------------------------
combined |       6    .9536233     .024666    .0604192    .8902172    1.017029
---------+--------------------------------------------------------------------
    diff |           -.0927534    .0298541               -.1756418   -.0098651
------------------------------------------------------------------------------
    diff = mean(A) - mean(control)                                t =  -3.1069
Ho: diff = 0                                     degrees of freedom =        4

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0180         Pr(|T| > |t|) = 0.0360          Pr(T > t) = 0.9820

. ttest SAP1, by(concentration2)

Two-sample t test with equal variances

   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       A |       3    .9068884    .0056056    .0097091    .8827696    .9310072
 control |       3           1           0           0           1           1
---------+--------------------------------------------------------------------
combined |       6    .9534442    .0209708    .0513677    .8995372    1.007351
---------+--------------------------------------------------------------------
    diff |           -.0931116    .0056056               -.1086751   -.077548
------------------------------------------------------------------------------
    diff = mean(A) - mean(control)                                t = -16.6105
Ho: diff = 0                                     degrees of freedom =        4

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0000         Pr(|T| > |t|) = 0.0001          Pr(T > t) = 1.0000

. ttest SAP2, by(concentration3)

Two-sample t test with equal variances

   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       A |       3    .9054441    .0096035    .0166337    .8641236    .9467646
 control |       3           1           0           0           1           1
---------+--------------------------------------------------------------------
combined |       6    .9527221    .0215751    .0528481    .8972614    1.008183
---------+--------------------------------------------------------------------
    diff |           -.0945559    .0096035               -.1212194   -.0678923
------------------------------------------------------------------------------
    diff = mean(A) - mean(control)                                t =  -9.8460
Ho: diff = 0                                     degrees of freedom =        4

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0003         Pr(|T| > |t|) = 0.0006          Pr(T > t) = 0.9997

. ttest SAP3, by(concentration4)

Two-sample t test with equal variances

   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       A |       3    .8871126    .0181715     .031474    .8089269    .9652984
 control |       3           1           0           0           1           1
---------+--------------------------------------------------------------------
combined |       6    .9435563    .0265183    .0649562    .8753889    1.011724
---------+--------------------------------------------------------------------
    diff |           -.1128874    .0181715               -.1633396   -.0624351
------------------------------------------------------------------------------
    diff = mean(A) - mean(control)                                t =  -6.2123
Ho: diff = 0                                     degrees of freedom =        4

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0017         Pr(|T| > |t|) = 0.0034          Pr(T > t) = 0.9983

. ttest PLB1, by(concentration5)

Two-sample t test with equal variances

   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       A |       3    .8185714    .0043438    .0075237    .7998814    .8372614
 control |       3           1           0           0           1           1
---------+--------------------------------------------------------------------
combined |       6    .9092857    .0406151    .0994864    .8048811     1.01369
---------+--------------------------------------------------------------------
    diff |           -.1814286    .0043438               -.193489   -.1693682
------------------------------------------------------------------------------
    diff = mean(A) - mean(control)                                t = -41.7670
Ho: diff = 0                                     degrees of freedom =        4

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

Significant difference in expression, CFE effective.

Compare controlwith CFE concentration A. Does CFE have effect on expression of virulence genes in *C. albicans* S7?

```
. ttest HWP1, by(concentration1)

Two-sample t test with equal variances
```

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
A	3	.8920671	.003183	.005513	.878372	.9057623
control	3	1	0	0	1	1
combined	6	.9460336	.0241765	.05922	.883886	1.008181
diff		-.1079329	.003183		-.1167702	-.0990956

```
    diff = mean(A) - mean(control)                                t = -33.9096
Ho: diff = 0                                     degrees of freedom =        4

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

. ttest SAP1, by(concentration2)

Two-sample t test with equal variances
```

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
A	3	.8698939	.0085749	.0148521	.8329992	.9067887
control	3	1	0	0	1	1
combined	6	.934947	.0293443	.0718784	.8595152	1.010379
diff		-.1301061	.0085749		-.1539138	-.1062984

```
    diff = mean(A) - mean(control)                                t = -15.1729
Ho: diff = 0                                     degrees of freedom =        4

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0001         Pr(|T| > |t|) = 0.0001          Pr(T > t) = 0.9999

. ttest SAP2, by(concentration3)

Two-sample t test with equal variances
```

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
A	3	.8294449	.0088309	.0152956	.7914485	.8674413
control	3	1	0	0	1	1
combined	6	.9147225	.0383412	.0939164	.8161632	1.013282
diff		-.1705551	.0088309		-.1950737	-.1460365

```
    diff = mean(A) - mean(control)                                t = -19.3134
Ho: diff = 0                                     degrees of freedom =        4

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

. ttest SAP3, by(concentration4)

Two-sample t test with equal variances
```

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
A	3	.962737	.026287	.0455304	.8496333	1.075841
control	3	1	0	0	1	1
combined	6	.9813685	.0144093	.0352954	.9443283	1.018409
diff		-.037263	.026287		-.1102473	.0357213

```
    diff = mean(A) - mean(control)                                t =  -1.4175
Ho: diff = 0                                     degrees of freedom =        4

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.1146         Pr(|T| > |t|) = 0.2293          Pr(T > t) = 0.8854
```

Significant reduction in expression of *HWP1*,*SAP1*,*SAP1* and *PLB1*. Negligible reduction in *SAP3* expression by CFE but no significant difference.

Compare control with CFE at concentration A. Does CFE have any effect on expression of virulence genes in *C. albicans* R3?

```
. ttest HWP1, by(concentration1)

Two-sample t test with equal variances

   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       A |       3    .8284284    .0056615    .009806    .804069    .8527878
 control |       3           1           0          0          1           1
---------+--------------------------------------------------------------------
combined |       6    .9142142     .038448   .0941781   .8153804    1.013048
---------+--------------------------------------------------------------------
    diff |           -.1715716    .0056615               -.1872904   -.1558528
------------------------------------------------------------------------------
    diff = mean(A) - mean(control)                                t = -30.3050
Ho: diff = 0                                     degrees of freedom =        4

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

. ttest SAP1, by(concentration2)

Two-sample t test with equal variances

   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       A |       3    .8645547    .0167142   .0289498   .7926395     .93647
 control |       3           1           0          0          1           1
---------+--------------------------------------------------------------------
combined |       6    .9322774    .0311952   .0764124   .8520874    1.012467
---------+--------------------------------------------------------------------
    diff |           -.1354453    .0167142               -.1818512   -.0890393
------------------------------------------------------------------------------
    diff = mean(A) - mean(control)                                t =  -8.1036
Ho: diff = 0                                     degrees of freedom =        4

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0006         Pr(|T| > |t|) = 0.0013          Pr(T > t) = 0.9994

. ttest SAP2, by(concentration3)

Two-sample t test with equal variances

   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       A |       3    .8256991    .0088811   .0153826   .7874866   .8639116
 control |       3           1           0          0          1           1
---------+--------------------------------------------------------------------
combined |       6    .9128496    .0391767   .0959629   .8121426   1.013557
---------+--------------------------------------------------------------------
    diff |           -.1743009    .0088811               -.1989589   -.1496428
------------------------------------------------------------------------------
    diff = mean(A) - mean(control)                                t = -19.6259
Ho: diff = 0                                     degrees of freedom =        4

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

. ttest SAP3, by(concentration4)

Two-sample t test with equal variances

   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       A |       3    .9258401    .0058574   .0101453   .9006378   .9510424
 control |       3           1           0          0          1           1
---------+--------------------------------------------------------------------
combined |       6      .96292    .0167883   .0411227   .9197644   1.006076
---------+--------------------------------------------------------------------
    diff |           -.0741599    .0058574               -.0904226   -.0578972
------------------------------------------------------------------------------
    diff = mean(A) - mean(control)                                t = -12.6609
Ho: diff = 0                                     degrees of freedom =        4

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0001         Pr(|T| > |t|) = 0.0002          Pr(T > t) = 0.9999

. ttest PLB1, by(concentration5)

Two-sample t test with equal variances

   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       A |       3    .9050508    .0006187   .0010716   .9023887   .9077129
 control |       3           1           0          0          1           1
---------+--------------------------------------------------------------------
combined |       6    .9525254    .0212331   .0520102    .897944   1.007107
---------+--------------------------------------------------------------------
    diff |           -.0949492    .0006187               -.096667   -.0932314
------------------------------------------------------------------------------
    diff = mean(A) - mean(control)                                t = -1.5e+02
Ho: diff = 0                                     degrees of freedom =        4

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

.
```

Reduction in expression of genes significant

Compare control with CFE at concentration A. Does CFE have affect expression of drug resistance genes in *C. albicans* SC5314?

```
. ttest CDR1, by(concentration1)

Two-sample t test with equal variances

   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       A |       3    .8912055    .0077915    .0134953    .8576814    .9247296
 control |       3           1           0           0           1           1
---------+--------------------------------------------------------------------
combined |       6    .9456028    .0245755    .0601973    .8824295    1.008776
---------+--------------------------------------------------------------------
    diff |           -.1087945    .0077915               -.1304271   -.0871618
------------------------------------------------------------------------------
    diff = mean(A) - mean(control)                                t = -13.9632
Ho: diff = 0                                     degrees of freedom =        4

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0001         Pr(|T| > |t|) = 0.0002          Pr(T > t) = 0.9999

. ttest CDR2, by(concentration2)

Two-sample t test with equal variances

   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       A |       3    .8683274    .0101776    .0176281    .8245369    .9121179
 control |       3           1           0           0           1           1
---------+--------------------------------------------------------------------
combined |       6    .9341637    .0297926    .0729767    .8575793    1.010748
---------+--------------------------------------------------------------------
    diff |           -.1316726    .0101776               -.15993   -.1034152
------------------------------------------------------------------------------
    diff = mean(A) - mean(control)                                t = -12.9375
Ho: diff = 0                                     degrees of freedom =        4

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0001         Pr(|T| > |t|) = 0.0002          Pr(T > t) = 0.9999

. ttest MDR1, by(concentration3)

Two-sample t test with equal variances

   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       A |       3    .9790863    .0514825    .0891702    .7575751    1.200597
 control |       3           1           0           0           1           1
---------+--------------------------------------------------------------------
combined |       6    .9895431    .0234938    .0575478    .9291504    1.049936
---------+--------------------------------------------------------------------
    diff |           -.0209137    .0514825               -.163852    .1220245
------------------------------------------------------------------------------
    diff = mean(A) - mean(control)                                t =  -0.4062
Ho: diff = 0                                     degrees of freedom =        4

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.3527         Pr(|T| > |t|) = 0.7054          Pr(T > t) = 0.6473
```

Significant reduction of *CDR1* and *CDR2* genes. Negligible reduction but no significant difference in *MDR1*

Compare control with CFE at concentration A. Does CFE have any effect on expression of drug resistant genes in *C. albicans* S7?

```
. ttest CDR1, by(concentration1)

Two-sample t test with equal variances
```

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
A	3	.9176497	.0089426	.015489	.8791728	.9561266
control	3	1	0	0	1	1
combined	6	.9588248	.0188434	.0461567	.9103864	1.007263
diff		-.0823503	.0089426		-.107179	-.0575217

```
    diff = mean(A) - mean(control)                                t =  -9.2088
Ho: diff = 0                                     degrees of freedom =       4

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0004         Pr(|T| > |t|) = 0.0008          Pr(T > t) = 0.9996

. ttest CDR2, by(concentration2)

Two-sample t test with equal variances
```

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
A	3	.8799866	.0016108	.0027899	.8730561	.8869171
control	3	1	0	0	1	1
combined	6	.9399933	.0268455	.0657577	.8709848	1.009002
diff		-.1200134	.0016108		-.1244856	-.1155412

```
    diff = mean(A) - mean(control)                                t = -74.5075
Ho: diff = 0                                     degrees of freedom =       4

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

. ttest MDR1, by(concentration3)

Two-sample t test with equal variances
```

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
A	3	1.01083	.0201284	.0348634	.9242248	1.097436
control	3	1	0	0	1	1
combined	6	1.005415	.0093218	.0228335	.9814528	1.029377
diff		.0108303	.0201284		-.0450551	.0667156

```
    diff = mean(A) - mean(control)                                t =   0.5381
Ho: diff = 0                                     degrees of freedom =       4

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.6905         Pr(|T| > |t|) = 0.6191          Pr(T > t) = 0.3095

.
```

Significant reduction in expression of CDR1 and CDR2 by CFE. No significant difference in MDR1, CFE has no effect

Compare control with CFE at concentration A. Does CFE have any effect on expression of drug resistance genes in *C. albicans* R3?

```
. ttest CDR1, by(concentration1)

Two-sample t test with equal variances
```

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
A	3	.9248396	.0111852	.0193733	.8767137	.9729654
control	3	1	0	0	1	1
combined	6	.9624198	.017535	.0429518	.9173446	1.007495
diff		-.0751604	.0111852		-.1062154	-.0441055

```
    diff = mean(A) - mean(control)                                t =  -6.7197
Ho: diff = 0                                     degrees of freedom =        4

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0013         Pr(|T| > |t|) = 0.0026          Pr(T > t) = 0.9987

. ttest CDR2, by(concentration2)

Two-sample t test with equal variances
```

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
A	3	.8715691	.0211273	.0365936	.7806655	.9624728
control	3	1	0	0	1	1
combined	6	.9357846	.0302324	.0740539	.8580697	1.013499
diff		-.1284309	.0211273		-.1870898	-.069772

```
    diff = mean(A) - mean(control)                                t =  -6.0789
Ho: diff = 0                                     degrees of freedom =        4

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0019         Pr(|T| > |t|) = 0.0037          Pr(T > t) = 0.9981

. ttest MDR1, by(concentration3)

Two-sample t test with equal variances
```

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
A	3	.965748	.0171522	.0297084	.8919481	1.039548
control	3	1	0	0	1	1
combined	6	.982874	.0108397	.0265518	.9550096	1.010738
diff		-.034252	.0171522		-.0818741	.01337

```
    diff = mean(A) - mean(control)                                t =  -1.9970
Ho: diff = 0                                     degrees of freedom =        4

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0583         Pr(|T| > |t|) = 0.1165          Pr(T > t) = 0.9417

.
```

Significant reduction of *CDR1 and CDR2* gene by CFE, CFE effective. No significant reduction of expression of MDR 1 by CFE

Compare control to CFE overall at concentration A. Does CFE affect expression of *HWP1*, *SAP1*, *SAP2 and SAP3* genes?

```
. ttest HWP1, by(concentration1)

Two-sample t test with equal variances
```

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
A	9	.875914	.0149505	.0448516	.841438	.91039
Control	12	1	0	0	1	1
combined	21	.9468203	.0150617	.0690216	.9154021	.9782386
diff		-.124086	.0128335		-.1509467	-.0972252

```
    diff = mean(A) - mean(Control)                                t =  -9.6689
Ho: diff = 0                                     degrees of freedom =       19

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

. ttest SAP1, by(concentration2)

Two-sample t test with equal variances
```

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
A	9	.8804457	.0087362	.0262086	.8603	.9005914
Control	12	1	0	0	1	1
combined	21	.9487624	.0137151	.0628503	.9201533	.9773716
diff		-.1195543	.0074991		-.1352501	-.1038585

```
    diff = mean(A) - mean(Control)                                t = -15.9425
Ho: diff = 0                                     degrees of freedom =       19

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

. ttest SAP2, by(concentration3)

Two-sample t test with equal variances
```

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
A	9	.8535294	.0137657	.0412972	.8217855	.8852732
Control	12	1	0	0	1	1
combined	21	.9372269	.0171809	.0787327	.9013882	.9730656
diff		-.1464706	.0118164		-.1712027	-.1217385

```
    diff = mean(A) - mean(Control)                                t = -12.3955
Ho: diff = 0                                     degrees of freedom =       19

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

. ttest SAP3, by(concentration4)

Two-sample t test with equal variances
```

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
A	9	.9252299	.014392	.043176	.8920419	.9584179
Control	12	1	0	0	1	1
combined	21	.9679557	.0101963	.0467252	.9466866	.9892247
diff		-.0747701	.012354		-.1006274	-.0489128

```
    diff = mean(A) - mean(Control)                                t =  -6.0523
Ho: diff = 0                                     degrees of freedom =       19

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

. ttest PLB1, by(concentration5)

Two-sample t test with equal variances
```

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
A	9	.8779005	.0181347	.0544042	.8360818	.9197193
Control	12	1	0	0	1	1
combined	21	.9476717	.0154573	.0708342	.9154283	.979915
diff		-.1220995	.0155668		-.1546811	-.0895179

```
    diff = mean(A) - mean(Control)                                t =  -7.8436
Ho: diff = 0                                     degrees of freedom =       19

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

.
```

Reduction in expression of genes statistically significant

Compare control to CFE overall at concentration A. Does CFE affect expression of *CDR1*, *CDR2* and *MDR1* genes?

```
. ttest CDR1, by(concentration1)

Two-sample t test with equal variances

   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       A |       9    .9112316    .0069492    .0208475    .8952068    .9272564
 Control |       9           1           0           0           1           1
---------+--------------------------------------------------------------------
combined |      18    .9556158    .0112802    .0478577    .9318167    .9794149
---------+--------------------------------------------------------------------
    diff |           -.0887684    .0069492               -.1035   -.0740368
------------------------------------------------------------------------------
    diff = mean(A) - mean(Control)                                t = -12.7740
Ho: diff = 0                                     degrees of freedom =       16

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

. ttest CDR2, by(concentration2)

Two-sample t test with equal variances

   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       A |       9    .8732944    .0070045    .0210135    .8571419    .8894468
 Control |       9           1           0           0           1           1
---------+--------------------------------------------------------------------
combined |      18    .9366472    .0157365    .0667643    .9034461    .9698483
---------+--------------------------------------------------------------------
    diff |           -.1267056    .0070045               -.1415545   -.1118567
------------------------------------------------------------------------------
    diff = mean(A) - mean(Control)                                t = -18.0891
Ho: diff = 0                                     degrees of freedom =       16

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

. ttest MDR1, by(concentration3)

Two-sample t test with equal variances

   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       A |       9    .9852215    .0179957     .053987    .9437234     1.02672
 Control |       9           1           0           0           1           1
---------+--------------------------------------------------------------------
combined |      18    .9926108    .0089113    .0378072    .9738097    1.011412
---------+--------------------------------------------------------------------
    diff |           -.0147785    .0179957              -.0529276    .0233706
------------------------------------------------------------------------------
    diff = mean(A) - mean(Control)                                t =  -0.8212
Ho: diff = 0                                     degrees of freedom =       16

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.2118         Pr(|T| > |t|) = 0.4236          Pr(T > t) = 0.7882

.
```

Reduction in expression of *CDR1* and *CDR2* genes highly significant. Slight reduction in expression of *MDR1*gene by CFE but not significant