Candida albicans: sex and survival in a rat model of colonisation

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Abstract

Unlike for most animal species, sex is not an obligatory reproductive mechanism for fungi, and for some fungi the complex diversity of sexual and asexual reproductive mechanisms has yet to be unravelled. A classic example of this diversity is *Candida albicans*, an opportunistic, commensal yeast found in normal oral and gastrointestinal flora, which is capable of causing infections in immune compromised hosts. It was long believed that *C. albicans* is asexual existing exclusively as a diploid yeast. However, with the identification of a mating type-like locus homologous to those in sexually reproducing fungi, demonstration of mating *in vitro*, and the discovery that a morphology switch, from white to opaque, promotes mating, it is now hypothesised that *C. albicans* may possess a cryptic sexual cycle. No evidence of meiosis has been found, however, and thus any matants are probably produced via a parasexual cycle. The question is: can such a cycle confer advantages, or does it simply represent the "remains" of a sexual mode of reproduction that is no longer effective for diversity generation but may have acquired other functions?

In this study I developed a rat model of oral colonisation in order to investigate whether sex confers an advantage to the survival of *C. albicans*, and how likely sex is to occur, *in vivo*.

Specific pathogen free Sprague Dawley rats were used and treated with immunosuppressant (dexamethasone) and antibiotic (doxycycline) throughout the course of the study. In this model, colonisation of oral surfaces was followed for 4 weeks post inoculation. Initially, colonisation increased until reaching a plateau 7 days post-inoculation which remained constant to the end of the sampling period. The commensal nature of *C. albicans* in this model was evidenced by: (i) healthy growth of the rats; (ii) lack of visible signs of oral disease; and (iii) no sub-epithelial penetration detected in histology of rat tongues following euthanasia.

Seven genetically manipulated *C. albicans* clinical isolates were used in this study. Strain pairs which had previously been demonstrated to be mating competent *in vitro* were selected for co-inoculation into the rat model. Strains of α mating type were marked with the NAT (nourseothricin) resistance cassette and strains of the compatible a mating type were marked with the MPA (mycophenolic acid) resistance cassette. Matants were detected in both oral and fecal samples from two *in vivo* co-inoculation experiments, inoculated with the same numbers of white phase cells. Two combinations of the *C. albicans* strains, namely *C. albicans* OD8916 with *C. albicans* W43 and *C. albicans* W17 with *C. albicans* W43, displayed mating *in vivo* in all experiments conducted. Detection of matants could be enhanced (~10 fold) by
changing the inoculum from white to opaque cells. For example, when rats were co inoculated with either white or opaque cells of the strain pair *C. albicans* W17α and W43a a total of 30 CFU and 520 CFU respectively matants were detected at day 7. However, after an initial increase, the number of matants observed in samples declined over time whereas overall yeast counts remained constant. The decline was probably not because of a lower growth rate of matants as the *in vitro* growth rates of recombinants recovered from *in vivo* experiments and their parents did not vary greatly. These growth curves were done two times to measure if growth rates varied considerably between the strains employed, as this will affect the out-competition by the co-inoculant. Neither was the decline in matant recovery because of the genetic modification of the strains, or marker loss, as co-inoculation in the rat model of strains containing one or both genetic markers did not affect the survivability of the strains *in vivo* and no marker loss in recovered isolates was observed. One explanation for the reduced detection of matants during an *in vivo* experiment is that the opaque (mating competent) cells switched rapidly to white, as demonstrated by plating of sequentially obtained samples on YPD agar plates containing Phloxine B, a dye that stains opaque cells pink. Another possible contributing factor is that one parent outcompeted the other over time, as indicated by a decrease in the number of the other parent recovered, thus reducing the possibility for mating to occur. To test if matants were less fit than their parents, rats were co-inoculated with a matant (obtained via an *in vivo* mating event) and each of its respective parents. By day 28 i.e. the last sampling point, very few recombinants were detected. The parent strain contributed as much as 95 % of the total yeast count and had clearly outcompeted the matant strain.

Four other strain combinations tested did not give matants. These strains did not switch to the opaque form readily as no or very few opaque cells were observed in samples post inoculum. Furthermore, one parent quickly outcompeted the other but, despite adjusting the numbers of the parents to give the less fit parent a better chance of survival, no mating was detected.

In conclusion, this study has demonstrated that mating of *C. albicans* does occur in the oral cavity *in vivo*, but that it appears to be a rare event. This possibly reflects both the inherent instability of mating-competent opaque cells and/or dynamic changes in the commensal populations of each parent, due to differences in colonisation abilities, thus reducing the chances of meeting and mating. The results also demonstrated that when mating was detectable *in vivo*, the resultant matants were not as fit as the parents, as they were quickly outcompeted by parental strains. This provides evidence to confirm the hypothesis that, within the experimental systems used, there appears to be no advantage conferred on *C. albicans* by sex.
Acknowledgements

“Anybody who has been seriously engaged in scientific work of any kind realizes that over the entrance to the gates of the temple of science are written the words: ‘Ye must have faith.’ It is a quality which the scientist cannot dispense with”.

Max Planck

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<tr>
<td>Avg</td>
<td>average</td>
</tr>
<tr>
<td>BC</td>
<td>Before Christ</td>
</tr>
<tr>
<td>cfu</td>
<td>Colony forming units</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees centigrade</td>
</tr>
<tr>
<td>dH₂O</td>
<td>Distilled water</td>
</tr>
<tr>
<td><em>et al.</em></td>
<td><em>et al.</em> (and others)</td>
</tr>
<tr>
<td>e.g.</td>
<td>For example</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>H1</td>
<td>High <em>in vitro</em> mating pair 1</td>
</tr>
<tr>
<td>H2</td>
<td>High <em>in vitro</em> mating pair 2</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency Virus</td>
</tr>
<tr>
<td>kg</td>
<td>Kilogram</td>
</tr>
<tr>
<td>kDa</td>
<td>KiloDalton</td>
</tr>
<tr>
<td>L</td>
<td>Litre</td>
</tr>
<tr>
<td>LOH</td>
<td>loss of heterozygosity</td>
</tr>
<tr>
<td>L1</td>
<td>low <em>in vitro</em> mating pair 1</td>
</tr>
<tr>
<td>L2</td>
<td>low <em>in vitro</em> mating pair 2</td>
</tr>
<tr>
<td>M1</td>
<td>medium <em>in vitro</em> mating pair 1</td>
</tr>
<tr>
<td>M2</td>
<td>medium <em>in vitro</em> mating pair 2</td>
</tr>
<tr>
<td>MAT</td>
<td>Mating locus</td>
</tr>
<tr>
<td><em>MTL</em></td>
<td>Mating type-like locus</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µl</td>
<td>Microliter</td>
</tr>
</tbody>
</table>
Definitions

**Fitness**: Throughout this thesis the author will employ the term “fitness”. In this thesis fitness refers to the ability of one particular strain to survive in the rat.
CHAPTER ONE

GENERAL INTRODUCTION
1.1 Introduction

Throughout this thesis I will use the term matant not recombinant. This is because no meiotic cycle has been observed in *C. albicans* as of yet and the return to diploid state is a result of chromosome loss. (Bennett and Johnson 2003; Forche, Alby et al. 2008)

1.1.1 Reproduction in fungi

There are two means by which fungi reproduce: asexual (clonal reproduction) and sexual recombination (Taylor 1999).

1.1.1.1 Asexual reproduction in fungi.

Depending on the type of fungus, asexual reproduction in fungi can be via budding, fragmentation or sporulation.

1.1.1.1.1 Budding

Budding is initiated by the formation of a ring of chitin around the site where the bud is going to appear. This chitin formation helps in the reinforcement and stabilization of the cell wall. The cell wall then weakens and extrudes as a result of enzymatic activity and turgor pressure. These cell wall processes are accompanied by mitotic events in the nucleus of the yeast. Once new cell wall material is incorporated between the parent and daughter nucleus and the daughter cell contents resulting from mitotic events in the nucleus are forced into the progeny cell and following the completion of the final phase of mitosis a cell plate forms between the parent and daughter cell due to the inward growth of the cell wall (Carlile 2000).

1.1.1.1.2 Fragmentation

Fungi can also reproduce by fragmentation. In this process a disrupted mycelium can grow into a new fungal colony on the condition that it contains the equivalent of the peripheral growth zone. (This so called peripheral growth zone is the region of mycelium behind the tip needed to support maximum growth of the hyphal tip). Classic examples of fungi that can reproduce via fragmentation are the commonly used fungi such as *Aspergillus oryzae*, *Penicillium notatum* and *Penicillium chrysogenum* (Savage and Vander Brook 1946; Li, Shukla et al. 2000)
1.1.1.3 Sporulation

Spore formation is by far one of the most commonly used modes of reproduction in fungi. Spores that are produced asexually are formed in the mitosporic or anamorphic phase (Figure 1.1) of the fungal life cycle (Gregory 2012).

**Figure 1.1:** Mieotic life cycle of fungi. The figure shows the generalised life cycle of a fungus and the stages where spores are produced. Michael J Gregory (Gregory 2012).

1.1.1.2 Sexual Recombination in fungi

All organisms share basic tenets in the sexual process, namely recognition of a potential mate, formation of a zygote following cell fusion, gamete formation as a result of meiosis and then changes in ploidy (Ni, Feretzaki et al. 2011). Unlike most animal species, fungi do not need to reproduce sexually in order to propagate (Sun and Heitman 2011). However, attention must also be drawn to the fact that fungi are very diverse in their mating mechanisms; fungi include groups like the mushrooms and toadstools where “thousands of varied mating types” may occur (Ni, Feretzaki et al. 2011); yeasts among which silent cassettes of genetic information allow interchange of sexual identity, and fungal species which display same sex mating. Equally fascinating is the lichen in which the sexual act may be prolonged for months or even years. This contributes to the fact that sex lives of fungi are by far among the most fascinating and diverse seen in the living kingdom. Fungi, at one end of the spectrum, possess several
outbreeding systems with numerous mating types, while at the other end of the spectrum they possess self-fertile inbreeding systems.

The aim of this section will be to give the reader a brief overview of sexual reproduction in fungi.

Sexual reproduction in fungi known to have a full sexual cycle is normally controlled by genes located in the genetic locus called a mating-type, or MAT, locus. In diploid cells the MAT locus is predominantly heterozygous; it codes for different but usually related genes on each of the two homologous chromosomes. The genes encoded by the MAT locus of different fungi generally fall into three specific categories: 1) DNA binding proteins responsible for the regulation of sexual cycle gene expression, 2) genes that encode mating pheromones, and 3) genes that encode receptors for mating pheromones (Herskowitz 1988; Hull, Raisner et al. 2000).

The MAT locus genes in *S. cerevisiae* have been particularly well characterized (Herskowitz, Rine et al. 1992). The mating type of a cell is dictated by two idiomorphs of the mating type locus, namely MATa and MATα (Figure 1.2). The MATa locus contains the MATa1 gene which is involved in the regulation of mating, and the MATα locus contains MATα1 and MATα2. In diploid MATa/α cells, the MATa1 and MATα2 gene products, a1 and α2, form a complex that represses MATa genes and mating, and a2 represses MATα genes (Figure 1.2). In haploid MATa cells, a1 activates MATa genes and α2 represses MATa genes. The mating type is controlled by the three transcriptional regulators a1, α1 and α2 via a simple combined circuit (Tsong, Miller et al. 2003). It is well known that α1 turns on α-specific genes in haploid cells while α2 represses a-specific genes, directing the cell to mate as an α cell. In haploid a cells, the a1 protein has no known function, but a-specific genes are expressed constitutively and α-specific genes are not. This could be because the α1 gene is absent thus directing the cell to mate as an a-cell. Cells that have mated express both a1 and α2 proteins, and these form a heterodimer that represses many of the mating genes, thereby blocking mating by the diploid a/α cell but permitting meiosis under favourable environmental conditions.
Mating in *S. cerevisiae* begins when each mating type produces and secretes a peptide pheromone. The mating type a cells produce pheromone-a and the mating type α produces pheromone-α. The presence of sex pheromones triggers a cascade that eventually leads to cell-cell fusion. In this yeast the binding of the peptide pheromone (Figure 1.3) to its cognate receptor, namely Ste2p in MATα cells and Ste3p in MATα cells, leads to a GDP/GTP exchange on the Gpa1p part of the α subunit of a G protein (heteromeric). The Ste4p and the Ste18p subunits then activate a series of protein kinases. It is believed that the G protein is linked to the cascade by Ste20p (Ramer and Davis 1993). Ste11p and Ste7p are then sequentially activated with Ste7p phosphorylating Fus3p/Kss1p (Errede 1993). Elion et al. (Elion, Satterberg et al. 1993) also reported that Ste5p regulates assembly of the Ste11p, Ste7p and Fus3p protein kinases into a complex that phosphorylates Ste12p and that Fus3p also phosphorylates the kinase inhibitor mediating cell cycle arrest namely, Far1p. Ste12p is responsible for the activation of mating specific genes (*FUS1*). The resultant arrest of vegetative cell growth with the activation of the MAP kinase pathway in two haploid or different mating types (a and α) cells thus leads to the fusion of two cells to form a diploid.
Since its classification nearly 80 years ago, the human opportunistic pathogen *C. albicans* has been designated as asexual yeast. However recent identification of genes homologous to genes that function in both mating and meiosis in *S. cerevisiae* have led researchers to believe that mating might occur in *C. albicans*. This has been demonstrated *in vitro* and *in vivo* (Magee and Magee 2000; Lachke, Lockhart *et al.* 2003; Dumitru, Navarathna *et al.* 2007). However, very little is known about why *C. albicans* undergoes sex. The biological significance of sex from a survival point of view is not yet investigated. A question the candidate will address in part through this body of work.
1.1.2 An introduction to \textit{C. albicans}

\subsection*{1.1.2.1 Taxonomy of the Candida genus}

The Mycological Herbarium classifies \textit{Candida} under the subphylum Saccharomycotina and family Saccharomycetaceae (NCBI). The characteristics of members are diverse with cells varying in shape, the presence of pseudo- or true mycelia and chlamydospores. It is also believed that ascospores, teliospores, ballistospores, arthrospores, and visible pigmentation due to carotenoids are absent. Odds \textit{et al.} (Odds 1994) described \textit{Candida} as ‘form genus’ which is essentially a group where yeasts are placed when they cannot be easily assigned elsewhere. The genus contains more than 150 species grouped on the basis of biochemical reactions, morphology and sexual stages (or lack thereof) (Odds 1994-2). It is a pleomorphic diploid yeast with a G+C DNA content of around 35 mol\% (Meyer and Phaff 1969). \textit{C. albicans} cells are capable of forming true mycelia and chlamydospores. It can grow on defined media consisting of a source of salts, carbon, nitrogen, phosphate and supplemental biotin (Hube, Sanglard \textit{et al.} 1998; Schoofs, Odds \textit{et al.} 1998; Coco, Bagg \textit{et al.} 2008).

\subsection*{1.1.2.2 Pathology of Candida infections.}

Candidiasis refers to infections caused by opportunistic fungal pathogens belonging to the genus \textit{Candida}. The disease can range from a minor infection of skin, nail and mucosa in healthy individuals to a florid invasive systemic infection in immuno-compromised patients (Bergbrant 1995). Superficial candidiasis may involve the epidermal and mucosal surfaces, including those of the oral cavity, pharynx, oesophagus, intestines, bladder and urinary tract, and vagina. The gastrointestinal tract and intravascular catheters are the major portals of entry for deep (or visceral) candidiasis. The kidneys, liver, spleen, brain, eyes, heart, and other tissues are the major organ sites involved in deep or visceral candidiasis (Walsh, Hiemenz \textit{et al.} 1996). Within the \textit{Candida} genus, \textit{C. albicans} is the most common species causing infection. Other species which cause candidiasis include \textit{Candida glabrata}, \textit{Candida krusei}, \textit{Candida parapsilosis} and \textit{Candia tropicalis} (Jarvis and Robles 1996). The most common manifestations of candidiasis are those of mucocutaneous candidiasis, characterized by recalcitrant nail and mucosal infections.

According to Odds (Odds 1994-2) \textit{C. albicans} infections can be differentiated on the type and site of infection (Table 1.1).
**Table 1.1**: Candida infections: sites and types adapted from (Odds 1994-2).

<table>
<thead>
<tr>
<th>Type of infection</th>
<th>Conditions</th>
<th>Infection site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superficial</td>
<td>Oropharyngeal</td>
<td>mouth and pharynx</td>
</tr>
<tr>
<td></td>
<td>Genital</td>
<td>vagina and penis</td>
</tr>
<tr>
<td></td>
<td>Paronychia, onchomycosis</td>
<td>nails</td>
</tr>
<tr>
<td></td>
<td>Intertrigo, Interdigital</td>
<td>skin and skin folds</td>
</tr>
<tr>
<td>Deep infections</td>
<td>Peritonitis</td>
<td>peritoneum</td>
</tr>
<tr>
<td></td>
<td>Oesophagitis</td>
<td>oesophagus</td>
</tr>
<tr>
<td></td>
<td>Pyelonephritis</td>
<td>urinary Tract</td>
</tr>
<tr>
<td></td>
<td>Osteoarthritis</td>
<td>joints</td>
</tr>
<tr>
<td>Systemic</td>
<td>Hepatosplenic candidiasis</td>
<td>multiple organs</td>
</tr>
</tbody>
</table>
1.1.2.3 Fungal infections and *C. albicans*: An overview

Since the early 1980s, fungi have emerged as major causes of human disease among the immunocompromised and those hospitalised with serious underlying disease. The morbidity and mortality associated with these infections are substantial, and it is clear that fungal diseases have become important public health problems [Figure 1.4; (Pfaller and Diekema 2007; Fortún 2012)].

![Graph showing episodes of candidemia per year](image)

**Figure 1.4**: Episodes of candidemia per year. Image obtained from Fortin *et al.* (Fortún 2012). There have been several factors which have contributed to the increase in fungal infections (Pfaller and Diekema 2007; Rodloff, Koch *et al.* 2011; Fortún 2012).

The most important factors are an ever-expanding population who are immunocompromised due to mucosal or cutaneous barrier disruption, defects in the number and function of neutrophils or in cell-mediated immunity, metabolic dysfunction, or extremes of age. Increasing use of broad-spectrum antibiotics, cytotoxic chemotherapies, and transplantation immunosuppression further increase the risk of both common and uncommon opportunistic fungal infections. In addition, as our population ages, environmental exposures to a variety of endemic fungal pathogens become more common and may further increase the risk of mycotic disease (Pfaller and Diekema 2007; Rodloff, Koch *et al.* 2011).
The list of opportunistic fungi causing serious, life-threatening infection increases every year (Ascioglu, Rex et al. 2002). In addition to Candida, the opportunistic fungi include yeasts other than Candida species such as Aspergillus, and Cryptococcus species, non-dematiaceous or hyaline moulds, and the pigmented or dematiaceous fungi. Despite this formidable list of opportunistic fungi, without question the single most important cause of opportunistic mycoses worldwide remains Candida (Rodloff, Koch et al. 2011). Seventy-seven patients with 78 ICU stays, representing 12% of all ICU stays, were found to harbour 180 isolates of Candida spp. Molecular typing revealed 27 C. albicans genotypes(Hammarskjold, Mernelius et al. 2013)

Candida species are human fungal pathogens and/or commensals. They are capable of initiating a variety of recurring superficial diseases, commonly on the oral and vaginal mucosae (Odds, Webster et al. 1988). A steadily increasing number of reports on superficial Candida infections associated with the administration of broad-spectrum antibiotics such as tetracycline was seen in the late 1950’s (Samaranayake and Samaranayake 2001). The extensive use of steroids, immunosuppressive agents in organ transplant recipients myeloablative radiation therapy (Wingard, Merz et al. 1991), and antineoplastic therapies in patients with hematologic malignancies contributed to the increasing morbidity associated with Candida in the following years. Since the first report of AIDS in 1981 and the joint report of human immunodeficiency virus (HIV) as the causative agent in 1983 (Barre-Sinoussi, Chermann et al. 1983; Gallo, Sarin et al. 1983), mucosal Candida infections have received profuse attention due to the morbidity it causes in AIDS patients. Until the introduction of triple therapy, it was observed that up to 90% of HIV-infected individuals suffered from oropharyngeal candidiasis (Pfaller and Diekema 2007; Rodloff, Koch et al. 2011). Of particular interest to us is the fact that HIV-infected patients appear to be more susceptible than immunocompetent individuals to oropharyngeal candidiasis (Samaranayake, Lamb et al. 1989).

C. albicans is the species most often associated with oral lesions, but other, less pathogenic species such as C. glabrata, C. tropicalis, C. parapsilosis, and C. krusei are also occasionally isolated. These become more prevalent resistance to azoles, which is the most used treatment for fungal infections. An important factor associated with candidiasis is the virulence of the infecting organism(Sherman, Prusinski et al. 2002) The specific features of the fungus that contribute to the development of oral candidiasis include its ability to adhere to and colonize the oral mucosa, secret hydrolytic enzymes, its ability to form cylindrical appendages termed germ tubes which develop into hyphae, and its cell surface hydrophobicity (Sherman,
Prusinski et al. 2002). In addition, phenotypic (hyphae, pseudo hyphae and coindial) and genotypic switching (Antony, Saralaya et al. 2007; Antony, Saralaya et al. 2009), secreted proteinases (Naglik, Challacombe et al. 2003), and phospholipases (Ibrahim, Mirbod et al. 1995) appear to play a subsidiary role in the pathogenicity. Invasive candidiasis (IC) is a persistent public health problem. IC is a condition when Candida enter the bloodstream and spread throughout the body. The mortality rates associated with this infectious disease have remained as high as 40% for more than a decade despite major advances in the field of antifungal therapy (Pfaller and Diekema 2007; Clancy and Nguyen 2012).

1.1.2.4 Predisposing factors for candida infection

The various factors that can render a host susceptible to candidiasis are listed in Table 1.2, which is derived from an extensive review of predisposing factors by Odds et al. (Odds 1994-2).

A number of medical procedures such as organ transplantation, antibiotic treatment and steroid use are believed to be risk factors for the development of candidiasis (Odds, Webster et al. 1988; Fridkin and Jarvis 1996).
Table 1.2: Factors predisposing a host to candidiasis.

<table>
<thead>
<tr>
<th>Type of factor</th>
<th>Possible role in infection</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medical factors</td>
<td>Drugs altering the natural microflora/promoting fungal colonisation</td>
<td>Antibiotics especially broad-spectrum antibiotics</td>
</tr>
<tr>
<td></td>
<td>Immunosuppression</td>
<td>Corticosteroids and immunosuppressive drugs</td>
</tr>
<tr>
<td></td>
<td>Mechanical disruption due to surgical procedures. e.g.</td>
<td>Voice prostheses, heart valves, intravascular catheters and blood transfusions.</td>
</tr>
<tr>
<td></td>
<td>Introduction of prostheses or other devices into tissue.</td>
<td></td>
</tr>
<tr>
<td>Mechanical factors</td>
<td>Local occlusion or tissue damage</td>
<td>Wearing dentures, thumb sucking, pacifier sucking</td>
</tr>
<tr>
<td></td>
<td>Trauma</td>
<td>Burns, wounds due to accidents, post op wounds</td>
</tr>
<tr>
<td></td>
<td>Route of infection</td>
<td>Surgery, mechanical ventilation renal failure/haemodialysis</td>
</tr>
<tr>
<td>Dietary factors</td>
<td>Immune suppression</td>
<td>Malnutrition, high carbohydrate diets, vitamin deficiency</td>
</tr>
<tr>
<td>Natural factors</td>
<td>Infections, debilitating disease and disorders</td>
<td>Microbial infections, endocrine dysfunctions, organ malignancies</td>
</tr>
<tr>
<td></td>
<td>Change in physiological status</td>
<td>Infancy, pregnancy or old age.</td>
</tr>
</tbody>
</table>

Table adapted from (Odds 1996) and Pfaller and Diekama(Pfaller and Diekema 2007).
1.1.2.5 Sites of C. albicans colonisation in humans

While Candida species have been known to chiefly colonise the gastrointestinal tract (GI) in humans as in other animals, they have been also known to colonise every tissue except hair (Pfaller and Diekema 2007). Odds noted that yeasts were present largely in the extremities of the GI tract and suggested that the GI tract is the primary source of C. albicans for establishment of infection in hosts predisposed to candidal invasion (Cole 1993). The belief that the gut serves as the major reservoir of C. albicans infections was further supported by studies conducted by Krause and colleagues (Krause, Matheis et al. 1969) who were the first to show that C. albicans can pass through the wall of the GI tract in humans to cause fungaemia. Their study was followed by experiments conducted on mice, dogs and monkeys (Stone 1974; Gordee, Zeckner et al. 1984; Kennedy 1985). It is now believed that the entry of C. albicans from the gut into the thoracic duct could be either by the process of bone resorption (the process by which osteoclasts break down bone and release the minerals, resulting in a transfer of calcium from bone fluid to the blood) or as a result of damage to the mucosal epithelium (Akpan 2002). The skin, stomach and vagina are other sites from which C. albicans is frequently isolated. It has been shown in a study of simultaneously cultured, molecular typed, vaginal and anorectal isolates from seven healthy women, that three women had unrelated strains, two had strains that were possibly the result of divergence from a single strain and two had identical strains at each site (Soll, Galask et al. 1991). This study supported the theory that colonisation of the vagina with C. albicans often originates from the GI tract. The vagina has yeast as part of its normal flora and colonisation of the vagina can occur as a result of C. albicans being present at a different site such as the GI tract or other sources such as skin and oral cavity (O'Connor and Sobel 1986; Odds, Webster et al. 1988). The stomach is another site from which Candida species have been isolated. The presence of particular species, however, appears to be dependent on the pH of the gastric juice. Knoke and colleagues (Knoke, Schulz et al. 1997) found that C. albicans and C. krusei were found in gastric juices with a pH < 3 while at a pH > 3 C. albicans and C. tropicalis were predominantly found. Their most important finding from my perspective was that in 59% of the healthy subjects C. albicans could be detected in their stomachs.
1.1.2.5.1 *C. albicans* in the oral cavity

The asymptomatic prevalence of *Candida* in the human oral cavity of healthy individuals ranges from 3-48% (Saltarelli 1975) and even higher in healthy children, 45-65% (Arendorf 1980; Xu and Mitchell 2003). For *C. albicans* alone a median carriage rate of 38.1% has been observed in surveys of community-dwelling outpatients (Odds 1988). *C. albicans* is also the most commonly isolated yeast species in patients with bloodstream infections in epidemiological studies (Morrell, Fraser *et al.* 2005). It was also seen that mean carriage in hospitalized individuals (without clinical candidiasis) was 40.6% (range, 6.0-69.6%) (Odds 1988). These data are important firstly because it indicates that *C. albicans* is present as a commensal in the oral cavity and not just the stomach, and secondly, these data also provide further evidence that poor health is a predisposing factor in the establishment of infection. In another study, Sharp *et al.* (Sharp, Odds *et al.* 1992) sampled 163 neonates in an intensive care and surgical unit. Twenty-one of the neonates initially carried *C. albicans* in their mouths, but only five yielded 6 or more yeast-positive cultures over the 17-week study period. Studies have also shown that 80-85% of *C. albicans* carriers have *C. albicans* on the tongue (Addy, Shaw *et al.* 1982). It has also been isolated from saliva with an incidence of 49% in healthy males and 25% in females (Oliver 1984). Recovery from saliva is often at very low numbers [less than 500 CFU/ml (Odds, Webster *et al.* 1988)] which may mean that *C. albicans* can be present but undetected in the mouth.

Although it is widely distributed in the oral cavity, *C. albicans* is found at the highest concentrations clustered at the midline in the middle and posterior third of the tongue in healthy individuals (Samaranayake, Lamb *et al.* 1989). Incidentally, it is in this site that the oral candidiasis is most common. Alterations in the oral cavity such as orthodontic appliances can also cause shifts in distribution of *C. albicans* (Addy, Shaw *et al.* 1982).

Orthodontic appliances also promote the growth of *C. albicans* causing an increase in the density of *C. albicans* at all sites of the oral cavity (Addy, Shaw *et al.* 1982).

1.1.2.6 Putative virulence factors of *C. albicans*

Although there are many reports in the literature of *C. albicans* virulence factors, many *Candida* researchers would say that *C. albicans* does not have virulence factors, rather it has properties that allow it to “take advantage” of a debilitated host. It regulates expression of genes as virulence factors to eventually cause disease when host defences are compromised. I
will proceed to briefly describe some of the most commonly investigated “so called” virulence factors.

1.1.2.6.1 Adhesion

The adhesion of \textit{C. albicans} to host tissue is a complex multifactorial process with the cell expressing several different adhesins in morphogenetically changing cell surfaces. Formation of a biofilm is a very important step in the process of colonisation. Some of the most well-studied adhesins are the agglutinin-like sequences (ALSs). ALSs are members of a family of seven or more glycosylated proteins. Als1p, Als3p and Als5p are present on the cell surface of hyphae and bind to human buccal epithelial cells, fibronectin, collagen, laminin, and endothelial cells (Hoyer, Payne \textit{et al.} 1998; Chaffin 2008). Als6p and Als9p bind to collagen and laminin respectively, while Als4p binds to endothelial cells and Als5p is additionally needed for cell aggregation (Sheppard, Yeaman \textit{et al.} 2004). \textit{C. albicans} also expresses an outer surface mannoprotein, known as the hyphal wall protein (Hwp1), that forms covalent bonds with human buccal epithelial cells (Chaffin, Lopez-Ribot \textit{et al.} 1998). Gale \textit{et al} characterised another protein Int1p (integrin-like protein) which binds to the extracellular matrix (ECM) ligands (such as fibronectin, laminin, and collagen I and IV) and induces morphological changes in response to extracellular signals (Hostetter 1996; Gale 1997). A number of adhesins, that covalently attach to the cell wall glucan, are termed glycosylphosphatidylinositol-dependent cell wall proteins (GPI-CWPs), and include those encoded by \textit{ALS1}, \textit{ALS2}, \textit{ALS4}, \textit{ALS5} (also referred to as \textit{ALA1}), \textit{HWP1}, and \textit{EAPI}. GPI-CWPs may mediate adhesion to organic and inorganic surfaces, extracellular matrix proteins, human endothelial cells, and epithelial cells (Filler 2006; Nobile and Mitchell 2006; Zhao, Daniels \textit{et al.} 2006).

1.1.2.6.2 Morphogenesis

Morphogenesis in \textit{C. albicans} is the reversible transition from a unicellular yeast form to filamentous pseudohyphae or hyphae. Yeast forms facilitate dissemination in tissues and to other hosts, while hyphal forms are important for tissue damage and invasion. \textit{C. albicans} changes from the yeast form to the hyphal form at a near-neutral pH a temperature of 37°C–40°C, CO2 concentration of approximately 5.5%, (Cutler 1991; De Bernardis, Mühlenschlegel \textit{et al.} 1998), and in the presence of inducers such as N-acetyl-D-glucosamine, serum and some amino acids (Mattia, Carruba \textit{et al.} 1982), while the reverse (hyphal to yeast) transition is provoked by lower temperatures, acidic pH, absence of serum, and higher concentration of
glucose (Cannon, Timberlake et al. 1994). In studies conducted by Hube et al. hyphal cells have been shown to induce phagocytosis by endothelial cells, helping Candida cells to escape from the bloodstream (Hube 2004). Several genes have been identified to regulate morphogenesis. Table 1.3 describes these genes and their functions.

**Table 1.3:** Genes that regulate phase transition.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECE1</td>
<td>hyphal elongation</td>
</tr>
<tr>
<td>RBF1</td>
<td>yeast–hypha transition</td>
</tr>
<tr>
<td>TUP1</td>
<td>negative regulator of filamentation</td>
</tr>
<tr>
<td>Rbp1</td>
<td>negative regulator of filamentation</td>
</tr>
<tr>
<td>Hst7p, Cph1p, Cst20p</td>
<td>unknown specific function, but null mutants shown defective hyphal formation.</td>
</tr>
<tr>
<td>EFG1</td>
<td>transcriptional activator as well as repressor, and is required for pseudohypal and hyphal morphogenesis</td>
</tr>
</tbody>
</table>

Adapted from (I. Ahmad; M. Owais; M 2010).

**1.1.2.6.3 Phenotypic switching**

*C. albicans* shows spontaneous, reversible morphological variation in its colony types (smooth, rough, star, stippled, hat, wrinkle, and fuzzy) at high frequency. This switching occurs under stress conditions such as changes in temperature, pH and the nutrient composition of the medium and results in changes in cell surface and metabolic, biochemical and molecular characteristics which enable *C. albicans* to become more virulent during infection (Soll 2002). The white-opaque system in strain WO-1 is the most studied phenotype switch. Study of gene expression during WO-1 switching revealed an association of *SAP1* and *SAP3* expression in opaque cells, in contrast to *SAP2, WH11* and *EFG1* expression in white cells (Soll 1997). This phenomenon, that opaque cells are more virulent than white cells, that
they are involved in biofilm formation and are more likely to undergo mating (Yang 2003), will be discussed in more detail in Chapter 3.

1.1.2.6.4 Phospholipases

Phospholipases (Cannon, Timberlake et al. 1994) are enzymes that hydrolyse ester linkages of glycopholipids. It has been suggested that they cause “direct host cell damage and lysis” (Ghannoum 2000). C. albicans’ phospholipases possess hydrolase activity and are classified on the basis of the ester bond they cleave, namely, phospholipase A, B, C, and D. Phospholipase B however also possesses lysophospholipase transacylase activity which results in the release of phospholipids. The phospholipid B glycoprotein (encoded by PLB1) has been isolated from hyphal tips during tissue invasion and was demonstrated to be required for virulence (Theiss, Ishdorj et al. 2006).

1.1.2.6.5 Proteinases

Proteinases are often used by a microorganism as a virulence factor in order to aid the cell to invade host tissue. C. albicans is known to express 10 different secreted aspartyl proteinases (encoded by SAP genes) (Hube 1998; Naglik, Challacombe et al. 2003). Saps hydrolyse host proteins such as albumin, haemoglobin, keratin, collagen, laminin, fibronectin, mucin, salivary Lactoferrin, interleukin-1, cystatin A, and immunoglobulin A (Hube 1998). In vitro studies have reported that SAP1, SAP2 and SAP3 are expressed by yeast cells, while SAP4, SAP5 and SAP6 are expressed in the hyphal phase (Hube 1998; Naglik, Albrecht et al. 2004). Both yeast and hyphal forms of C. albicans express SAP9 and SAP10 (Albrecht, Felk et al. 2006).

1.1.2.6.6 Biofilm formation

The prevalence of C. albicans in nosocomial fungal infections can be attributed to their ability to form biofilms on catheters, endotracheal tubes, pacemakers and other prosthetic devices (Douglas 2003). These devices provide Candida cells with a platform on which to form biofilms and also enable dissemination of the yeast cells by providing a route through host defences. Dental plaque is a well-known example of a biofilm which, although mostly bacterial, also often includes Candida cells (Filoche, Wong et al. 2010). Biofilm formation on oral tissues and surfaces is favoured by a high concentration of glucose, serum, and salivary proteins (Calderone, Suzuki et al. 2000). During weakened immunity, hematogenous dissemination of Candida cells from biofilms to internal organs could occur, resulting in
candidemia and septicemia. Several studies have now directly linked biofilm formation to progression towards disease caused by *Candida* species (Hawser and Douglas 1994; Chandra, Kuhn *et al.* 2001; Jin, Samaranayake *et al.* 2004). In order for cells to form biofilms, they first have to adhere to the host or material surface. This adherence is mediated by hydrophobic interactions, electrostatic forces, and adhesion–ligand interactions, involving multiple adhesion molecules. As discussed earlier several adhesins are expressed by *Candida* cells and they perform different functions including a role in biofilm formation (Douglas 2003).

### 1.1.2.7 From commensal to pathogen

With the acceptance of Louis Pasteur’s germ theory in the late 1860s, it was accepted that a microorganism would be considered a pathogen if it met the specifications of Koch’s postulates. (Casadevall and Pirofski 2000) However, scientists later delving into the study of microorganisms found that some microorganisms were classified as pathogens even though they did not cause disease in the host, while some were classified as non-pathogens even though they did cause disease in their host. These conclusions were formed when it was found that normal individuals were colonised by a large number of pathogenic microorganisms in various sites such as their mouth gut and skin and yet did not suffer from disease. These findings gave rise to new ideas and terminology with regard to host-microorganism relationships (Casadevall and Pirofski 2000).

The online medical dictionary describes the term ‘pathogen’, from its Greek origins in the Greek words *pathos* which means suffering, and *genein* ([http://medical-dictionary.thefreedictionary.com/pathogen](http://medical-dictionary.thefreedictionary.com/pathogen)), which means give birth to or beginning. The word *pathogen* therefore refers to an organism giving rise to suffering or disease. However, by the early twentieth century it became more and more obvious that pathogenicity was not a stable or a non-variable trait of most microorganisms. Acquiring a pathogen *de novo* was not necessary to cause a microbial disease. Many microorganisms that were found to exist in normal healthy individuals were found to be responsible for disease in sick individuals (Henrici 1934). Henrici noticed that a small number of individuals in a community developed cerebrospinal meningitis (caused by *Neisseria meningitidis*), whereas others carried the bacteria but remained healthy (i.e., were carriers); however, the majority were neither sick nor carriers (Henrici 1934).

However the carrier state had not be taken into account by Koch’s postulates and this state therefore made what used to be a clear line between pathogens and non-pathogens very blurry
and confusing. It was Park and Williams (Park 1917) who described the carrier state as a commensal (parasite that causes no harm) development by the pathogenic microorganism. Furthermore, the recognition that microorganisms, understood to be non-pathogens, caused disease in certain hosts challenged the classifications of saprophyte (an organism that lives on dead organic matter) and commensal. As a result, additional terms were added to the lexicon in an attempt to find terminology that could accommodate the new medical and scientific findings. One example of this new terminology ‘opportunistic microorganism’ was defined by von Graevenitz as “one that utilizes the opportunity offered by weakened defence mechanisms to inflict damage to the host” (von Graevenitz 1977) but does not exclude pathogenicity for a normal host when a large inoculum or specific virulence factors can overcome normal defences. This term therefore included microorganisms such as *C. albicans*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Streptococcus pyogenes* that are present in the host in large numbers without causing infection. This brings us to the definition of colonisation and infection. Casadevall and Pirofski (2000) define a host to be colonised when the presence of a microorganism in a host does not cause infection. In 1934 Henrici defined infection as “The invasion of the body tissues by microorganisms resulting in disease” (Henrici 1934).

### 1.1.2.8 The good turn bad and ugly: The *C. albicans* journey

*C. albicans* is a classic example of an opportunistic pathogen. Reports of the human – *Candida* relationship go as far back as 377 BC as it is thought that oral lesions reported by Hippocrates may have been caused by *Candida* species (Odds, Webster *et al.* 1988).

*C. albicans* is an extremely successful commensal of human beings. It can infect a wide assortment of body sites and is almost always isolated from the oral cavity (Hube 2004). It is different from other medically important fungi, such as *Histoplasma capsulatum*, *Cryptococcus neoformans* and *Aspergillus fumigatus*, in that it is rarely found in environmental niches such as soil. Also, it does not cause disease through the inhalation of fungal spores or mycelial fragments. It is frequently isolated from human mouth, yet few carriers develop clinical signs of candidiasis. The occurrence of several forms of oral candidiasis however reveals the ability of the yeast to colonize the host, and then, in the presence of predisposing factors such as antibiotic consumption and immune deficiency (where the host’s immune defences are compromised) develop into infection (Cannon, Holmes *et al.* 1995). Oral candidiasis manifests in the form of yeast overgrowth with penetration of oral tissues which often presents as white lesions in different sites within the
oral cavity. While tissue invasion may be assisted by secreted hydrolytic enzymes, hypha formation, and contact sensing, it is the host’s immune competence that ultimately determines whether clearance, colonization, or candidiasis occurs (Cannon, Holmes et al. 1995).

In his review discussing the invasiveness of *C. albicans*, Bernard Hube proposes several primary events that are responsible for the progression of commensal colonisation to superficial infection or invasion. He also describes the mechanisms that enable *C. albicans* to counteract specific environmental pressures in the host (Hube 2004). He summarised these by saying that disease occurs only when immunity is attenuated with little or no profound alteration in the pathogen transcript profile, and that the gene expression of *C. albicans* is regulated by both, permanent host–pathogen interplay and by genetic programs. An example of its adaptability is seen when *C. albicans* yeast cells are exposed to blood plasma. They change their growth pattern and rapidly switch to hyphal growth (dimorphism). This transition is now known to be regulated by a complex transcriptional program that modulates the morphology while also inducing the expression of several hyphal-associated genes encoding surface proteins (*HWP1, HYR1* and *ALS3*), secreted proteinases (*SAP4, SAP5* and *SAP6*) or detoxification enzymes such as superoxide dismutases (*SOD5*) (Hube, Monod et al. 1994; Brown and Gow 1999; Nicholls, Straffon et al. 2004). This interplay between host environmental factors and *Candida* not only allows its immediate adaptation to changing environmental conditions, but also prepares cells for subsequent steps of infection (Hube 2004). Colonisation by *C. albicans* is a pre-requisite for infection. In the oral cavity, more people are naturally colonised by *C. albicans* than infected by it (Casadevall and Pirofski 2000; Nair, Anil et al. 2001). This therefore highlights the need to study more closely the behaviour and survivability of *C. albicans* in its commensal state in the host and thus demonstrating the importance of using a method or *in vivo* model of colonisation as opposed to a model of infection.
1.1.3 Animal models


The Merriam-Webster dictionary defines an animal model as ‘an animal sufficiently like humans in its anatomy, physiology, or response to a pathogen to be used in medical research in order to obtain results that can be extrapolated to human medicine’.

The use of animals for research has always been a significant entity in the field of scientific research and experimentation. Experimentation using animal models has been recorded as early as the 3rd and 4th century BC in the writings of Greek-philosophers-physicians. In their book on animal models Cohen and Loew (Loew 1984) described Aristotle (384-322 BC) as one of the first to experiment with the dissection of animals and, in 304 BC, Erasistratus is thought to be the first person to perform dissections on living animals. Since then, animal experimentation has led to a new understanding of the mechanisms involved in physiology, behaviour, genetic disposition, and disease progress and treatment regimens in humans. The report of the Royal Society stated that “virtually every medical achievement in the twentieth century has relied on the use of animals in some way”, thus highlighting the importance of the use of animals in medical research (The Royal Society. The Royal Society: London 2003). In this section the candidate will aim to discuss some of the historical and current applications of animal models in scientific research.

1.1.3.1 Animal models help

The human race has a lot to be grateful for when it comes to the use of animals in scientific research. Almost all medical achievements in the past century can be accredited to the use of animals in some way. Developments in the treatment of diseases such as diabetes, leukaemia and heart surgery transplants among others are now possible because of animal studies. In support of this I will now give a few examples of some significant medical advances that have been developed through the use of research on animals.
1.1.3.1.1 Use of animal models in medicine.

Of all the people who develop kidney failure one in three would die without a kidney transplant or regular dialysis on a kidney machine, both being techniques that were developed using animal models such as dogs.

Animal models have also been developed to test the efficacy of potential lifesaving agents against cancer. In oncology research the discovery of some 24 significant biomedical advances in the past 30 years would not have been possible without animal research (Frankie 2005). It is now widely believed that without laboratory animals cancer researchers would lose a fundamental method for obtaining data required to make appropriate decisions about potential new therapies that would impact significantly on human populations (Dey, De et al.). The introduction of effective vaccines in the late 1950s has brought polio under control (Larson and Ghinai 2011) and almost eradicated the disease as a public health problem in industrialised countries (Frankie 2005; Callaway 2011). The World Health Organisation describes polio as an infectious disease found to predominantly affect young children. The poliomyelitis virus is transmitted via the fecal-oral route. The virus enters through the mouth and on entering the bloodstream can invade the central nervous system, destroying nerve cells in the limbs, trunk and the brainstem, resulting in paralysis and sometimes death. Research on this disease required the use of living nerve tissue to ensure that the virus used for vaccine production caused paralysis typical of polio for which no tissue culture is available. Additionally the development of this vaccine requires a live attenuated virus which has been known to revert back to its virulent form thus making the use of animals the only practical way of predicting the potential virulence of each batch of the polio vaccine.

Antihistamines are another example of an important result from animal investigations (Bender 1966). The immunosuppressive drugs now widely used post-surgery for all organ transplantations to prevent rejection, are also a result of animal studies. Cyclosporin is a classic example of an immunosuppressive drug used to prolong the survival of transplanted kidneys (Borel 2002).

The prime objective of animal models of behaviour is to aid in the study and understanding of cellular and molecular mechanisms involved in various behaviours (Castellucci 2008). Animals are now used to study alcoholism (McBride and Li 1998), social stress (Blanchard, McKittrick et al. 2001) and drugs used to treat behavioural disorders (Gainetdinov, Mohn et al. 2001).
1.1.4 Aims of this study

This general introduction has highlighted the importance of \textit{C. albicans} as a human pathogen and the crucial role \textbf{colonisation} plays in the ability of \textit{C. albicans} to invade host tissue and cause disease (Cannon, Timberlake \textit{et al.} 1994; Cannon, Holmes \textit{et al.} 1995). I have also attempted to highlight the major role animal models play in our ability to understand disease progression.

Additionally, rapid growth and reproduction of \textit{C. albicans} is required in order to quickly form a biofilm and establish itself as an opportunistic pathogen. While it is known that \textit{C. albicans} reproduces predominantly asexually, the biological reason for sexual recombination is largely unknown.

Hence the overall goals of this thesis are

1. Develop an \textit{in vivo} model of oral \textit{C. albicans} colonisation in order to investigate \textit{C. albicans} \textit{in vivo} mating.
2. Investigate whether mating of \textit{C. albicans} occurs \textit{in vivo}.
3. Investigate whether mating \textit{in vivo} might be advantageous to survival in the host.

1.1.5 Hypotheses

1. Mating does occur \textit{in vivo} and can be detected in a rat model of colonisation.
2. That the occurrence of mating \textit{in vivo} does not contribute to the \textit{in vivo} survival of \textit{C. albicans}. 
CHAPTER TWO:

Development of a rat model of human oral colonisation by *C. albicans*
2.1 Introduction

2.1.1 Animal models of Candida oral pathogenesis and colonisation

An animal disease model can be described as a living non-human model system that is used specifically for the purpose of understanding, and research into, a disease or the effectiveness of a drug in the treatment of a disease. As well as ethical dilemmas involved in the experimentation on humans, variables such as dietary and social habits, immune status and other physiological factors e.g., hormones and salivary flow along with cultural, racial and ethnic variations in human behaviour justify the use of an appropriate animal model rather than humans directly (Samaranayake and Samaranayake 2001).

Ideally, the animal used to model human disease would be as close as possible on the evolutionary tree to humans. The closer it is, the better the predictive value of the animal model (Kari, Rodeck et al. 2007). The animal model would also provide highly reproducible, and easily measurable, data that reflects the process under investigation, whether it be a disease caused by a single organism, a polymicrobial disease, or the effect of a drug in the treatment of a disease.

The study of oral candidiasis is particularly complex because the organism involved is a commensal and an opportunistic pathogen. Hence there is a need for an animal model of colonisation as well as models of infection in order to provide an accurate idea of the behavioural patterns of C. albicans in its human host.

Several animal models are in use today including systemic and mucosal Candida infection models and only recently murine oral colonisation models have been used. Here I will discuss a selection of these with reference to oral Candida infection and colonisation

2.1.1.1 Monkey model (Macaca irus)

Macaca irus is commonly known as the crab-eating macaque and is referred to in laboratories as the cynomolgus monkey. Although primates would appear to be an ideal animal model for experimental Candida infections, considering their relatively close genetic relatedness to humans, both ethical issues and their high costs in maintenance serve as a deterrent to their use in experiments. The oral microflora of monkeys, especially M. irus, is qualitatively and quantitatively very comparable to that of humans (Loftin, Brown et al. 1980) and C. albicans
is frequently found in the oral cavity of monkeys (Bowen 1974). Monkeys are also able to keep acrylic plates resembling denture prostheses in place, a prerequisite for experimental studies on Candida-associated denture stomatitis (Olsen and Haanaes 1977). However, they have also shown that artificial oral infestation of monkeys with Candida is difficult and unpredictable. Hence, the monkey oral candidiasis model has been largely replaced by the use of smaller mammals such as hamsters, rats and mice (Samaranayake and Samaranayake 2001).

### 2.1.1.2 Hamster model (Mesocricetus auratus)

The cheek pouch of the male BIO 87.20 hamster commonly known as the Syrian hamster has been used in studies investigating experimental oral candidiasis. McMillan and Cowell (McMillan and Cowell 1985), observed that a single inoculation of the organism (10⁷ CFU per ml) was adequate to cause infection of the hamster cheek pouch mucosa. They also found that it was easy to retain the inoculum within the cheek pouch by a simple artificial ligation of the cheek pouch with sutures following inoculation with Candida. These workers used the latter technique to study Candida infection of the hamster cheek pouch after induction of epithelial hyperplasia by turpentine (in liquid paraffin) application. The disadvantages of the hamster cheek pouch are its low oxygen tension and the lack of a natural salivary flow, which only poorly mimics the oral milieu. Hence, this model is rarely used (McMillan, Smillie et al. 1982; Veys, Baert et al. 1994; Baert, Veys et al. 1996) as an infection model and had never been used as a colonisation model.

### 2.1.1.3 Mouse Model (Mus musculus)

Mice are easily obtained in large numbers and are cheap to maintain. The immunobiology of the healthy murine oral mucosa has also been characterized by several groups (Deslauriers, Neron et al. 1985) (Yanagi, Hisajima et al. 2008), thus making it possible to use this model to study the adaptive immune responses of mucosal tissues to candidal infection.

Most recently mice have been used as a colonisation model by Matsubara et al for oral colonisation (Matsubara 2012) and by Rahman et al for concurrent oral and vaginal colonisation (Rahman, Mistry et al. 2012). In the Matsubara model of oral colonisation the mice are immunesuppressed by intraperitoneal administration with cyclophosphamide two times before inoculation with C. albicans. The first time is 4 days before the inoculation and the second, 1 day before. The mice are then intraperitoneally anesthetised in order to be
inoculated with *C. albicans* by topical application using a sterile swab of the yeast suspension containing viable cells. After the initial inoculation of *C. albicans* in the oral mucosa of the animals, immunosuppression continues every 4 days until the end of the experiment. In the concurrent oral and vaginal colonisation model the mice were administered a dose of oestradiol valerate per mouse per week both subcutaneously with and intramuscularly. The mice needed to be anaesthetised in order to be inoculated orally and vaginally, using a pipette, suspension of *C. albicans*.

A model most recently in use is the mouse model of oropharyngeal candidiasis which uses mice that are made susceptible to oral infection by a cortisone acetate injection and then inoculated with a swab saturated with *C. albicans* sublingually (Solis and Filler 2012).

In contrast to the rat, *Candida* is not a normal resident of the oral cavity of the conventional laboratory mouse (Yanagi, Hisajima et al. 2008). Conventional infant mice can be colonized by topical inoculation of the oral mucosal surfaces with $10^8$ *C. albicans* yeast per millilitre (Lacasse 1993). However, the fact that *C. albicans* does not exist in mice as part of their natural flora and the small size of their oral cavities, means that it is not feasible to take sequential samples thus limiting the use of mice to end-point measurements.

### 2.1.1.4 Rat model (*Rattus norvegicus*)

Sprague-Dawley (SD) rats and Wistar rats are the two types of rat most commonly used to investigate oral Candida infections (Samaranayake and Samaranayake 2001). The low maintenance cost and the relatively large size of the oral cavity, which easily permits inoculation and sample collection are highly advantageous for their use in studies investigating experimental oral candidiasis. Additionally, the tongue of the rat is fairly easily colonized and/or infected by *Candida*, resulting in conditions such as median rhomboid glossitis and atrophic candidiasis (Samaranayake and Samaranayake 2001); [Figure 2.1].
Figure 2.1: Topographic distribution of common oral lesion sites in human and rat oral candidiasis. Simplified schematic diagram comparing the topographic distribution of common lesion sites in human oral candidiasis (A) and experimental candidal infection in a rat model (B); the most common sites of infection are shaded. The clinical variants of the disease and their preponderant sites are as follows: 1: Erythematous candidiasis; 2: Pseudomembranous candidiasis; 3: Hyperplastic candidiasis; 4: Candida-associated denture stomatitis; 5: Candida-associated angular chelitis; 6: Candida-associated median rhomboid glossitis. (Samaranayake and Samaranayake 2001).

*Candida* infections in SD rats can be induced within a few weeks without traumatizing the mucosal epithelium, and a number of investigators claim this model to be satisfactory since it is known to yield consistently reproducible data (Russell and Jones 1973; Allen and Beck 1983; Allen and Beck 1987; Núñez, Novio *et al.* 2010; Martins, Junqueira *et al.* 2011). The bulk of these studies involved the addition of antibiotic to food to aid the formation of lesions. Clinical and histological outcomes in experimental candidiasis in SD rats are comparable to those of humans. Small white patches of "thrush" could be observed on the keratinized lingual mucosa and/or sometimes on the cheek mucosa.

Russell and Jones experimentally induced oral candidiasis in rats and also found that rats are likely to harbour *C. albicans* in the oral cavity, but to a lesser extent than in humans (Russell and Jones 1973); [Figure 2.2].
Figure 2.2: Buccal sulcus of rat infected with *C. albicans*. Candidal mycelial elements (as shown by arrow) penetrate the keratinised layer of the epithelium. There is intercellular oedema and inflammatory-cell infiltration of the deeper layers of the epithelium (Russell and Jones 1973).

More recently in use is a rat model of denture stomatitis developed by Nett *et al.*, and another model by Johnson *et al.* In the model developed by Nett *et al* rats were immunosuppressed with a single dose of cortisone (200 mg/kg subcutaneously), prior to placement of denture appliances, on the day of infection. For a subset of experiments, either ampicillin sodium-sulbactam sodium at 100 mg intraperitoneally twice a day or aztreonam at 50 mg/kg subcutaneously was administered 48 h prior to denture placement to reduce enteric bacterial colonization common to rodents due to coprophagy, or the consumption of faeces (Nett 2010). In the model developed by Johnson *et al.*, the rats were first anesthetized by intraperitoneal injection with 90 mg ketamine/kg of body weight plus 10 mg/kg xylazine and then inoculated by removing a portion of the denture system spreading a pelleted yeast paste on the palate of the rat and then replacing the denture (Johnson 2012). Both these models resulted in sustained colonization of the denture and palate for 8 weeks post-inoculation.

This natural presence of oral *Candida* mimics the human ecosystem since 30 to 50% of humans carry oral yeasts (Samaranayake and Samaranayake 2001). Additionally, rats also have larger oral cavities than mice, thus permitting sequential samples enabling the study of colonisation over a length of time. The use of this model for studies investigating the oral colonisation by *Candida* would also require the use of Specific Pathogen Free (SPF) rats in order to overcome the issue of naturally colonised rats.
2.2 Experimental aims of this chapter

In this chapter I will aim to develop a model of oral colonisation by:

1. Testing the appropriate amount of immunesuppression required to achieve oral colonisation.

2. Testing the appropriate amount of inoculum required to achieve oral colonisation.

3. Testing the model for reflecting variations in the ability of different strains to colonise the *in vivo* model.
2.3 Methods and materials

2.3.1 Strains and culture conditions

The yeast strains used were: *C. albicans* ATCC 10231; *C. albicans* ATCC 36082; and clinical isolates. *C. albicans* 412S was provided by Dr David Perlin, Public Research Health Institute, New Jersey, USA (Table 2.1). All strains were grown on YPD medium containing 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose and 1.5 % (w/v) agar or CHROMagar™ Candida (Fort Richard laboratories, Auckland, NZ; which contained Chromopeptone 1% (w/v), glucose 2% (w/v), chromogen mix 0.2 % (w/v), chloramphenicol 0.05 % (w/v), ampicillin 0.1% (where stated) and 1.5 % (w/v) agar). Yeast were grown at 30° C prior to inoculation of animals.

Table 2.1: Strains used in the development of an oral model of colonisation.

<table>
<thead>
<tr>
<th>Strain name</th>
<th>Source</th>
<th>Isolated from</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. albicans</em> ATCC 10231</td>
<td>USA</td>
<td>man with bronchomycosis</td>
</tr>
<tr>
<td><em>C. albicans</em> ATCC 36082</td>
<td>USA</td>
<td>human blood</td>
</tr>
<tr>
<td><em>C. albicans</em> 412S</td>
<td>USA</td>
<td>Vagina</td>
</tr>
</tbody>
</table>

2.3.2 Preparation of inoculum

*C albicans* cells, from stock stored in YPD + 15% (w/v) glycerol stored at -80°C, were streaked on a YPD agar plate and incubated at 30°C for 18 h. This was done to adapt the cells to growth on a surface rather than as a planktonic culture. The cells were then harvested from the plate by washing it with 1 ml sterile distilled water and then pipetting the cells into a 1.5 ml microfuge tube. An additional 0.5 ml of sterile water was used to wash off the remaining cells. These were then pooled with the first wash. The cells were centrifuged at 2400 x g for 3 min and the supernatant discarded. The pellet was resuspended in 1 ml sterile water (if the cell pellet was large it was resuspended in 1.5 ml). This wash was repeated 2 times and finally the pellet was resuspended in 1 ml sterile water. The OD$_{540}$ was adjusted using a spectrophotometer and a haemocytometer to the required cell concentration (3 x 10$^8$ cells/ml).
2.3.3 Animal care and handling

This study was approved by the University of Otago Animal Ethics Committee (60/07 and 97/10; Appendix 1) as per the provisions set out in the Animal Welfare Act 1999. The specific pathogen free SD rats were obtained from the Hercus Taieri Resource Unit. The study employed the use of male Sprague-Dawley rats (6 weeks old, approx 200 g) housed together fed standard rat pellets and water, containing doxycycline and dexamethasone, ad libitum. On arrival they were kept in their cages for 48 hours to acclimatise to their new environment. They were then weighed, allocated to their respective study groups and their tails tattooed with their ID numbers. All rats were then swabbed (see section 2.2.4) to ensure that they were free of endogenous *C. albicans* prior to the commencement of the study. The rats’ weights were monitored to ensure they showed a healthy growth rate and they were examined to ensure they were not stressed by looking for signs of pink and/or ruffled fur.

2.3.4 Inoculation and sampling of rats.

The rats’ oral cavities were inoculated with 0.1 ml inoculum (i.e. $3 \times 10^7$ cells; see section 2.3.2), 3 times, at 48 hour intervals using an automatic pipette with a sterile tip (Figure 2.3). The oral cavities were then swabbed twice per week, starting four days after the last inoculation, for a period of four weeks (Figure 2.4). The swabs were suspended in 1 ml sterile saline in 15 ml Falcon tubes (Becton, Dickinson and Company, Auckland, NZ.) by vortexing for 1 min. Two 50 µl and two 100 µl portions were plated on CHROMagar® (Fort Richard laboratories, Auckland, NZ) and incubated at 30°C for 48 hours. Following incubation it was seen that all colonies were green. The *C. albicans* colonies, which appear green on CHROMagar®, were then counted to determine the number of colony forming units (CFU) per swab.
Figure 2.3: Rat handling regime for all aspects of the *in vivo* model. A: The inoculation phase of the study. B: The regime for the sampling phase of the study.

Figure 2.4: Summary of the rat experimental model. The rats were swabbed on entry into the holding facility (week -2) to ensure they were free of *Candida*. Rats were inoculated on three occasions with $3 \times 10^7$ *C. albicans* 10261 cells. Sampling commenced at week 0, twice a week for four weeks post-inoculation. The grey squares indicate the inoculation points. The red labels indicate sampling points. The letters in the squares indicate the days of the week. * The different timing and amounts of dexamethasone and doxycycline administered will be described in later chapters.
2.4 Results

2.4.1 Determination of appropriate inoculum size

The initial objective was to determine conditions under which rats were colonised for several weeks with significant numbers of yeasts, but no clinical symptoms of conditions and with minimum immunosuppression. First the appropriate inoculum was determined.

Five groups of rats were inoculated with different numbers of *C. albicans* cells under different immunosuppression conditions and one non-immunosuppressed condition. Four of the five groups of rats were inoculated with two different concentrations of *C. albicans* ATCC 10231. One group (E) was not inoculated with *C. albicans* and treated as the control group (Table 2.2). Groups A and C were inoculated with 100 µl of a *C. albicans* 10361 cell suspension at 3 x 10^8 cells/ml. Groups B and D were inoculated with 100 µl of a *C. albicans* 10231 cell suspension at 1 x 10^6 viable cells/ml.

Table 2.2: Variation in the inoculum size: Inoculation of rats was repeated two more times at 48h intervals as described in Section 2.3.4

<table>
<thead>
<tr>
<th>Rat Group</th>
<th>Inoculum (Number of cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>3 x 10^7</td>
</tr>
<tr>
<td>B</td>
<td>1 x 10^6</td>
</tr>
<tr>
<td>C</td>
<td>3 x 10^7</td>
</tr>
<tr>
<td>D</td>
<td>1 x 10^6</td>
</tr>
<tr>
<td>E</td>
<td>0</td>
</tr>
</tbody>
</table>

Rats were sampled twice a week and the level of colonisation calculated (Figure 2.5). Both the levels of immunosuppression and the inoculum size affected the level of colonisation. The biggest effect on the colonisation was immunosuppression; colonisation in immunosuppressed rats was on average about 100-fold higher than in rats without immunosuppression. The inoculum size affected colonisation to a lesser extent, but inoculation with 3 x 10^7 cells consistently resulted in higher levels of colonisation than inoculation with 1 x 10^6 cells. It was seen that the colonisation pattern also varied (represented as error bars in the figures in this chapter or more clearly visible in Figures 3.10) within a
group of rats. This could be due to the sample size collected which in turn could vary depending on the size of the oral cavity, the efficiency of sampling (e.g. the rat moved during the sampling process) and in the cases where fecal pellets were sampled the size of the fecal pellet also varied.

Visual examination of the oral cavity (Figure 2.6) coupled with histology of the rats’ tongues (Figure 2.7) showed no signs of oral lesions or invasive oral candidiasis even in immunosuppressed rats inoculated with larger number of cells. From the study it was therefore concluded that $3 \times 10^7$ cfu was the better inoculum size and it was preferable to use immunosuppressed rats. This was because these conditions gave consistently high levels of colonisation without symptoms of invasive candidiasis.

**Figure 2.5:** Effect of inoculum size on oral colonisation in the rat model. Group A and C were inoculated with $3 \times 10^7$ cells. Group B and D were inoculated with $1 \times 10^6$ cells. Group E was the control uninoculated group. Group A and B were treated with immunosuppressant and antibiotic while group C and D were untreated. The first number in the bracket indicated the amount of dexamethasone (µg/ml) used in week 1 and the second number in the bracket indicates the amount used from week 2 through to week 6 of the study.
Figure 2.6: Appearance of rat tongues. Pictures of rats’ tongues showing no visible oral lesions or damaged tissue. The tongues, examined after euthanasia, were from representative rats (rats 1 and 2, Group A - immunosuppressed and largest inoculum).

Figure 2.7: Histology of rat tongues stained with PAS. The dark pink to red stained cells seen in A, B and C, are *C. albicans* cells and filaments on the surface of the tongue (rat 1, Group A after euthanasia on week 6). There is no penetration of the sub epithelial tissue in any of the rats’ tongues. Figure D is from an uninoculated control rat (Group E). (Histology and PAS staining was kindly undertaken by the histology unit at the School of Dentistry, Otago University and Dr. Marina Bakri)
2.4.2. Determination of appropriate immunosuppression conditions

Having established that the appropriate inoculum size was $3 \times 10^7$ cells (given on three occasions 48 h apart), groups of rats were treated with varying concentrations of the immune suppressant dexamethasone, supplied in the drinking water, to determine the best conditions to establish the stable colonisation. Antibacterial treatment (doxycycline) was also added to the drinking water, to eliminate competition from the host flora. Several immunosuppression conditions were investigated in this experiment. This was done to determine the minimum amount of immunosuppression required to give sufficiently high level of colonisation of the oral mucosa without infection (deep tissue penetration). The inoculum for this study was prepared as described in Section 2.3.2. The immunosuppression conditions investigated in this experiment are shown in Table 2.3. Group A rats were treated with 0.5 μg/ml of dexamethasone for the entire length of the study. Group B was treated with 0.25 μg/ml of dexamethasone during week 1-2 and 0.5 μg/ml of dexamethasone for weeks 3-6. Group C was treated with 0.25 μg/ml of dexamethasone throughout the entire length of the study. Group D was not treated with immunosuppressant. All the groups were treated with 1.25 mg/ml doxycycline during week 1 and 0.125 mg/ml doxycycline for the rest of the study (Figure 2.8). Samples obtained were then processed as described in Section 2.3.4.

Table 2.3: 1) Treatment regime for each group; amount of inoculum, immunesuppressant and antibiotic, over the 6 week period.

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Dexamethasone (μg/ml)</th>
<th>Doxycycline (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Week 1</td>
<td>Week 2</td>
</tr>
<tr>
<td><strong>A</strong></td>
<td>$3 \times 10^7$</td>
<td>0.5/1.25</td>
</tr>
<tr>
<td><strong>B</strong></td>
<td>$3 \times 10^7$</td>
<td>0.25/1.25</td>
</tr>
<tr>
<td><strong>C</strong></td>
<td>$3 \times 10^7$</td>
<td>0.25/1.25</td>
</tr>
<tr>
<td><strong>D</strong></td>
<td>$3 \times 10^7$</td>
<td>0/1.25</td>
</tr>
</tbody>
</table>

37
Figure 2.8: Effect of immunosuppression on oral colonisation in the Rat Model. All groups were inoculated with $3 \times 10^7$ Cells/ml. Numbers in brackets indicate concentration of dexamethasone over weeks 1-2, and 3-6.

The graph above (Figure 2.8) shows the group A and B which was treated with 0.5 µg/ml and 0.25 µg/ml dexamethasone during week 1 and 2 and 1.25 µg/ml dexamethasone from week 3-6 showed the highest levels of colonisation, approximately $10^6$ cfu/swab, at 4.5 weeks post inoculation. Group C which was treated with 0.25 µg/ml dexamethasone (week 1-2) and 1.25 µg/ml dexamethasone (week 3-6) also showed lower levels of colonisation with $10^5$ cfu/ml. The control group D with no dexamethasone treatment in the first two weeks showed a decrease in C. albicans colonisation after week 2 even though it was treated with 1.25 µg/ml dexamethasone for the following weeks. All groups were treated with 1.25 mg/ml doxycycline.

Antibiotic and immunosuppression conditions were similar for Group E and F (Table 2.4, Figure 2.9) with the rats receiving 0.5 µg/ml of dexamethasone in week 1, 2 and 1.25 µg/ml dexamethasone for weeks 3 to 6. The antibiotic concentration was 1.25 mg/ml doxycycline for week 1 and 0.125 mg/ml doxycycline for week 2 to week 6. Group G, H and I received no immune suppression for 1 and 2, and received 1.25 µg/ml dexamethasone for week 3-6.
Table 2.4: 2) Treatment regime for each group; amount of inoculum, immunesuppressant and antibiotic, over the 6 week period.

<table>
<thead>
<tr>
<th>Group</th>
<th>Inoculum</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4</th>
<th>Week 5</th>
<th>Week 6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$3 \times 10^7$</td>
<td>0.5/1.25</td>
<td>0.5/.125</td>
<td>1.25/.125</td>
<td>1.25/.125</td>
<td>1.25/.125</td>
<td>1.25/.125</td>
</tr>
<tr>
<td>E</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td></td>
<td>0/1.25</td>
<td>0/.125</td>
<td>1.25/1.25</td>
<td>1.25/1.25</td>
<td>1.25/1.25</td>
<td>1.25/1.25</td>
</tr>
<tr>
<td>H</td>
<td></td>
<td>0/1.25</td>
<td>0/0.125</td>
<td>1.25/0.125</td>
<td>1.25/1.25</td>
<td>1.25/1.25</td>
<td>1.25/1.25</td>
</tr>
<tr>
<td>I</td>
<td></td>
<td>0/1.25</td>
<td>0/0.125</td>
<td>1.25/0.125</td>
<td>1.25/1.25</td>
<td>1.25/1.25</td>
<td>1.25/1.25</td>
</tr>
</tbody>
</table>

It was observed that the same level of colonisation, approximately $10^5$ cells/swab, was obtained with both 0.25 $\mu$g/ml dexamethasone with 0.5 $\mu$g/ml dexamethasone. Since immunosuppression of the rats puts a lot of stress on the animal and makes them susceptible to superinfection, an ideal model would subject rats to a minimum amount of immunosuppression that allows maximum colonisation; therefore I decided to use 0.25 $\mu$g/ml dexamethasone for future studies. All rats were weighed to ensure healthy weight gain and checked for signs of sickness or stress (pink and or ruffled fur) due to immune suppression. It was observed that the rats appeared healthy and grew at the normal rate and no morbidity or mortality was observed.
Figure 2.9: Effect of immunosuppression on oral colonisation in the Rat Model. All groups were inoculated with $3 \times 10^7$ Cells/ml. Numbers in brackets indicate concentration of dexamethasone over weeks 1-2, and 3-6.
2.4.3 Comparing the variability in colonisation with different *C. albicans* strains.

*The work presented in this Section was undertaken by Professor Richard Cannon as part of his Research and Study leave. Although the work was not completed by the candidate, it is included as it was at critical for the in vivo model. It highlights the difference in colonisation patterns between strains which is further reinforced in Sections 3.7.3 – 3.7.5.*

It was possible to compare the colonisation of rats with *C. albicans* ATCC 10231 to that by other strains in concurrent experiments undertaken by Prof. Richard Cannon during his research and study leave in the USA at the PHRI. He tested the ability of different strains to colonise the mouths of rats. If the model reflected human colonisation each strain would colonise differently which might reflect their virulence. This study also helped ascertain the robustness of the model to determine whether the model worked irrespective of where the rats are obtained from or where in the world the experiment is conducted. The inoculum was prepared as described in section 2.3.2 and samples processed as described in section 2.3.4. The strains tested in the Public Health Research Institute Newark, NJ, USA. (PHRI) were *C. albicans* 36082 (Figure 2.10) and *C. albicans* 412S (Figure 2.11).
**Figure 2.10**: Oral colonization with *C. albicans* ATCC 36082. The rats in groups A, B, C, D, and F were inoculated with $3 \times 10^7$ cells/ml. Group E was the control uninoculated group. Group A, B, C, D, and E were treated with immunosuppressant and antibiotic while group F was untreated. The numbers in the brackets indicate the amount of dexamethasone (μg/ml) used in week, weeks 2-6.
Figure 2.11: Oral colonization with *C. albicans* 412S. The rats in groups A, B, C, D and F were inoculated with $3 \times 10^7$ cells/ml. Group E was the control uninoculated group. Group A, B, C, D, and E were treated with immunosuppressant and antibiotic while group F was untreated. The numbers in the brackets indicate the amount of dexamethasone (μg/ml) used in week 1, weeks 2-6.

As seen in Figure 2.12, colonization was strain-specific with the strain *C. albicans* 10231 resulting in approximately $10^5$ cfu/swab at week 3. Strain *C. albicans* ATCC 36082 showed a colonization level of about $10^2$ cfu/swab at week 3 after inoculation. Strain *C. albicans* 412S showed colonization of about $10^3$ - $10^4$ cfu/swab. It is clear from these data that each strain varies in their pattern of colonization of the oral cavity, with some strains being able to reach larger numbers e.g. *C. albicans* 10231, hence colonise better than others e.g. *C. albicans* ATCC 36082.
Figure 2.12: Variation in colonisation ability of different strains. The figure shows a variation in colonisation patterns of different strains. The blue triangles represent *C. albicans* 10231, the orange squares represent *C. albicans* 412S and the purple diamonds represent *C. albicans* 36082.
2.5 Discussion.

Animal models are very effective tools for the study of oral candidiasis. Several models of oral infection are in place and used to study the various aspects of infection and pathogenesis (Russell and Jones 1973; Allen and Beck 1983; Samaranayake and Samaranayake 2001) but none have been developed to study asymptomatic oral colonisation. Clancy et al. discussed the advantages of using a murine model for candidiasis, wherein they injected the mice intravenously through the tail vein (Clancy, Cheng et al. 2009). This was considered an advantage because they achieved high levels of disseminated mucosal candidiasis. In the case of the oral cavity Kubota et al., in order to achieve oral/oesophageal candidiasis, immunosuppressed mice and then inoculated the oral cavity with the aid of swabs saturated with *C. albicans* (Kamai, Kubota et al. 2001). Hisajima, Abe et al. in order to achieve oral/oesophageal candidiasis immunosuppressed mice intravenously, anesthetised the mice and then inoculated the oral cavity with the aid of swabs saturated with *C. albicans* (Hisajima 2008). A model most recently in use is the mouse model of oropharyngeal candidiasis. This model uses mice that are made susceptible to oral infection by a cortisone acetate injection and then inoculated with a swab saturated with *C. albicans* sublingually (Solis and Filler 2012). However, although mice are cost effective and easy to maintain, their oral cavities are small and this also impacts on the ability to obtain multiple samples from the same animal over a period of time. Additionally, swabbing the mouths may also remove a large proportion of the colonising yeast leading to a gradual reduction in colonisation levels. Furthermore, all these models require intravenous injections which are not convenient and not pleasant for the handler or the mouse as an oral administration. As far back as 1973 Russell et al. used a rat model to study oral candidiasis. This model however, required repeated inoculation throughout the length of the experiment (Russell and Jones 1973). Repeated inoculation does not truly mimic oral colonisation. It precludes the investigation of the ability of the organism to establish itself by overcoming host defences and colonising the host tissue. It is important to note that *C. albicans* is an opportunistic pathogen and normally exists as a commensal in the human body. Its ability to colonise the human host is therefore an important factor in determining its survival in the human host. This emphasises the need to develop a model for asymptomatic colonisation, which is the most common situation in humans, rather than use the invasive infection models that are frequently used.
This study therefore set out to design an animal model of asymptomatic colonisation rather than infection. Criteria for the model were that it was as simple as possible, to ensure reproducibility with relatively low cost of maintenance. It should achieve high levels of colonisation for extended periods of time with a minimal amount of immunosuppression so as to cause minimal morbidity to the animals. Lastly the model should be robust and the resultant colonisation should be reproducible.

It was decided to use the rat in this study. As mentioned earlier in the introduction, two species of rats, Sprague-Dawley (SD) and Wistar have been widely used in experimental oral Candida infections. We used SD rats as they are known for their calmness and ease of handling. The two main advantages using rats are the relatively low maintenance cost and the size of the oral cavity. The oral cavity easily permits inoculation and sample collection, and the fact that the tongue of this animal is fairly easily colonised by Candida, (Samaranayake and Samaranayake 2001) makes the rat an obvious choice for a model to study oral colonisation by C. albicans.

In contrast to the mouse infection model described by Elahi et al. (Elahi, Pang et al. 2000) that did not achieved similar amounts of colonisation but only for a week post inoculation, the model developed in this study achieves consistent colonisation of up to $10^6$ cfu, it allows multiple measurements from one animal and allows us to conduct a time course study of colonisation as well as end point examination, as opposed to only end point examination of the tissue burden and damage being possible in their murine model. Most importantly the SD rat colonisation model is exactly that, a ‘colonisation’ model. The rat model described by Russell et al. (Russell and Jones 1973) required repeated inoculation throughout the experiment, whereas the SD model involves inoculation only during week -1 of the experiment, following which C. albicans becomes established by itself and therefore displays true colonisation. This enables one to study a more natural co-existence of C. albicans with its host, in contrast to the models described by other workers (Russell and Jones 1973; Dwivedi, Mallya et al. 2009). The method for inoculation and drug delivery is simple and non-invasive. The yeast cells are pre-conditioned to surface growth by growing the C. albicans on agar plates. This was done because studies shown that C. albicans exhibits contact-dependent cellular behaviours such as invasive hyphal growth and biofilm development (Kumamoto 2005). Following this, the rats are inoculated with a simple suspension of the required amount of cells in water. This is a simpler procedure compared to other models that requires an anaesthetic and an intraperitoneal injection (Elahi, Pang et al.
or the model used by Russell et al. which requires continuous inoculation throughout the study (Russell and Jones 1973).

Other advantages of this model include the ability of the investigators to alter the extent or levels of colonisation by simply adjusting the drug regime, the simplicity of delivery of the immunosuppressant and antibiotic in the water and the size of the inoculum. This model also displayed strain-specific variation in the degree of colonisation. This means the ability of *C. albicans* to colonise varied with the type of strain used in the study. *C. albicans* 36082 and *C. albicans* 10231 were clinical isolates from the blood and lung, respectively, and *C. albicans* 412S was clinical isolate from the vagina. This strain-specific variation could also prove to be disadvantageous as there is a possibility that some strains may not colonise the oral cavity very well and certain strains would then recover higher levels of immunosupression depending on their degree of virulence. This would cause strain on health of the rat. The SD model is also advantageous for ethical reasons - the animals are healthy and show no signs of disease or stress due to the experimental conditions.

To summarise: the SD rat colonisation model is one that is cost-effective and low maintenance, one that allows multiple measurements from a single animal (permitting time-course studies), the drug delivery and inoculation is very simple and non-invasive. This model also permits various levels of colonisation in addition to strain-specific variation which indicates similarity to human colonisation. This model could be very beneficial for use in the study of competition between different organisms, thus permitting us to study the comparative survival of mutants and their parents. Additionally, it could also be used to test the ability of compounds to eradicate *C. albicans* from already colonised rats. Lastly, it could also be used to study host-*C. albicans* interactions that progress to oral infection.
CHAPTER THREE:

*C. albicans* mating *in vivo*
3.1. Introduction

3.1.1. *C. albicans*: growth and morphology

Although *in vivo* *C. albicans* mainly exists as a biofilm the candidate used the measurement of *in vitro* growth liquid culture as a simple way of evaluating and comparing the growth properties of the strains studied in the thesis. This type of growth is described below.

3.1.1.1 The yeast growth cycle

Yeast growth in batch culture is represented by three main phases, the lag phase, the exponential phase and the stationary phase [Figure 3.1] (Richards 1928). Upon inoculation into fresh growth medium, a culture of yeast cells enter a brief lag phase during which they are biochemically active but not dividing. The lag phase is the initial growth phase during which the number of cells remains relatively constant preceding rapid growth. This initiates the second exponential phase of growth. In this phase the cells grow most rapidly. The exponential phase depends on several factors: the organism itself, the growth medium, and the temperature are all important factors in determining the generation time (the time it takes the culture to double, denoted $t$). The third phase of growth is the stationary phase during which metabolism slows and is followed by cessation of rapid cell division, possibly due to a depletion of a nutrient such as the carbon source and/or an environmental limitation such as a reduction in pH or oxygen depletion.

![Figure 3.1: A typical logarithmic yeast growth curve where Nt is the population size at time t (Richards 1928).](image-url)
3.1.1.2 C. albicans: morphology and vegetative reproduction

*C. albicans* grows and reproduces most commonly by budding (Figure 3.2). This process is initiated when mother cells attain a critical cell size which coincides with the onset of DNA synthesis. The cell wall then weakens and there is then an extrusion of cytoplasm aided by turgor pressure from the cell. A chitinous ring forms at the junction between the mother cell and the newly emerging bud, which, when closed, finally results in the formation of a daughter cell.

A very striking feature of *C. albicans* is that it can exist in several morphological forms. *C. albicans* mainly exists in three forms, namely yeast cells (previously called blastopores), pseudohyphal cells and true hyphal cells. Another morphology, chlamydospores, occurs under some depleted nutrient conditions. When grown in rich medium at 30°C, *C. albicans* grows as yeast cells which are ovoid in shape and daughter cells separate from the mother cells, [Figure 3.2a] (Berman and Sudbery 2002). Pseudohyphal cells are formed when the daughter bud elongates but still remains attached to the mother cell after formation of the septum (Figure 3.2b). This results in the formation of filaments composed of elongated cells with constrictions at the septa. Hyphal forms of *C. albicans* are observed as long and highly polarized cells, with parallel sides and no obvious constrictions between cells [Figure 3.2] The growth rate of the different strains used in the current study was measured in order to elucidate whether they would differ drastically and therefore give us an indication of how the strains might behave in vivo.

![Figure 3.2: Differences between a) yeast, b) pseudo hyphae and c) true hyphae adapted from Arnaud et al. (Arnaud, Costanzo et al. 2009).](image)
3.1.2 Mating type locus and ploidy: effect on the growth of C. albicans

Until the late 1990s it was believed that C. albicans was an asexual yeast, then Hull and Johnson (Hull and Johnson 1999) demonstrated the existence of a mating type-like locus MTL. It was described as being similar to the MAT locus in S. cerevisiae. There are two alleles, MTLα and MTLα, at the MTL locus in most C. albicans strains. This report was quickly followed by the work of Magee and Magee. (Magee and Magee 2000) who constructed MTLα and MTLα strains and demonstrated mating of C. albicans in vitro. Following the discovery of the MTL locus, several studies were done to investigate the effect of ploidy on the virulence of C. albicans in vivo. Most clinical isolates obtained are MTL heterozygous. It was shown that spontaneous MTL-homozygosis is achieved in vitro primarily by the loss of one homologue of chromosome 5, which carries the MTL locus, followed by duplication of the retained homologue resulting in uniparental disomy (Wu, Pujol et al. 2005). Wu et al. (Wu, Lockhart et al. 2007) studied the effects of homozygosity on the virulence of C. albicans. In their experiments they demonstrated that α/α strains were far more virulent than either their α/α or α/α offspring. In support of this study, an experiment conducted by Ibrahim et al. (Ibrahim, Magee et al. 2005) found that tetraploids formed as a result of a mating process are less virulent than diploid cells and can change ploidy during the process of infection. They also studied the effect of mating type configuration on the virulence of C. albicans, and concluded that it does not necessarily affect the ability of C. albicans to infect its host. To date, no studies have been conducted to investigate the effect of LOH (loss of heterozygosity) at the MTL on the growth of strains in vivo. In this chapter we will attempt to investigate the effect of the presence of a resistance marker on the growth and survival of C. albicans and also the effect of LOH on the growth of C. albicans in vitro. Additionally, we will attempt to investigate whether mating occurs in vivo and its effect on the survivability of generated matants.

3.1.3 Sexual recombination in yeasts

The depth of knowledge of sexual recombination in the fungal phylum ascomycetes is variable in that mating and meiosis have been thoroughly investigated in some fungi such as S. cerevisiae, but for other species the process is still mostly a mystery. For instance it is still not clear whether Candida tropicalis can mate or whether C. albicans undergoes meiosis (Sai,
Holland et al. 2011). Sexual recombination in fungi has been discussed in detail in chapter 1 (Section 3.1.3). Here the candidate will compare what is known to date about mating in C. albicans with sexual recombination in S. cerevisiae.

### 3.1.4. Mating in C. albicans

Following its classification nearly 80 years ago, the human pathogen C albicans was designated as an asexual yeast. However, the identification of a mating type locus in 1999 (Hull and Johnson 1999; Magee and Magee 2000) and the completion of the C. albicans whole genome sequencing (Jones, Federspiel et al. 2004) allowed the identification in C. albicans (Rossignol, Lechat et al. 2008) of homologs of S. cerevisiae genes that function in both mating and meiosis. These included homologues of GPA1 (which encodes the $\alpha$-subunit of a heterotrimeric G protein), STE6 (which encodes an ABC transporter), STE12 (which encodes a transcriptional regulator), STE20 (which encodes a kinase), and DMC1 [which encodes a meiosis-specific recombination enzyme] (Johnson 2003). Recent comparisons, however, have revealed a number of differences in the way genes are regulated for mating in C. albicans when compared to sexual recombination in S. cerevisiae.

S. cerevisiae contains a cassette system that includes two silent loci, namely the homothallic mating locus (HML) and the homothetic mating locus (HMR) [Figure 3.3]. As seen in Figure 3.3, in S. cerevisiae there are three loci containing mating type genes, two of which, namely the HML and the HMR, are silent. The HML locus contains the MATa1 and MATa2 genes; the other contains the MATa1 and the MATa2 genes. The third locus, i.e. the MAT locus, contains either the MATa or the MATa genes which are expressed.. In contrast, C. albicans is normally heterozygous for mating type at one locus [MTL]. While S. cerevisiae changes mating type at the expression locus with no loss of the alternate mating type information, C. albicans loses the information of one of the two mating types when it expresses a mating type.
The Mating Type-Like locus (MTL) genes in *C. albicans* are intact and code for well-conserved proteins that have been shown to complement *S. cerevisiae* mutants. Their presence in *C. albicans* suggested either that it has a hidden sexual cycle or that *C. albicans* now lacks a sexual cycle and these conserved gene products have been co-opted for different purposes. As described in Chapter 1, the mating type of a haploid cell is defined by the genotype (a or α) of the MAT locus. The *S. cerevisiae* MAT locus switches its form reversibly (from a to α or from α to a) by site-specific recombination with a copy of either the silent HML or the silent HMR gene cassette (Figure 3.3A). Mating then occurs between an a and an α cell which results in the diploid heterozygote a/α. In the MAT-heterozygous diploids, expression of both MATa1 and MATα2 in the same cell suppresses mating (i.e. unlike *C. albicans* it does not form tetraploid cells) and facilitates meiosis through the binding of aMata1p-Mata2p repressor complex.
**Figure 3.4:** Comparing *S. cerevisiae* and *C. albicans* mating loci. **a)** The mating-type (MAT) locus in *Saccharomyces cerevisiae*. **b)** The mating-type-like (MTL) locus in *C. albicans*. Both panels show the configuration in a/α cells. The *C. albicans* MTL locus contains genes that encode nine proteins: three regulatory proteins a1, α1 and α2 (conserved with *S. cerevisiae* and coloured the same), two phosphatidylinositol kinases (PIKs), two oxysterol binding protein-like proteins (OBPs), and two poly (A) polymerases (PAPs). Diagram reproduced from Johnson *et al.* (Johnson 2003).

In early 2000, Hull *et al* (Hull, Raisner *et al.* 2000) demonstrated that the diploid *C. albicans* strain CAI4 contained MTLα1 on one copy of chromosome 5 and MTLα1 and MTLα2 on the homolog (Figure 3.4b). These researchers (Hull, Raisner *et al.* 2000) then demonstrated fusion between MTLα and MTLα strains (α/α and α/a, respectively) *in vivo*. They genetically altered *C. albicans* CAI4 (a URA3 null mutant of the fully sequenced strain SC5314) to create two types of “a” strains and two types of “α” strains. For the a strains, either the entire MTLα locus was deleted to give an MTLα/mtlαΔ strain or only the α1 and α2 genes were deleted to give anMTLα/mtlα1mtlα2 strain. The α strains were also similarly constructed either by deleting the entire MTLα locus to obtain mtlαΔ/MTLα strains or by deleting only the α1 gene to give a mtlα1/MTLα strain. Auxotrophic ade2/ade2 (Ade−Ura+) and ura3/ura3 (Ade+Ura−) derivatives of these strains were then constructed to allow successful mating events to be detected by selecting for cells with the ability to grow on media lacking adenine and uracil (Ade+Ura+ prototrophs). They then introduced combinations of the auxotrophic strains into tail veins of mice and demonstrated mating events by screening kidneys of euthanized mice.
for the recombinant Ade⁺Ura⁺ prototrophs. Additionally, Magee and Magee (Magee and Magee 2000) used a medium containing sorbose to induce loss of heterozygosity in a/α heterozygotes and demonstrated mating in vitro wherein they showed that selectable prototrophic conjugates resulted from complementation of the various auxotrophic markers in two sets of strains. Miller and Johnson (Miller and Johnson 2002) later showed that the homozygous MTLα and MTLa strains engineered from strain CAI4 underwent white-opaque switching and that mating occurred $10^6$ times more efficiently when both strains were in the opaque phase, than when one or both strains were in the white phase. In 2002, Lockhart et al. (Lockhart, Pujol et al. 2002) showed that approximately 3% of 220 clinical isolates of C. albicans, representing the major clades worldwide, were homozygous for the mating type locus, they also demonstrated that all tested clinical strains that underwent white-opaque switching were homozygous for MTL, and that heterozygotes did not undergo white-opaque switching. Tsong et al. (Tsong, Miller et al. 2003) discovered that two homeodomain proteins MTLα1 and MTLα2 form a heterodimer to repress the white-opaque switch and seven genes analogous to the homozygote specific genes set (HSGS) of S. cerevisiae analogue, four of which relate to pheromone response and 2 are ORFs of unknown function. They tested mating behaviour in a collection of diploid isogenic strains bearing all 16 possible combinations of the four genes a1, a2, α1 and α2. They knocked out each ORF leaving the up-stream and downstream of the DNA sequences of the coding regions intact. Subsequently Ura⁻Ade⁺ auxotrophs (constructed from the knocked out strains) were tested for mating with two Ura⁺Ade⁻ tester strains: an α strain and an a strain. Of the 16 strains that were tested for their ability to undergo white-to-opaque switching, it was seen that all but four strains, namely the only ones that retain both α1 and α2, could efficiently switch. Thus α1 and α2 control white-opaque switching. On the other hand, MTLα2 and MTLα1 were shown to induce mating. The work also suggested that there is a direct link between mating and white-opaque switching with the opaque cells being the mating-competent forms of C. albicans. Together, these results demonstrate for the first time that the mating type locus regulates switching and that switching facilitates mating.

Work conducted by Dumitru et al. (Dumitru, Navarathna et al. 2007) has demonstrated mating in vivo between auxotrophic C. albicans strains under anaerobic conditions in the colon and cecum of rats orally inoculated with MTLα/a cells in their drinking bottles at a concentration of $10^5$ cells/ml for 3 hours, followed by MTLα/a cells for 3 hours, these bottles were switched every 3 hours for 24 hours. The mice were then allowed to rest under normal
conditions for 3 days before euthanasia and the removal of their colons and ceca, which were then screened for prototrophic mutants. Their results indicated that mating in the gastrointestinal tract could support anaerobic mating to an extent that could be detectable in the mammalian host.

3.1.5 *C. albicans*: phenotypic switching, diploids and pheromone signalling

Mating in *C. albicans* is driven by sex-specific pheromones. The α-pheromone is a 13-amino acid peptide, produced by proteolytic processing of the MFα gene product (Bennett, Uhl et al. 2003). In *C. albicans*, mature α pheromone is targeted by the Bar1 protease (an aspartyl protease), secreted by a cells to inactivate the α-pheromone thus enabling non mating cells to overcome pheromone-induced cell-cycle arrest (Manney 1983). Bar1p activity could also sharpen the gradient of α-pheromone thereby promoting accurate partner recognition (Barkai, Rose et al. 1998). Ene and Bennett, (Ene and Bennett 2009) demonstrated that Bar1p also regulates a novel program of homothallic mating in *C. albicans*. It is now known that opaque a cells secrete both a and α pheromones; inhibition of self-mating requires that Bar1p degrades α-pheromone to prevent autocrine a activation of the mating response (Alby and Bennett 2009). In the absence of Bar1p, self-fertilization of opaque a cells can occur, leading to the formation of same-sex tetraploid a-a cells.

The α cells secrete α-pheromone and respond to a-pheromone via the Ste3p receptor and the a cells produce a-pheromone and respond to α-pheromone via the Ste2p receptor (Alby and Bennett 2011). This pheromone binding activates a conserved MAPK cascade (Figure 3.5) which leads to the formation of mating projections (Figure 3.5b) followed by cell–cell conjugation (Bennett and Johnson 2005).
Figure 3.5: Models of the pathways regulating the pheromone-induced (a) opaque and (b) white responses. Red circles denote the differences between the two pathways. Note that the same α-pheromone activates the same Ste2 receptor. Diagram reproduced from Sahni et al. (Sahni, Yi et al. 2009).

Figure 3.6: Shmoo morphology of opaque cells. Photomicrograph reproduced from Cullen et al. (Cullen and Sprague 2002) showing elongated polarised mating projection called shmoos.

Typically only opaque cells (Shmoos; Figure 3.6) exhibit the mating response to pheromones, however white cells can also respond by undergoing increased aggregation and biofilm formation (Slutsky, Staebell et al. 1987). Experiments by Sahni et al. and Yi S et al. in 2008
have demonstrated that both opaque and white cells use the same conserved MAPK cascade for pheromone signalling (Yi 2009; Sahni, Yi et al. 2010). While the same α-pheromone activates the same receptor which in turn activates the dissociation of the α subunit from the βγ subunits of the G protein complex, the key difference is that while signalling activates the Cph1p/Ste12p transcription factor in opaque cells, the Tec1 transcription factor is activated in white cells. A key difference in the activation of the G protein complex in opaque cells is that it requires only the IC3 loop (intracellular loop 3) and the intracellular carboxy terminal tail (I tail), while in white cells it requires the IC1 loop, the IC3 loop and the carboxy terminal tail (Yi 2009). Another key difference is that while white cells require an extra receptor domain to activate the MAP kinase pathway, IC1 loop-dependency can be circumvented via STE4 overexpression (encoding G protein b subunit) which suggests that IC1 the loop plays a role in Ste2p function, not downstream in the activation of the white-specific transcription factor.

In 2009 Yi S et al. (Yi 2009) also found that while pheromone signalling by white a cells also requires a specific region in the Ste2p receptor, it is not necessary for signal transduction in opaque cells.

The mating phenomenon in C. albicans poses some very interesting questions regarding its biological significance in the host-commensal or host-parasite relationship. Questions the candidate will investigate in the following chapter.
3.2. Experimental aims for this chapter

1. To test whether the presence of a genetic selection marker has an adverse effect on cell survival in vivo.

2. To test whether mating occurs in vivo in a rat model of oral colonisation.

3. To investigate the genetic nature and in vivo fitness of any progeny obtained from in vivo mating.

4. For strain combinations in which no in vivo mating was observed, to determine whether slower growth rates were a factor contributing to the lack of mating.

While several studies have investigated both in vivo and in vitro mating in C. albicans, previous studies of in vivo mating have used a disseminated infection model (Yanagi, Hisajima et al. 2008; Núñez, Novío et al. 2010; Martins, Junqueira et al. 2011) and the biological significance of such recombination events from a host-commensal perspective remains largely unknown. Bearing this in mind, it was decided to investigate whether mating occurred in vivo in the rat model of oral colonisation.

A requirement for tracking the survival in vivo of the two parental strains and of any matants that resulted from in vivo mating was the use of a system to genetically mark each parent. It was decided to use a group of strains that had been developed by colleagues in Massey University for a study of in vitro mating by C. albicans. Pairs of parental strains were marked with either of two antibiotic resistance genes, namely genes conferring mycophenolic acid resistance and nourseothricin resistance (Zhang et al. unpublished material).

The overall aim of this chapter was to elucidate the biological significance of mating in C. albicans. It was to address the implications of the presence of a mating type like locus in C. albicans i.e. does it represent an evolutionary impasse - a residual set of genes; or does C. albicans need to maintain some of its sexual machinery, which perhaps has been adapted for other purposes such as biofilm formation.
Figure 3.7: Schematic for the investigation of mating in the *in vivo* colonisation model.
3.3 Methods for the investigation of strain survival in vivo

3.3.1 Investigation of the effect of an introduced genetic marker on strain survival in vivo

To determine the effect of genetic markers on the fitness of the strains in vivo, an in vivo competition experiment was designed that tested the survival of unmarked vs. single marked strains (strains with one antibiotic resistance marker, namely MPA resistance or NAT resistance) [Table 3.1]. CaNAT1 which is a gene conferring resistance to nourseothricin (Shen, Guo et al. 2005), and the other is a mycophenolic acid resistance marker (MPA') (Beckerman, Chibana et al. 2001). The strains were constructed and provided by Dr. Ningxin Zhang, Massey University. (See Appendix 5 for details of the strains’ construction).

Table 3.1: Strains used for in vivo survival studies.

<table>
<thead>
<tr>
<th>Strain Type</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. albicans W43</td>
<td>Clinical isolate</td>
</tr>
<tr>
<td>C. albicans W43-pNZ4</td>
<td>Clinical isolate, MPA resistant</td>
</tr>
<tr>
<td>C. albicans OD8916</td>
<td>Clinical isolate</td>
</tr>
<tr>
<td>C. albicans OD8916-pNZ12</td>
<td>Clinical isolate, NAT resistant</td>
</tr>
<tr>
<td>C. albicans W17</td>
<td>Clinical isolate</td>
</tr>
<tr>
<td>C. albicans W17-pNZ12</td>
<td>Clinical isolate, NAT resistant</td>
</tr>
</tbody>
</table>

3.3.2 Preparing yeast for inoculation

The inoculum was prepared as described in Chapter 2, Section 2.3.2, however this time 1.5 X 10^7 cells for each parent was used in the final inoculum (so that the total number of cells in the inoculum was optimal for the colonisation model). Additionally, the inoculum was diluted and plated on YPD agar plates containing chloramphenicol (0.05% w/v) to confirm that the parent ratio in the inoculum were approximately 1:1.
3.3.3 Sample processing to determine the presence of yeast and the ratio of each strain of the inoculated strains

The rats were sampled once a week for 4 weeks post-inoculation. The sample obtained, namely the oral swab and fecal pellet, was resuspended in 1 ml sterile saline and vortexed to resuspend cells adhering to the cotton. Following vortexing, 3 x 100 μl portions were plated on YPD medium containing chloramphenicol. Plates were then incubated at 30˚C for 48 h. The colonies appearing on the agar plates were counted and 20 colonies were picked at random and tested by PCR, as shown in Table 3.3 and Table 3.4, to detect the presence of markers, namely MTLa, MTLα, MPAr and NATr (Table 3.2). The PCR products were viewed on a UV trans-illuminator after separation using agarose gel electrophoresis (1% agarose). The data obtained gave an indication of the proportion of each parent from the clones tested. These numbers were then used to calculate the total number of each strain present within the sample.

Table 3.2: Primers used for detection of markers.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Forward primer sequence</th>
<th>Reverse primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mycophenolic acid resistance (MPAr)</td>
<td>TACCTATGCACTACT</td>
<td>ATCGTTAGTCAACTTT</td>
</tr>
<tr>
<td></td>
<td>ACTACTC</td>
<td>TGCAAC</td>
</tr>
<tr>
<td>Nourseothricin resistance (Natr)</td>
<td>TGCCTCTTACCAACT</td>
<td>CTAACACATACCCACC</td>
</tr>
<tr>
<td></td>
<td>ATTTCA</td>
<td>GTCCA</td>
</tr>
<tr>
<td>Mating-type like locus a (MTLa)</td>
<td>TTGAAGCGTGAGAG</td>
<td>ATCAATTCCCTTTTCTC</td>
</tr>
<tr>
<td></td>
<td>GCTAGGAG</td>
<td>TTCGATTAGG</td>
</tr>
<tr>
<td>Mating-type like locus alpha (MTLa)</td>
<td>TTCGAGTACATTCTG</td>
<td>TGTAACATCACCTCAA</td>
</tr>
<tr>
<td></td>
<td>GTCGCG</td>
<td>TTGTACCCCGA</td>
</tr>
</tbody>
</table>

The expected sizes of the products are NAT: 850 bp, MPA: 1800 bp, MTLa: 750bp, MTLa: 500bp

+: primers sequences were obtained from Dr. Ningxin Zhang, Massey University and the primers obtained from Invitrogen® Ltd.
3.3.4 PCR reaction components and cycling conditions

Colony PCR was performed using the tip of a sterile pin to pick up a small amount of the test colony. This was resuspended in the amount of nuclease free water required by the reaction mixture and placed on a heating block to heat for 10 min at 70°C. The reaction was set up in an Eppendorf Mastercycler® PCR Cycler (Eppendorf) according to the manufacturer’s instructions for TaKaRa Ex Taq™ and is as described in Table 3.3 and Table 3.4.

**Table 3.3:** Reaction mixture for PCR.

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease free water</td>
<td>12.2</td>
</tr>
<tr>
<td>10×Ex Taq Buffer</td>
<td>2</td>
</tr>
<tr>
<td>dNTP Mixture (2.5 mM each)</td>
<td>1.6</td>
</tr>
<tr>
<td>Primer 1</td>
<td>1</td>
</tr>
<tr>
<td>Primer 2</td>
<td>1</td>
</tr>
<tr>
<td>Primer 3</td>
<td>1</td>
</tr>
<tr>
<td>Primer 4</td>
<td>1</td>
</tr>
<tr>
<td>Enzyme</td>
<td>0.2</td>
</tr>
</tbody>
</table>
**Table 3.4:** PCR conditions in the thermal cycler.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>95°C</td>
<td>5 min</td>
</tr>
<tr>
<td>95°C</td>
<td>30 sec</td>
</tr>
<tr>
<td>55°C</td>
<td>30 sec</td>
</tr>
<tr>
<td>55°C</td>
<td>30 sec</td>
</tr>
<tr>
<td>72 °C</td>
<td>1 min</td>
</tr>
<tr>
<td>4°C</td>
<td>∞</td>
</tr>
</tbody>
</table>
3.4 Methods for investigation of recombination in vivo

3.4.1 Preparing a suspension of yeast for inoculation with white, opaque or uneven number of colonies.

*C. albicans* strains (Table 3.5) were streaked on YPD agar plates (for white cells) and incubated at 30°C for 18 h. For experiments where the rats were inoculated with opaque cells, *C. albicans* strains were streaked on YPD agar containing phloxine B (5 μg/ml). Cells from a pink colony (indicating opaque cells) were re-streaked on YPD agar containing Phloxine B, and incubated at 30°C for 18 h. The cells, white or opaque, were then harvested from the plate by washing with 1 ml sterile distilled water and pipetting the cells into a 1.5 ml microfuge tube. An additional 0.5 ml of sterile water was put on the plate to wash off the remaining cells. These were then pooled with the first wash. The cells were washed by centrifugation (x3) and the pellet was resuspended in 1 ml sterile water. The OD_{540} was adjusted using the spectrophotometer (calibrated for cell count using a haemocytometer) to give a final concentration of 1.5 X 10⁷ cells/ml for each parent in the inoculum. The inoculum was also plated onto YPD agar to obtain the total number of viable cells in the inoculum. The number obtained from this was taken as the time 0 (t=0) count of colony forming units. (In experiments where an uneven number of parents were used in the inoculum the cell concentrations were adjusted to those indicated in the appropriate results section).

3.4.2 Sample processing and screening for matants

The rats were sampled once a week for 4 weeks post-inoculation. The samples obtained, (oral swab or fecal pellet) were resuspended in 1 ml sterile saline. The fecal pellet was vortexed to obtain a homogenous mixture. The oral swab was also vortexed to resuspend cells adhered to the cotton. Following vortexing, 2 x 5μl and 2 x 50 μl portions were plated out on YPD-Phloxine B (Figure 3.8). In addition, three 100 μl portions of the samples were plated on double selection medium (DSM) consisting of 0.67 % (w/v) yeast nitrogen base with amino acids, 2 % (w/v) glucose, 1.5 % (w/v) bacteriological agar, mycophenolic acid (MPA) 5 μg/ml, nourseothricin (NAT) 100 μg/ml. This medium enabled me to screen for potential matants that were resistant to both drugs. It was seen that both large and small (pin-point) colonies were obtained on the double selection plate. Initially both the large and the small colonies were tested and it was seen that all small colonies were parents that had the NAT"
marker only, i.e. they were not matants. However of the large colonies some were matants while some were not. For this reason we decided to consider large colonies as putative matants. The large colonies were picked and streaked to single colonies on YPD and individual colonies were tested by PCR to detect MPA¹ and NAT¹ markers and also MTLα and MTLα markers.

Figure 3.8: YPD + Phloxine (5 µg/ml) plate showing opaque colonies stained pink. Rat samples were plated on a YPD plate containing Phloxine B and incubated at 37°C for 48 h.
| Experiment | \( C.\ albidans \) (Parent 1) | | \( C.\ albidans \) (Parent 2) | |
| --- | --- | --- | --- |
| Strain pairs with high frequency mating \textit{in vitro}\* | OD8916\(\alpha\) | NAT\(^e\) | W43 | MPA\(^f\) |
| | W17\(\alpha\) | NAT\(^e\) | W43 | MPA\(^f\) |
| Strain pairs with Medium frequency mating \textit{in vitro}\* | YSU63\(\alpha\) | NAT\(^e\) | W43 | MPA\(^f\) |
| | Au90\(\alpha\) | MPA\(^f\) | HUN97 | NAT\(^f\) |
| Strain pairs with Low/No frequency mating \textit{in vitro}\* | Au90\(\alpha\) | pNZ4 | FJ11 | NAT\(^f\) |
| | Fj11\(\alpha\) | NAT\(^e\) | W43 | MPA\(^f\) |
| Strain pairs with High frequency mating \textit{in vitro} but inoculated with opaque cells | OD8916\(\alpha\) | NAT\(^e\) | W43 | MPA\(^f\) |
| | Fj11\(\alpha\) | NAT\(^e\) | W43 | MPA\(^f\) |
| Strain pairs with Medium frequency mating \textit{in vitro} but inoculated with opaque cells | YSU63\(\alpha\) | NAT\(^e\) | W43 | MPA\(^f\) |
| | Au90\(\alpha\) | MPA\(^f\) | HUN97 | NAT\(^f\) |
| Strain pairs with Low/No frequency mating \textit{in vitro}\* but inoculated with opaque cells | Au90\(\alpha\) | MPA\(^f\) | FJ11 | NAT\(^f\) |
| | Fj11\(\alpha\) | NAT\(^e\) | W43 | MPA\(^f\) |

\( \text{pNZ11:MTLa} + \) Nourseothricin (NAT) resistance marker
\( \text{pNZ4:MTLa} + \) Mycophenolic (MPA) resistance marker
\* \textit{In vitro} mating frequencies as determined by Zhang \textit{et al.} (unpublished material)
3.5 Methods for in vitro growth curves

3.5.1 Parental and matant strains

The strains used were *C. albicans* OD8916*, C. albicans* W43+, *C. albicans* W17*, *C. albicans* HUN97*, *C. albicans* YSU63*, *C. albicans* Au90+, *C. albicans* FJ11*, matants obtained from *in vivo* and *in vitro* mating of *C. albicans* OD8916* vs. *C. albicans* W43+ and *C. albicans* W43+ vs. *C. albicans* W17. All strains, except for the matants, were obtained from Dr Jan Schmid (Massey University, Palmerston North, New Zealand). Matants were obtained from *in vivo* mating studies conducted in this study. (*-NAT*: +: MPA)

3.5.2 Culture conditions and inoculum preparation

All growth curves were done in YPD liquid medium at 37°C in a shaking incubator. Yeasts for the inoculum were grown at 37°C on YPD agar. Growth was determined by measuring the OD₅₄₀ using a Beckman Coulter spectrophotometer (Amersham Biosciences New Zealand Ltd, Auckland, NZ). The matants were obtained from oral swabs or fecal pellet following plating on DSM as described in section 2.3.2 and stored as a concentrated suspension in YPD containing 15% glycerol at -80°C until required. The suspensions were then thawed and streaked onto YPD as above.

3.5.3 Measuring culture growth and calculating growth rate and doubling time

The cell suspension was diluted 1 in 10 in sterile YPD (this diluent was the same batch as the growth medium), diluted 1 in 10 again to obtain a 1 in 100 dilution. This enabled an accurate measure of OD₅₄₀ (the OD reading has to be between 0.05 – 0.5 OD) for accuracy. The OD₅₄₀ reading was noted. This cell suspension was then used to inoculate two flasks containing 100 ml YPD culture to an initial OD₅₄₀ of 0.1. The culture flasks were then incubated at 200 rpm and 37°C. Samples (1 ml) were taken at 30 minute intervals for OD₅₄₀ measurement against a blank of YPD for 12 hours (with dilution in YPD where required to obtain a reading below 0.5). A graph was then plotted of the OD₅₄₀ vs. time. Using the slope of plotted graph the doubling time of each culture was calculated over the period where exponential growth was observed. Doubling time was calculated as follows
X (number of doublings) = (log(absorbance at time T2) – log(absorbance at time T1)) ÷ log 2

Doubling time per hour = X ÷ (T2 - T1)

Doubling time = 1 ÷ doubling time per hour

3.5.4 Flow cytometry analysis of DNA content

Glycerol stocks were made of matants obtained by me from an in vivo mating experiment and shipped to Massey University to be analysed using flow cytometry.

The FACS analysis was undertaken by Dr. Ningxin Xhang at Massey University

Cells from glycerol stocks were patched on YPD agar and incubated overnight at 30°C. This was then used to inoculate YPD broth at an initial OD600 of approximately 0.2. The culture flask was then incubated at 30°C at 150rpm. Cells were harvested when the OD was between 1 and 2. The cells were then fixed in 70% ethanol at 4°C for 1 hour. They were then collected by centrifugation at 13,000Xg at room temperature for 5 min and the supernatant carefully aspirated. The cells were then washed twice with 750 µl of 5X TE buffer (pH 8.0) to remove trace amounts of ethanol, which can inhibit subsequent RNase treatment. During the second wash, cells were sonicated for 5min (40kHz). They were then treated with RNAse A for 2 hr at 37°C (Sigma) [2 mg/ml in 50 mM TrisCl, 0.5 mM EDTA, pH 8] followed by incubation for 1h with Pepsin (Sigma) [5 mg/ml in 55 mM HCL], and subsequently washed with 50 mM TrisCl pH 7.5, 5 mM EDTA as described by Hull et al.. (Hull, Raisner et al. 2000). A sample of 50 µl cell suspension was then placed in an Eppendorf tube containing 0.5 ml 1 µM Sytox Green (Invitrogen) in 50 mM TrisCl pH 7.5, 5 mM EDTA and incubated at 4°C overnight in the dark. Flow cytometry analysis was performed on a BD FACSCalibur (BD biosciences) using an excitation wavelength of 488 nm (15 m W argon-ion laser). Emission from Sytox Green was measured using a 530/30 band pass filter. The sample was collected at the rate of 12 µl/min equating to a rate of >500 events per second. A minimum of 50,000 events were collected. DNA content in a sample was calculated as the average of median values of M1 and M3 peaks. M1 and M3 peaks are peaks in the DNA content distribution corresponding to exponentially growing cells in their G1 and G2 phases, respectively. M2 corresponds to S
(stationary) phase cells. The peak regions were selected visually based on the shape of the histogram.

### 3.6 Methods for the investigation of survival of matants in vivo

To better understand the fitness of matants in the *in vivo* model an *in vivo* competition experiment was designed that tested the survival of matants vs. their corresponding parent. The matants tested were those obtained from the *in vivo* mating model as part of this study.

**Table 3.6**: Strains used for *in vivo* matant survival studies.

<table>
<thead>
<tr>
<th>Study</th>
<th><em>C. albicans</em> (Strain 1)</th>
<th><em>C. albicans</em> (Strain 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>strain</td>
<td>marker</td>
</tr>
<tr>
<td>Survival between parents and recombinants obtained <em>in vivo</em></td>
<td>OD8916α</td>
<td>pNZ11</td>
</tr>
<tr>
<td></td>
<td>W43a</td>
<td>pNZ4</td>
</tr>
<tr>
<td></td>
<td>W17α</td>
<td>pNZ11</td>
</tr>
<tr>
<td></td>
<td>W43a</td>
<td>pNZ4</td>
</tr>
</tbody>
</table>

### 3.6.1 Preparing yeast for inoculation

The inoculum was prepared as described in Chapter 3, Section 3.3.2, however this time 1.5 X 10^7 cells for each parent was used in the final inoculum (so that the total number of cells in the inoculum was optimal for the colonisation model). Additionally, the inoculum was diluted and plated on YPD agar plates containing chloramphenicol to confirm that the parent ratio in the inoculum were approximately 1:1. A total viable count was obtained.
3.6.2 Sample processing to determine the presence of yeast and the ratio of each strain of the inoculated strains

The rats were sampled once a week for 4 weeks post-inoculation. The oral swab was resuspended in 1 ml sterile saline and vortexed to resuspend cells adhering to the cotton. Following vortexing, 3 x 100 μl portions were plated on YPD medium containing chloramphenicol (0.05%). Plates were then incubated at 30˚C for 48 h. The colonies appearing on the agar plates were counted and 20 colonies were picked at random and tested by PCR, as shown in Tables 3.2 – 3.4, to detect the presence of markers, namely MTLα, MTLα, MPAfr and NATfr. The PCR products were viewed on a UV trans-illuminator after separation using agarose gel electrophoresis (1% agarose). The data obtained gave an indication of the proportion of the clones tested that were each parent. These numbers were then used to calculate the total number of each strain present within the sample. Although the focus of this thesis is the rat oral model of colonization, the data from fecal pellets were also used to evaluate levels of gut colonisation. However, colony numbers recovered varied depending on the size of the pellet and consistency of the pellet. Such data were therefore not appropriate for full quantitative analysis, but were presented in the thesis because they were a further indication of the occurrence of mating in vivo.
3.7 Results

3.7.1 Effect of the presence of introduced genetic markers on strain survival in vivo

Detailed below in Table 3.7 are the strains that were used for determining the effect of marker on in vivo survival.

Table 3.7: Strains used for in vivo survival studies.

<table>
<thead>
<tr>
<th>Survival Experiment</th>
<th>Strain 1</th>
<th>Strain 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unmarked vs. single marked</td>
<td><em>C. albicans W43</em></td>
<td><em>C. albicans W43-pNZ4</em></td>
</tr>
<tr>
<td></td>
<td><em>C. albicans OD8916</em></td>
<td><em>C. albicans OD8916--pNZ12</em></td>
</tr>
<tr>
<td></td>
<td><em>C. albicans W17</em></td>
<td><em>C. albicans W17-pNZ12</em></td>
</tr>
</tbody>
</table>

Mycophenolic resistance marker (MPA) +: Nourseothricin resistance marker (NAT)

3.7.1.1 Confirmation that strain ratios can be determined using PCR

Examples of the bands seen in PCRs conducted to detect the presence MTL and Antibiotic resistance markers are shown in Figure 3.9 and Figure 3.10. A 1 Kb plus Nucleic Acid marker (*: Invitrogen™ Nucleic Acid Markers) was used to determine product size.

Figure 3.9: PCR amplification of the MTL locus. A: Specific PCR amplification of the MTLa locus. B: Specific PCR amplification of the MTLa locus. Lanes 3, 4, 5 and 6 are mutants and show the presence of both markers. Lane 1 is C. albicans W43a which only has the MTLa marker and lane 2 is C. albicans OD8916 which only has MTLa marker.
Figure 3.10: PCR to detect presence of MPA and NAT markers. A: PCR amplification specific for MPA marker B: PCR amplification specific for NAT marker. Lane 3, 4, 5 and 6 are matants and show presence of both markers. Lane 1 is *C. albicans* W43-pNZ4 which only has the MPA resistance marker and lane 2 is *C. albicans* OD8916-pNZ11 which only has the NAT resistance marker.

The figures above (Figure 3.9 and Figure 3.10) show that PCR could be used to determine whether the colony analysed was either parent of a matant. As seen in Figure 3.9 Lane 1 is *C. albicans* W43a which only has the *MTLa* marker and lane 2 is *C. albicans* OD8916 which only has *MTLa* marker, while Lanes 3, 4, 5 and 6 are matants and show the presence of both markers. Similarly, Figure 3.10 shows that Lane 1 is *C. albicans* W43-pNZ4 which only has the MPA resistance marker and lane 2 is *C. albicans* OD8916-pNZ11 which only has the NAT resistance marker, while lane 3, 4, 5 and 6 are matants and show presence of both markers.
3.7.1.2 **Determination of the effect of an introduced genetic marker on strain survival in vivo**

When rats were co-inoculated with equal numbers of viable cells of two strains that were identical other than the presence of a single selection marker, the unmarked strain survived better than its marked counterpart. Three separate strains (*C. albicans* W43, *C. albicans* OD8916 and *C. albicans* W17) were examined in this manner.

For *C. albicans* W43 (Figure 3.11) [marked with MPA resistance] as early as a week after inoculation the marked strain started showing a minor drop in population with 44% (2.5E+02) of the total strains being marked strains. By 21 days post inoculation this difference in percentage is markedly higher with the marked strains only making up 20% (1.0E+03) of the total yeast population in the oral cavity.

Interestingly, for *C. albicans* OD8916 (Figure 3.12) the marked strain was recovered in greater numbers with nearly 90% of the total strains (2.0E+01) recovered by the end of the study being the marked ones. The number of yeast obtained were very erratic and fluctuated, this could be due to several factors including sampling errors and a change in the status of the animal (growth, immune status, eating habits).

For *C. albicans* W17 (Figure 3.13) it was seen that percentage the of the unmarked strains recovered was higher than that of the marked strain, with the unmarked strain making up to 55% (1.6E+03) of the total cell population (2.8E+03) by day 14 post inoculation. The percentage of the unmarked *C. albicans* strain further increased to up to 90% of the total cell population (3.2E+03) by day 21 post inoculation.
Figure 3.1: Relative survival in the rat oral cavity of unmarked *C. albicans* W43 and a marked derivative. Three rats were inoculated with a mixture of the two strains (1:1) and oral swabs taken at the times indicated. Results are expressed as means of the percentage of the total CFU recovered from swabs by PCR analysis as described in section 3.3.4.
Figure 3.12: Relative survival in the rat oral cavity of unmarked *C. albicans* OD8916 and a marked derivative. Three rats were inoculated with a mixture of the two strains (1:1) and oral swabs taken at the times indicated. Results are expressed as means of the percentage of the total CFU recovered from swabs by PCR analysis as described in section 3.3.4.
Figure 3.13: Relative survival in the rat oral cavity of unmarked *C. albicans* W17 and a marked derivative. Three rats were inoculated with a mixture of the two strains (1:1) and oral swabs taken at the times indicated. Results are expressed as a percentage of the total CFU recovered from swabs by PCR analysis as described in section 3.3.4.
3.7.2 Co-inoculation of rats with strain pairs having a high mating frequency *in vitro*

Several parameters were examined during this series of studies as follows: the white opaque count, the proportion of each parent present in the recovered sample and the presence of markers in recovered possible matants. Two pairs of strains that had shown high *in vitro* mating frequency by Zhang *et al.* were used to co-inoculate the rats.

These pairs were A: *C. albicans* OD8916α-pNZ11 and *C. albicans* W43-pNZ4;

B: *C. albicans* W17α-pNZ11 and *C. albicans* W43-pNZ4

In separate experiments cells were inoculated as either white or opaque derivatives. All results are presented as the proportion of each parent as calculated by randomly picking 20 colonies and identifying each parent by PCR amplifying both the *MTL* locus and the resistance markers.

The experiment using white cells of *C. albicans* OD8916α-pNZ11 and *C. albicans* W43-pNZ4, performed three times (Appendix 4, Table 3.13). However the data shown is that from the third study as the white-opaque count was able to be measured only for this study.

To summarise the findings of this study it was seen that *C. albicans* OD8916-pNZ11 was outcompeted by *C. albicans* W43-pNZ4 by day 28, in both the oral and the fecal samples, both strains were present at nearly equal numbers at day 21 in the oral samples while *C. albicans* OD8916-pNZ11 was detected in only one of the three rats. It is possible there OD8916 was present but that it was not detected in the sample size tested.

Matants were obtained in this experiment at seven days post inoculum (described in more detail in Section 3.7.5). No matants were obtained in subsequent samplings. The total numbers of yeast recovered were maintained at $10^4$ CFU per sample from the oral swab and fecal pellet samples in the experiment using *C. albicans* W43 and *C. albicans* OD8916.

While the candidate has attempted to explain the detailed cfu/samples counts for the figures that follow, for ease of understanding please refer to Appendix 4, Table A4.1. Please also see Appendix 2 Figure A2.1 – A2.6 for the white opaque proportions for this study.
Following inoculation of the rats (three rats were used per experiment) with the pairs of strains which showed high mating *in vitro* it was seen that, as expected, one parent was fitter than the other. In the oral cavity in the experiment with *C. albicans* OD8916α-pNZ11 (P1) and *C. albicans* W43-pNZ4 (P2) [Figure 3.14] it was seen that at day 7, rat 1 had of 370 cfu/swab of P1 and 300 cfu/swab of P2. At day 14 it was seen that P1 was 660 cfu/swab and P2 was 440 cfu/swab. At day 21 P1 was 3238 cfu/swab and P2 was 6013 cfu/swab and in the final sample it was seen that in rat 1, P1 was 214 cfu/swab and P2 was 4057 cfu/swab.

In rat 2 a similar trend was seen with P1 calculated to be 380 cfu/sample and P2 showed 465 cfu/swab at day 7. At day 14, P1 was 245 cfu/sample and P2 was 200 cfu/swab. At day 21 post inoculation P1 was at 1834 cfu/swab and P2 was at 2751 cfu/swab and lastly at day 28 P1 showed 563 cfu/swab and P2 was calculated to be 5081 cfu/swab.

Finally in rat 3, at the first sampling point (day 7) P1 was calculated to be 300 cfu/swab and P2 was calculated to be 200 cfu/swab. At day 14, P1 numbers were at 668 cfu/swab and P2 numbers were at 1002 cfu/swab. P1 was calculated to be 3274 cfu/swab at day 21 and P2 was 4911 cfu/swab at the same time point. At day 28 post inoculation P1 was calculated to be 344 cfu/swab and P2 was calculated to be 6541 cfu/swab.

An interesting, albeit expected, observation to make at this point is that matings where both the parents were opaque gave more matants at earlier samplings than when both parents inoculated were white. However, for the purposes of this thesis it is important to note that, although more matants were obtained, the increase in mating events did not contribute to the overall fitness of the matants.
Figure 3.14: Isolation of yeast from oral samples following co-inoculation with *C. albicans* W43 (*MTLa-MPAr*) and *C. albicans* OD8916 (*MTLa-NATr*). Rats were co-inoculated with white cells of *C. albicans* W43 (red) and *C. albicans* OD8916 (blue). The R in the figure is the rat number, triangles represent rat 1, squares rat 2 and circles rat 3.

In the fecal pellet in the same experiment (Figure 3.15) it was seen that at day 7, rat 1 had a calculated value of 745 cfu/sample of P1 and 745 cfu/sample of P2. At day 14 it was seen that P1 was 1050 cfu/sample and P2 was 1050 cfu/sample. At day 21 P1 was 355 cfu/sample and P2 was 6745 cfu/sample and in the final sample it was seen that in rat 1, P1 was not detected and P2 was at 22200 cfu/sample.

In rat 2, it was seen that P1 was calculated to be 2691 cfu/sample and P2 showed 3289 cfu/sample at day 7. At day 14, P1 was 2134 cfu/sample and P2 was 1746 cfu/sample. At day 21 post inoculation P1 was not detected and P2 was at 3155 cfu/sample and lastly at day 28 P1 not detected within the sample size and P2 was calculated to be 5795 cfu/sample.

Finally in rat 3, at the first sampling point (day 7) P1 was calculated to be 3734 cfu/swab and P2 was calculated to be 3055 cfu/swab. At day 14, P1 numbers were at 3640 cfu/swab and P2 numbers were at 5460 cfu/swab. P1 was calculated to be 19980 cfu/swab at day 21 and P2...
was 2220 cfu/swab at the same time point. At day 28 post inoculation P1 was calculated to be 2680 cfu/swab and P2 was calculated to be 50920 cfu/swab.

At this stage the candidate feels it important to point out that the focus of this thesis is the rat ORAL model of colonisation. The data from the faecal pellet was also used to describe the levels of colonisation, the candidate has to point out that it was inconsistent as it varied depending on the size of the pellet and consistency of the pellet. They have been commented on in the text describing the graphs in the results section of the thesis.

Figure 3.15: Isolation of yeast from fecal samples following co-inoculation with *C. albicans* W43 (*MTLa-MPA*) and *C. albicans* OD8916 (*MTLa-NAT*). Rats were co-inoculated with white cells of *C. albicans* W43 (red) and *C. albicans* OD8916 (blue). The R in the figure is the rat number, triangles represent rat 1, squares rat 2 and circles rat 3.

It was then decided to investigate whether inoculation of the rats with opaque cells to would increase the frequency of obtaining recombination events *in vivo*. It was also decided to measure the proportion of white to opaque cells present at each sampling point. It was seen that both parents were recoverable even 28 days post inoculation in only one of the two rats in as high as $10^4$ Cfu/sample in the oral swab (Figure 3.16) and $10^3$ in the fecal sample 28 days post inoculation (Figure 3.17).
Figure 3.16: Isolation of yeast from oral samples following co-inoculation with *C. albicans* W43 (MTLa-MPA<sup>+</sup>) and *C. albicans* OD8916 (MTLa-NAT<sup>4</sup>). Rats were co-inoculated with opaque cells of *C. albicans* W43 (red) and *C. albicans* OD8916 (blue). The R in the figure is the rat number, triangles represent rat 1, squares rat 2 and circles rat 3.

Following inoculation of the rats (three rats were used per experiment) with opaque cells of the pairs of strains which showed high mating *in vitro* it was again seen that one parent was fitter than the other. In the oral cavity in the experiment with *C. albicans* OD8916α-pNZ11 (P1) and *C. albicans* W43-pNZ4 (P2) [Figure 3.15] it was seen that at day 7, rat 1 had of 2000 cfu/swab of P1 and 1500 cfu/swab of P2. At day 14 it was seen that P1 was 1833 cfu/swab and P2 was 2750 cfu/swab. At day 21 P1 was 9585 cfu/swab and P2 was 9585 cfu/swab and in the final sample it was seen that in rat 1, P1 was 47900 cfu/swab and P2 was 2521 cfu/swab.

In rat 2 a similar trend was seen with P1 calculated to be 1500 cfu/sample and P2 showed 1536 cfu/swab at day 7. At day 14, P1 was 3638 cfu/sample and P2 was 2425 cfu/swab. At day 21 post inoculation P1 was at 1168 cfu/swab and P2 was at 778 cfu/swab and lastly at day 28 P1 showed 62500 cfu/swab and P2 was calculated to be 6944 cfu/swab.
Finally in rat 3, at the first sampling point (day 7) P1 was calculated to be 1408 cfu/swab and P2 was calculated to be 1250 cfu/swab. At day 14, P1 numbers were at 1113 cfu/swab and P2 numbers were at 2783 cfu/swab. P1 was calculated to be 7700 cfu/swab at day 21 and P2 was 5133 cfu/swab at the same time point. At day 28 post inoculation P1 was calculated to be 52600 cfu/swab and P2 was calculated to be 2768 cfu/swab.

For counts in tabular format please refer to Appendix 4, Table A4.1.

The proportion of white to opaque cells was also measured (by plating samples on YPD agar containing the dye Phloxine B) for both the oral swab and the fecal pellet (Appendix 2, Figure A2.1); the proportion of white cells increases until it is ~96%, by day 28, for both oral and fecal samples.

In the fecal pellet in the same experiment (Figure 3.17) it was seen that at day 7, P1 was not detected in rat 1 and P2 had a calculated value of 2980 cfu/sample. At day 14 and subsequent samplings it was seen that P1 was undetected, while P2 was calculated to be 4200 cfu/sample, 4250 cfu/sample and 1100 cfu/sample at day 14, day 21 and day 28 respectively.

In rat 2 it was seen that P1 was calculated to be 3331 cfu/sample and P2 showed 10873 cfu/sample at day 7. At day 14, P1 was 3818 cfu/sample and P2 was 8622 cfu/sample. At day 21 post inoculation P1 was not detected and P2 was at 4250 cfu/sample and lastly at day 28 P1 not detected within the sample size and P2 was calculated to be 2240 cfu/sample.

Finally in rat 3, at the first sampling point (day 7) P1 was calculated to be 10873 cfu/swab and P2 was calculated to be 15089 cfu/swab. At day 14, P1 numbers were at 9700 cfu/swab and P2 numbers were at 15167 cfu/swab. P1 was calculated to be 1444 cfu/swab at day 21 and P2 was 13000 cfu/swab at the same time point. At day 28 post inoculation P1 was calculated to be 26000 cfu/swab and P2 was calculated to be 1368 cfu/swab. Please refer to Appendix 2, Figure A2.1 for white to opaque proportions in this study.
Figure 3.17: Isolation of yeast from fecal samples following co-inoculation with *C. albicans* W43 (MTLa-MPA\(^\alpha\)) and *C. albicans* OD8916 (MTLa-NAT\(^\delta\)). Rats were co-inoculated with opaque cells of *C. albicans* W43 (red) and *C. albicans* OD8916 (blue). The R in the figure is the rat number, triangles represent rat 1, squares rat 2 and circles rat 3.

When the experiment was repeated using *C. albicans* W43 and *C. albicans* W17 it was seen that the total yeast numbers reached > 10\(^3\) cfu/sample for both the oral (Figure 3.18) and the fecal samples (Figure 3.19) when white cells were used as inoculum. It was also seen that *C. albicans* W17 outcompeted *C. albicans* W43 in both the oral cavity and the fecal pellet in all rats by day 21 except for rat2 in the oral cells where *C. albicans* W43 recovered in numbers but was not detected at day 28.
Figure 3.18: Isolation of yeast from oral samples following co-inoculation with *C. albicans* W43 (*MTLa*-MPA<sup>¢</sup>) and *C. albicans* W17 (*MTLa*-NAT<sup>¢</sup>). Rats were co-inoculated with white cells of *C. albicans* W43 (red) and *C. albicans* W17 (green). The R in the figure is the rat number, triangles represent rat 1, squares rat 2 and circles rat 3.

In the oral cavity in the experiment with *C. albicans* W17α-pNZ11 (P1) and *C. albicans* W43-pNZ4 (P2) (Figure 3.18) it was seen that at day 7, rat 1 had of 340 cfu/swab of P1 and 280 cfu/swab of P2. At day 14 it was seen that P1 was 1500 cfu/swab and P2 was not detected. At day 21 P1 was 4000 cfu/swab and P2 was not detected and in the final sample it was seen that in rat 1, P1 was 3200 cfu/swab and P2 was still undetected.

In rat 2 a similar trend was seen with P1 calculated to be 675 cfu/sample and P2 showed 75 cfu/swab at day 7. At day 14, P1 was 4100 cfu/sample and P2 was undetected. At day 21 post inoculation P1 was at 12128 cfu/swab and P2 was at 638 cfu/swab and lastly at day 28 P1 showed 12400 cfu/swab and P2 was undetected in the sample.

Finally in rat 3, at the first sampling point (day 7) P1 was calculated to be 182 cfu/swab and P2 was calculated to be 10 cfu/swab. At day 14, P1 numbers were at 997 cfu/swab and P2 numbers were at 52 cfu/swab. P1 was calculated to be 2256 cfu/swab at day 21 and P2 was
not detected at the same time point. At day 28 post inoculation P1 was calculated to be 1515 cfu/swab and P2 was undetected in the sample.

In the fecal pellet for the same experiment (Figure 3.19) it was seen that at day 7, P1 was calculated to be 1464 cfu/sample in rat 1 and P2 had a calculated value of 163 cfu/sample. At day 14 P1 was calculated to be 143 cfu/sample and P2 was calculated to be 8 cfu/sample. At subsequent samplings it was seen that P2 undetected, while P1 was calculated to be 3893 cfu/sample, and 3065 cfu/sample at day 21 and day 28 respectively. In rat 2 it was seen that P1 was calculated to be 162 cfu/sample and P2 showed 29 cfu/sample at day 7. At day 14, P1 was calculated to be 162 cfu/sample and P2 was 205 cfu/sample. At day 21 post inoculation P2 was not detected and P1 was at 630 cfu/sample and lastly at day 28, P2 was not detected within the sample size and P1 was calculated to be 3252 cfu/sample. In rat 3, at the first sampling point (day 7) P1 was calculated to be 2187 cfu/swab and P2 was calculated to be 243 cfu/swab. At day 14, P1 numbers were at 998 cfu/swab and P2 numbers were at 53 cfu/swab. P1 was calculated to be 9233 cfu/swab at day 21 and P2 was undetected at the same time point. At day 28 post inoculation P1 was calculated to be 1243 cfu/swab and P2 was still undetected.
Figure 3.19: Isolation of yeast from fecal samples following co-inoculation with *C. albicans* W43 (MTLa-MPA°) and *C. albicans* W17 (MTLa-NAT°). Rats were co-inoculated with white cells of *C. albicans* W43 (red) and *C. albicans* W17 (green). The R in the figure is the rat number, triangles represent rat 1, squares rat 2 and circles rat 3.

Again it was decided to inoculate the rats with opaque cells to investigate whether this would increase the frequency of mating events and hence the number of matants detected in the samples for these strains (Figure 3.20 and Figure 3.21).

In the oral cavity in the experiment where rats were inoculated with opaque cells of *C. albicans* W17α-pNZ11 (P1) and *C. albicans* W43-pNZ4 (P2) [Figure 3.20] it was seen that at day 7, rat 1 had of 344 cfu/swab of P1 and 281 cfu/swab of P2. At day 14 it was seen that P1 was 1505 cfu/swab and P2 was not detected. At day 21 P1 was 479 cfu/swab and P2 was calculated to be 25 cfu/swab and in the final sample it was seen that in rat 1, P1 was 950 cfu/swab and P2 was calculated to be at 50cfu/swab.

Unfortunately, no *C. albicans* were able to be recovered from rat 2 at sampling point 1. However at day 14 it was seen that a small number of cells were recovered and P1 was calculated to be only 10cfu/swab (only one colony was recovered) and at day 21 post
inoculation P1 numbers increased to give 836 cfu/swab and P2 was at 44 cfu/swab and lastly at day 28 P1 showed 779 cfu/swab and P2 was calculated to be 41 cfu/swab.

Finally in rat 3, at the first sampling point (day 7) P1 was calculated to be 576 cfu/swab and P2 was calculated to be 384 cfu/swab. At day 14, P1 numbers were at 3487 cfu/swab and P2 numbers were at 184 cfu/swab. P1 was calculated to be 3990 cfu/swab at day 21 and P2 was not detected at the same time point. At day 28 post inoculation P1 was calculated to be 1045 cfu/swab and P2 was undetected in the sample.

The proportion of white to opaque cells was also measured for both the oral swab and the fecal pellet (Appendix 2, Figure A2.2); the proportion of white cells increases until it is ~100%, by day 21, for both oral and fecal samples.

Figure 3.20: Isolation of yeast from oral sample following co-inoculation with *C. albicans* W43 (*MTLa-MPAr*) and *C. albicans* W17 (*MTLa-NATr*). Rats were co-inoculated with opaque cells of *C. albicans* W43 (red) and *C. albicans* W17 (green). The R in the figure is the rat number, triangles represent rat 1, squares rat 2 and circles rat 3.
In the fecal pellet for the same experiment (Figure 3.21) it was seen that at day 7, P1 was calculated to be 367 cfu/sample in rat 1 and P2 had a calculated value of 448 cfu/sample. At day 14 P1 was calculated to be 15000 cfu/sample and P2 was calculated to be 460 cfu/sample. At day 21 and day 28 samplings it was seen that P2 was at 126 cfu/sample and 82 cfu/sample respectively, while P1 was calculated to be 2389 cfu/sample, and 1553 cfu/sample at day 21 and day 28 respectively. In rat 2 it was seen that P1 was calculated to be 2292 cfu/sample and P2 showed 3438 cfu/sample at day 7. At day 14, P1 was 1520 cfu/sample and P2 was 80 cfu/sample. At day 21 post inoculation P2 was not detected and P1 was at 1320 cfu/sample and lastly at day 28, no *C. albicans* detected within the sample size. In rat 3, at the first sampling point (day 7) both P1 and P2 were calculated to be at similar numbers (~7000 cfu/swab). At day 14, P1 numbers were at 3995 cfu/swab and P2 numbers were at 705 cfu/swab. P1 was calculated to be 2605 cfu/swab at day 21 and P2 was undetected at the same time point. At day 28 post inoculation P1 was calculated to be 3705 cfu/swab and P2 was calculated to be 195 cfu/sample. Please refer to Appendix 2, Figure A2.2 for white to opaque proportions in this study.
Figure 3.21: Isolation of yeast from fecal sample following co-inoculation with \textit{C. albicans} W43 (\textit{MTLa}-\text{MPA}^{5}) and \textit{C. albicans} W17 (\textit{MTLa}-\text{NAT}^{5}). Rats were co-inoculated with opaque cells of \textit{C. albicans} W43 (red) and \textit{C. albicans} W17 (green). The R in the figure is the rat number, triangles represent rat 1, squares rat 2 and circles rat 3.
3.7.3 Co-inoculation of rats with strains having medium, low or zero *in vitro* mating frequency

Competition experiments were also carried out using strains that showed low or no mating in *in vitro* experiments (Zhang 2012). The combination of the strains used and the type parameters adjusted in the inoculum to try and obtain better mating *in vivo* are shown in Table 3.8.
Table 3.8: *In vivo* recombination of strains with low or medium *in vitro* recombination frequency*.

<table>
<thead>
<tr>
<th>Experiment</th>
<th><em>C. albicans</em> (Strain 1)</th>
<th><em>C. albicans</em> (Strain 2)</th>
<th>Recombinants Obtained</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain pairs with medium frequency <em>in vitro</em> mating</td>
<td>YSU63α pNZ11</td>
<td>W43 pNZ4</td>
<td>No</td>
</tr>
<tr>
<td>Au90a pNZ4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium frequency mating strains, but inoculated with opaque cells</td>
<td>YSU63α pNZ11</td>
<td>W43 pNZ4</td>
<td>No</td>
</tr>
<tr>
<td>Au90a pNZ4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium frequency mating strains, but inoculated with unequal numbers of each parent</td>
<td>YSU63α+ pNZ11</td>
<td>W43* pNZ4</td>
<td>No</td>
</tr>
<tr>
<td>Au90α+ pNZ4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strains showing low or no <em>in vitro</em> mating</td>
<td>Au90a pNZ4</td>
<td>FJ11α pNZ11</td>
<td>No</td>
</tr>
<tr>
<td>W43a pNZ4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strains showing low or no <em>in vitro</em> mating, but inoculated with opaque cells</td>
<td>Au90a pNZ4</td>
<td>FJ11α pNZ11</td>
<td>No</td>
</tr>
<tr>
<td>W43a pNZ4</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Inoculum contained 2 x 10^6 cells. \(^\ast\): inoculum contained 1 x 10^6 cells.
While *in vitro* recombination experiments obtained matants with these strains (Zhang 2012), no matants were detected in this study after the *in vivo* co-inoculation of the oral cavity (Table 3.5). No opaque cells were detected post inoculation in any of the studies investigating strains with medium *in vitro* mating frequencies. Yeast numbers in the fecal pellet were as high as $10^4$ CFU/sample for *C. albicans* YSU63 (Figure 3.22) and *C. albicans* W43 was outcompeted by *C. albicans* YSU63, 7 days post-inoculation (Figure 3.22 and Figure 3.23).

![Oral Samples](image)

**Figure 3.22:** Isolation of yeast from oral samples following co-inoculation with *C. albicans* W43 (*MTLa*-MPA*) and *C. albicans* YSU63 (*MTLa*-NAT*). Rats were co-inoculated with white cells of *C. albicans* W43 (red) and *C. albicans* YSU63 (yellow). The R in the figure is the rat number, triangles represent rat 1, squares rat 2 and circles rat 3.

As no matants were observed in these experiments, the candidate will not go into the specific details of the colony numbers of each of the parents in these set of studies. What the candidate expects to achieve through the showing of these data is the trend in the proportion of parents at each time point. For more details on the actual colony counts please refer to Appendix 4, Table A4.2.

As with the oral samples, the fecal samples also showed that *C. albicans* YSU63 outcompeted *C. albicans* W43 in rat 1 by day 14, and in rat 2 and rat 3 by day 21 of the study.
For more details on the actual colony counts please refer to Appendix 4, Table A2.2.

**Figure 3.23:** Isolation of yeast from fecal sample following co-inoculation with *C. albicans* W43 (*MTLa*-MPA<sup>+</sup>) and *C. albicans* YSU63 (*MTLa*-NAT<sup>+</sup>). Rats were co-inoculated white cells of *C. albicans* W43 (red) and *C. albicans* YSU63 (yellow). The R in the figure is the rat number, triangles represent rat 1, squares rat 2 and circles rat 3.

In experiments where the oral cavities of rats were inoculated with opaque cells only, a different trend was seen in the oral (Figure 3.24) and in the fecal samples (Figure 3.25). In the oral cavity both *C. albicans* YSU63 and *C. albicans* W43 were present in equal amounts up to 21 days post-inoculation. At the final sampling point (day 28) *C. albicans* W43 was outcompeted by *C. albicans* YSU63 in two rats. A different trend was seen in fecal samples. *C. albicans* YSU63 was outcompeted by *C. albicans* W43 in one rat while the numbers of both strains were similar in the other two rats.
Figure 3.24: Isolation of yeast from oral sample following co-inoculation with *C. albicans* W43 (MTLa-MPA<sup>−</sup>) and *C. albicans* YSU63 (MTLa-NAT<sup>+</sup>). Graph of yeast present in oral samples from rats co-inoculated with opaque cells of *C. albicans* W43 (red) and *C. albicans* YSU63 (yellow). The R in the figure is the rat number, triangles represent rat 1, squares rat 2 and circles rat 3.
Figure 3.25: Isolation of yeast from fecal sample following co-inoculation with *C. albicans* W43 (MTLa-MPA<sup>t</sup>) and *C. albicans* YSU63 (MTLa-NAT<sup>t</sup>). Graph of yeast present in fecal samples from rats co-inoculated with opaque cells of *C. albicans* W43 (red) and *C. albicans* YSU63 (yellow). The R in the figure is the rat number, triangles represent rat 1, squares rat 2 and circles rat 3.

For more details on the actual colony counts please refer to Appendix 4, Table A4.3.

In the following set of experiments, rats were inoculated with twice as many of the parent (Table 3.8) that was outcompeted by the other parent in the previous experiments (*C. albicans* W43).
Figure 3.26: Isolation of yeast from oral sample following co-inoculation with *C. albicans* W43 (*MTLa*-MPA) and *C. albicans* YSU63 (*MTLa*-NAT). Graph of yeast present in oral samples from rats co-inoculated with opaque, uneven numbers of *C. albicans* W43 (red) and *C. albicans* YSU63 (yellow). The R in the figure is the rat number, triangles represent rat 1, squares rat 2 and circles rat 3.

The co-inoculation of rats with *C. albicans* YSU63 and *C. albicans* W43 (Figure 3.26 and Figure 3.27) it was shows that the numbers of the parent that was inoculated with a higher number (*C. albicans* W43) remained almost the same as the other parent, in all rats in the oral sample, until day 21 post-inoculation, and dropped to a ten-fold lower number of cells by day 27 post-inoculation. In the fecal sample a similar trend was seen with all rats showing similar number of parents 14 days post inoculum. At 21 days post inoculum *C. albicans* W43 was recovered in lesser numbers than the *C. albicans* YSU63. However both parents were still recovered at the last sampling point in two of the three rats in the test group. For more details on the actual colony counts please refer to Appendix 4, Table A4.3.
Figure 3.27: Isolation of yeast from fecal sample following co-inoculation with *C. albicans* W43 (*MTLa-MPA*') and *C. albicans* YSU63 (*MTLa-NAT*'). Graph of yeast present in oral samples from rats co-inoculated with opaque, uneven numbers of *C. albicans* W43 (red) and *C. albicans* YSU63 (yellow). The R in the figure is the rat number, triangles represent rat 1, squares rat 2 and circles rat 3.

As above, no matants were detected in subsequent experiments using low to zero *in vitro* mating experiments. I have therefore included the figures and the colony counts in the Appendix and referred to them in the following text supplying. This will give the reader an overview of how each parent survived in the *in vivo* model of colonisation.

In the experiment with *C. albicans* HUN97 and *C. albicans* Au90, (Appendix 2, Figure A2.7-A2.8) it was seen that *C. albicans* Au90 survived better than its co-inoculant. Although the amount of was almost of *C. albicans* HUN97 in the inoculum was the same as *C. albicans* Au90 the recovery of *C. albicans* Au90 throughout the course of the study was erratic, with a higher number of *C. albicans* HUN97 being detected at day 7 and day 21 in rat 2 and rat 3 and at day 14 in rat 1. (For more details on the colony counts for all experiments employing the use of *C. albicans* HUN97 and *C. albicans* Au90 please refer to Appendix 4, Table A4.4)
Graphs of white to opaque proportion are not shown because no opaque isoforms were detected post inoculation, in both the oral cavity, and the fecal pellet for this experiment.

In experiments where the oral cavity is inoculated with only opaque cells it was seen that in the oral cavity (Appendix 2, Figure A2.9) \textit{C. albicans} Au90 outcompeted \textit{C. albicans} HUN97. In the fecal sample (Appendix 2, Figure A2.10) a trend similar to that in the oral cavity was seen wherein \textit{C. albicans} Au90 outcompeted \textit{C. albicans} HUN97. Especially in Rat 3 none of the latter were detected in the fecal sample from day 14 onwards. On counting the white to opaque numbers it was seen that all opaque cells switched \textit{en masse} to white cells except for the fecal pellet in the recombination study of \textit{C. albicans} HUN97 and \textit{C. albicans} Au90 where it was seen that up to 20\% of the cells were opaque 14 days post sampling. No mutants were detected at all for both the studies.

In the experiment where the rats were inoculated with two times more (Table 3.8) of the parent that showed to be outcompeted by the other in previous studies (\textit{C. albicans} HUN97). In the co-inoculation with \textit{C. albicans} Au90 and \textit{C. albicans} HUN97 (Appendix 2, Figure A2.11-A2.12) it was seen that similar numbers of co-inoculants were recovered in all rats till day 21 post inoculation. The numbers of the parent \textit{C. albicans} HUN97 reduced slightly on the last sampling point (day 27 post inoculation). At this stage it would be interesting to point out that both parents were recovered at the last sampling point in all the three animals.
In experiments conducted to investigate whether mating occurs in vivo between strains showing a low or no frequency of mating in vitro, it was seen that while the colonising yeast numbers were as high as $10^4$ CFU/sample in both the combinations investigated, one parent was quickly outcompeted by the other parent. In both studies conducted C. albicans FJ11 survived better than the other parental strains namely C. albicans AU90 and C. albicans W43. For the experiment involving C. albicans Au90 and C. albicans FJ11 (Appendix 2, Figure A2.13 and Figure A2.14), C. albicans FJ11 outcompeted C. albicans Au90 with none of the latter strain detected in rat 1 at 14 days post-inoculation, in rat 2 at 21 days post-inoculation and at the endpoint no C. albicans Au90 cells were detected at all. Graphs of white to opaque proportions are not presented at this stage because no opaque colonies were detected in any of the samples post inoculation, as seen in the earlier studies, all opaque cells switched en masse to white after being inoculated into the oral cavity of the Rat. (For colony counts of all experiments employing the use of C. albicans FJ11 and C. albicans Au90 as a combination please refer to Appendix 4, Table A4.5).

For experiments where the inoculum consisted of only opaque cells, once again it was seen that C. albicans FJ11 survived better than C. albicans Au90 (Appendix 2, Figure A2.15 and Figure A2.16). In rat 1 and rat 2 C. albicans Au90 cells were not detected in the oral sample at the last sampling point (day 28) or at day 14 (sample 2) in rat 3.

In the fecal samples (Appendix 2, Figure A2.16) as well it was seen that C. albicans FJ11 outcompeted C. albicans Au90. With no cells of the latter detected in rat 2 and rat 3 at sampling point 3 (day 21) and sampling point 4 (day 28) post inoculation.

In the experiment using C. albicans W43 and C. albicans FJ11 it was seen that the latter outcompeted C. albicans W43 in both the oral (Appendix 2, Figure A2.17) and fecal (Appendix 2, Figure A2.18) samples. However it is interesting to note that C. albicans FJ11 established itself in the oral model in the expected numbers ($>10^3$ CFU sample) only 21 days post-inoculation (For colony counts of all experiments employing the use of C. albicans FJ11 and C. albicans W43 as a combination please refer to Appendix 4, Table A4.6).

In an experiment using opaque cells, however, it was seen that C. albicans FJ11 established itself fairly early on in the study, with numbers reaching the expected values of approximately $10^4$ cfu/sample in both the oral (Appendix 2, Figure A2.19) and fecal (Appendix 2, Figure A2.20) samples. In rat 3 no C. albicans W43 cells were seen 14 days post-inoculation or in
subsequent samples. In rat 1, although the strain cell numbers were seen to recover at day 21 (sample 3) post-inoculation, none were detected at day 28.

At this stage it was indicated by Dr. Ningxin Xhang (Zhang 2012) that the C. albicans strain FJ11 is unable to undergo mating because of the presence of a mutation in the mating locus. Following this discovery it was decided not to investigate in vivo mating using this strain any further.
3.7.4 Investigation of mating *in vivo*

A major finding of this work was the discovery of mating *in vivo* in the rat oral colonisation model. Initially the rats were inoculated with the white form of *C. albicans* strains and the samples were screened for matants. Table 3.9 is a summary of all the investigations conducted in order to ascertain whether mating occurs *in vivo*.

**Table 3.9:** Investigating mating *in vivo*.

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Parent 1</th>
<th>Parent 2</th>
<th>Matants obtained</th>
</tr>
</thead>
<tbody>
<tr>
<td>White cells</td>
<td>OD8916</td>
<td>W43</td>
<td>Yes</td>
</tr>
<tr>
<td>White cells</td>
<td>W17</td>
<td>W43</td>
<td>Yes</td>
</tr>
<tr>
<td>White cells</td>
<td>YSU63α</td>
<td>W43</td>
<td>No</td>
</tr>
<tr>
<td>White cells</td>
<td>Au90a</td>
<td>HUN97</td>
<td>No</td>
</tr>
<tr>
<td>White cells</td>
<td>Au90a</td>
<td>FJ11α</td>
<td>No</td>
</tr>
<tr>
<td>White cells</td>
<td>W43a</td>
<td>FJ11α</td>
<td>No</td>
</tr>
<tr>
<td>Opaque cells</td>
<td>OD8916</td>
<td>W43</td>
<td>Yes</td>
</tr>
<tr>
<td>Opaque cells</td>
<td>W17</td>
<td>W43</td>
<td>Yes</td>
</tr>
<tr>
<td>Opaque cells</td>
<td>YSU63α</td>
<td>W43</td>
<td>No</td>
</tr>
<tr>
<td>Opaque cells</td>
<td>Au90a</td>
<td>HUN97</td>
<td>No</td>
</tr>
<tr>
<td>Opaque cells</td>
<td>Au90a</td>
<td>FJ11α</td>
<td>No</td>
</tr>
<tr>
<td>Opaque cells</td>
<td>W43a</td>
<td>FJ11α</td>
<td>No</td>
</tr>
<tr>
<td>Unequal number of opaque cells</td>
<td>YSU63α</td>
<td>W43</td>
<td>No</td>
</tr>
<tr>
<td>Unequal number of opaque cells</td>
<td>Au90a</td>
<td>HUN97</td>
<td>No</td>
</tr>
</tbody>
</table>
Below in Figure 3.28 shows an example of a PCR amplification for the detection of matants from an *in vivo* sample.

**Figure 3.28**: Detection of matants in *in vivo* by PCR amplification. PCR amplification of MAT (top image) and MPA/NAT resistance markers (lower image) for matants obtained in the co-inoculation of *C. albicans* W43a and *C. albicans* OD8916 and the matants obtained in the co-inoculation of *C. albicans* W43a and *C. albicans* W17. C1 and C2 are the singly marked parental controls. Lanes marked P or M indicate PCR fragments from representative parental strains or matant strains, respectively.

In the experiments where the rat model was inoculated with white cells of *C. albicans* OD8916 and *C. albicans* W43a, it was seen that matants were obtained at 7 days post inoculation (Sampling point 1) [Figure 3.28] at a total calculated value of 150 CFU/sample in the oral sample and 80 CFU/sample in the Fecal pellet. The oral cavity gave a calculated value of 30 CFU/sample at day 14 (sampling point 2) while the fecal cavity gave a calculated value of 10 CFU/sample. In a second experiment, a total calculated value of 40 CFU/sample
of matants were obtained in the oral cavity and 10 CFU/sample at the first sampling point and 10 CFU/sample of matants were detected in the oral cavity and 20 CFU/sample in the fecal cavity, 14 days (sampling point 2) after inoculation. In a third replicate experiment conducted it was seen that 70 CFU/sample of matants were obtained in the oral cavity, while in the fecal cavity 150 CFU/sample were obtained at the first sampling point, followed by 80 CFU/sample in the oral cavity and 90 CFU/sample in the fecal sample at the second sampling point and for the first time at sampling point 3 a calculated value of 10 CFU/sample of matants were obtained in the oral cavity and 30 CFU/sample of matants were detected in the fecal sample.

Inoculating the oral cavity with opaque only cells showed matants were detected early in the sampling process (day 7) post inoculation, with a total calculated value of 321 CFU/sample in the oral cavity and a calculated value of 230 CFU/sample in the fecal cavity. At sampling point 2 matants were detected at a calculated value of 154 CFU/sample in the oral cavity and 150 CFU/sample in the fecal sample and at sampling point 3 at a calculated value of 30 CFU/sample in the oral cavity and no matants were seen in the fecal sample from this point onwards (Appendix 4, Table A4.1).

In the experiment where the rat model was inoculated with white cells of \textit{C. albicans} W17 and \textit{C. albicans} W43a it was seen that the oral cavity gave a calculated value of 40 CFU/sample matants while the fecal pellet had 10 CFU/sample matants However when the oral cavity was inoculated with opaque cells it was seen that matants detected at day 7 (sample 1) were calculated to be 297 CFU/sample in the oral cavity while the fecal cavity showed 149 CFU/sample (Appendix 4, Table A4.2 ). No matants were found for the subsequent sampling points.

3.7.5 Flow cytometry of matants obtained \textit{in vivo}

When the ploidy of the matants (Table 3.10) were calculated using cells grown in the stationary phase it was seen that all matants showed higher DNA content that the average of the two parents’ combined DNA content. It is also observed that the DNA content of the oral samples were more than that of the fecal samples a point that will be discussed later (see Future Directions).
Table 3.10: Ploidy of Matants obtained from the \textit{in vivo} model.

<table>
<thead>
<tr>
<th>Matant strain (\textit{C. albicans})</th>
<th>Calculated ploidy</th>
</tr>
</thead>
<tbody>
<tr>
<td>OD8916XW43 (FS1)</td>
<td>1.77</td>
</tr>
<tr>
<td>W17 x W43 (FS1)</td>
<td>1.40</td>
</tr>
<tr>
<td>OD8916XW43 (FS2)</td>
<td>2.40</td>
</tr>
<tr>
<td>W17 x W43 (FS2)</td>
<td>2.00</td>
</tr>
<tr>
<td>OD8916XW43 (OS2)</td>
<td>2.80</td>
</tr>
<tr>
<td>W17 x W43 (OS1)</td>
<td>2.19</td>
</tr>
<tr>
<td>OD8916XW43 (OS2)</td>
<td>1.84</td>
</tr>
<tr>
<td>OD8916XW43 (OS2)</td>
<td>3.41</td>
</tr>
</tbody>
</table>

F: fecal sample, O: oral sample and the number indicates the sampling point at which it was obtained.
3.7.6 Investigation of the loss of markers by matants in the oral cavity

It was decided to investigate whether due to the genetic load on putative matants, some of the matants might induce marker loss in order to better survive in the host. It was therefore decided to check for possible marker loss by inoculating the oral cavity of the rats with matants obtained as a result of an *in vivo* mating event.

In this experiment model organism was inoculated with $3 \times 10^6$ cells of the matants obtained from *in vivo* matings (namely *C. albicans* OD8916 x *C. albicans* W43, and *C. albicans* W17x *C. albicans* W43) of *C. albicans*, in order to investigate whether matants lose either or both of their antibiotic resistance markers *in vivo*. The data for this study is shown in Table 3.11. It was observed that minimal marker loss was detected in either of the matants tested.
Table 3.11: Investigation of loss of markers of matants \textit{in vivo} obtained from a mating event between \textit{C. albicans} OD8916 and \textit{C. albicans} W43.

<table>
<thead>
<tr>
<th></th>
<th>Counts For Sample 1/ WEEK 3</th>
<th>Counts For Sample 2/ WEEK 4</th>
<th>Counts For Sample 3/ WEEK 5</th>
<th>Counts For Sample 4/ WEEK 6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Avg CFU/ml on YPD + CM /0.1ml (OD8916xW43)</td>
<td>No tested</td>
<td>No with +ve PCR for marker tested</td>
<td>number showing marker loss</td>
</tr>
<tr>
<td>oral swabs</td>
<td>Rat1 1.39E+02</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Rat2 7.30E+01</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Rat3 1.28E+02</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Rat3 1.28E+02</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
</tbody>
</table>
3.7.7 Investigation of fitness of matants *in vivo*

In the survivability experiment using *C. albicans* W43 and its corresponding matant (Figure 3.29) it was seen that in as early as a week post inoculation the numbers of matants begin to fall while its corresponding parent takes over. By day 14 the parents make up as much as 80% of the total *C. albicans* population. Only a slight variation was seen between rats study with parents making up 60% of the total *C. albicans* population in rat 3, at day 7 post inoculation, while in R1 and R2 parents made up 70% and 80% of the total population respectively. Parents totally outcompeted the matants by Day 28 of the study. No matants were recovered from oral cavity in R1 and R2.

![Percentage recombinants vs. parents](image)

**Figure 3.29**: Survivability of *C. albicans* OD8916xW43 (matants) obtained *in vivo* versus *C. albicans* W43. Rats were inoculated with a mixture of parents and matants in a ratio of 1:1 and oral swabs were taken at 7, 14, 21, and 28 days.
In the study investigating the survivability of *C. albicans* OD8916 parent with its corresponding matant (Figure 3.30) it was seen that matant numbers reduced dramatically in as early as 7 days post posy inoculum. In R1 the matants made up only 25% of the total *C. albicans* population in the oral cavity. While in R2 and R3 the matants made up 10% of the total *C. albicans* population. By day 21 all the cells harvested were parents. Which indicates that *C. albicans* OD8916 totally outcompeted its corresponding matant. In Rat 2 no matants were recovered in as early as 14 days post inoculation.

![Figure 3.30: Survivability of *C. albicans* OD8916xW43 (matants) obtained *in vivo* versus *C. albicans* OD8916. Rats were inoculated with a mixture of parents and matants in a ratio of 1:1 and oral swabs were taken at 7, 14, 21, and 28 days.](image)

In the study where *C. albicans* W17 was tested for survival against its corresponding matants (Figure 3.31, Figure 3.32) it was seen that in the case where the parent was *C. albicans* W17 matant numbers were lowered considerably in as early as day 7 post inoculum with matants making up as little as 10% in R1, 10% in R2 and as low as only 5% of the total CFU population in R3. It was seen that by sample 2 (day 14) all the cells isolated were parents indicating that recombinants are not as fit as parents in the *in vivo* scenario.
Figure 3.31: Survivability of *C. albicans* W17xW43 (matants) obtained *in vivo* versus *C. albicans* W17. Rats were inoculated with a mixture of parents and matants in a ratio of 1:1 and oral swabs were taken at 7, 14, 21, and 28 days.

In the study where *C. albicans* W43 parent was inoculated with a matant resulting from a cross between *C. albicans* W43 and *C. albicans* W17 it was observed that the matants were 25% of the total population in R1 and R3 and only 20% of the total population in R2. It was observed that by the end of the study matants only made of 105 of the total population in R1 and R3 and 5% of the total population in R2. This clearly indicates that the matants are less fit than the parents when tested for survival in the *in vivo* model.
Figure 3.32: Survivability of *C. albicans* W17xW43 (matants) obtained *in vivo* versus *C. albicans* W43. Rats were inoculated with a mixture of parents and matants in a ratio of 1:1 and oral swabs were taken at 7, 14, 21, and 28 days.
### 3.7.8 In vitro growth rates

One explanation for the inability to detect matants when rats were co-inoculated with two parental strains is that one of the parents has a much lower growth rate. In other words if one parent was able to grow faster in vitro than the other, this would then reduce the probability of both parents achieving sufficient relative concentrations to promote mating. The second reason for measuring growth rates was to compare those of matants to those of their parents. This may give an indication of the fitness of matants relative to their parents. Therefore the in vitro growth rates of *C. albicans* strains were measured.

#### 3.7.8.1 In vitro doubling times of *C. albicans* strains

The growth of *C. albicans* strains in YPD was measured at 37°C (Appendix 3, Figure A3.1). The doubling times for the strains during exponential growth were calculated (Table 3.12). The doubling times varied between strains with *C. albicans* W17, *C. albicans* OD8916 X W43 and *C. albicans* W17 X W43* showing higher doubling times, (slower growth) than the other strains. The slowest growing strain in these experiments was *C. albicans* FJ11.
Table 3.12: *In vitro* doubling times for *C. albicans* strains used in mating experiments.

<table>
<thead>
<tr>
<th>Experiment</th>
<th><em>C. albicans</em> (Strain 1)</th>
<th></th>
<th><em>C. albicans</em> (Strain 2)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Name</td>
<td>Marker</td>
<td>Doubling time (h)</td>
<td>Name</td>
</tr>
<tr>
<td>High frequency mating</td>
<td>OD8916α</td>
<td>pNZ11(^1)</td>
<td>1.13</td>
<td>W43a</td>
</tr>
<tr>
<td></td>
<td>W17α</td>
<td>pNZ11</td>
<td>1.31</td>
<td>W43a</td>
</tr>
<tr>
<td>Medium frequency mating</td>
<td>YSU63α</td>
<td>pNZ11</td>
<td>1.00</td>
<td>W43a</td>
</tr>
<tr>
<td></td>
<td>Au90a</td>
<td>pNZ4</td>
<td>1.16</td>
<td>HUN97α</td>
</tr>
<tr>
<td>Low/NO frequency mating</td>
<td>Au90a</td>
<td>pNZ4</td>
<td>1.16</td>
<td>FJ11α</td>
</tr>
<tr>
<td></td>
<td>Fj11α</td>
<td>pNZ11</td>
<td>1.55</td>
<td>W43a</td>
</tr>
</tbody>
</table>

\(^1\): Nourseothricin resistant, MPA; \(^2\): Mycophenolic Acid resistant.
When comparing the growth (Table 3.13) of singly-marked parents with the corresponding matant (Table 3.13), it was observed that the doubling time of the matants was higher than that of the corresponding parents i.e. they grew more slowly. For example, the doubling time of *C. albicans* OD8916 X W43 was calculated to be 1.43 h, while those of its corresponding parents, *C. albicans* W43 and *C. albicans* OD8916 were both calculated to be 1.13 h. In the case of recombinant *C. albicans* W17 X W43 the doubling time was 1.26 and that of the parents was 1.306 for *C. albicans* W17 and as above, 1.130 for *C. albicans* W43. In all cases except for *C. albicans* W17 the parents grew faster than their corresponding matants.
Table 3.13: *In vitro* doubling times for *C. albicans* parental strains and matants (*).

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Parent name</th>
<th>Marker</th>
<th>Doubling time</th>
<th>Matant</th>
<th>Marker</th>
<th>Doubling time</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>In vitro</em> matants vs. parents</td>
<td>OD8916α</td>
<td>pNZ11</td>
<td>1.13</td>
<td>OD8916X W43*</td>
<td>pNZ4</td>
<td>1.43</td>
</tr>
<tr>
<td></td>
<td>W43a</td>
<td>pNZ11</td>
<td>1.13</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>W17α</td>
<td>pNZ11</td>
<td>1.31</td>
<td>W17 X W43*</td>
<td>pNZ4</td>
<td>1.26</td>
</tr>
<tr>
<td></td>
<td>W43a</td>
<td>pNZ4</td>
<td>1.13</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>In vivo</em> Matants vs. parents</td>
<td>OD8916α</td>
<td>pNZ11*</td>
<td>1.13</td>
<td>OD8916X W43*</td>
<td>pNZ4</td>
<td>1.21</td>
</tr>
<tr>
<td></td>
<td>W43a</td>
<td>pNZ11</td>
<td>1.13</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>W17α</td>
<td>pNZ11</td>
<td>1.31</td>
<td>W17 X W43*</td>
<td>pNZ4</td>
<td>1.34</td>
</tr>
<tr>
<td></td>
<td>W43a</td>
<td>pNZ4</td>
<td>1.13</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.8 Discussion

3.8.1 Ability to detect *C. albicans* matants *in vivo* depends on the interplay between yeast-dependant and host-dependent factors

There are several factors that could affect the frequency at which matants are detected in the *in vivo* rat model (Figure 3.33).

To begin with, in order for mating to occur, two mating-competent cells need to come into contact. There are several factors in the *in vivo* rat model that will affect the ability of two mating competent *C. albicans* to come into contact. Firstly, mating competency of a particular *C. albicans* cell depends on whether it is in the white or opaque form. It has been shown that opaque cells of *C. albicans* mate at a much higher frequency ($10^6$ times more frequently) than white cells (Miller and Johnson 2002). It is also known that different strains of *C. albicans* switch from white to opaque form at different frequencies (Slutsky, Staebell *et al.* 1987). Therefore in this model, mating between two *C. albicans* strains depends on the number of opaque cells present and the frequency with which they switch from white to opaque phenotype and *vice versa*. Additionally, it is known that some mating pairs mate *in vitro* at a higher frequency than others; this is another factor that would affect the mating of two *C. albicans* strains in the *in vivo* model.

Other factors that influence the ability to detect mating in the *in vivo* model are those affecting the number of non-matants at the time of sampling. The ability to detect matants would be influenced by the proportion of cells sampled that are matants, which is in turn dependent on the *in vivo* growth rates of parental cells and matants, and the effectiveness with which the rat immune defences remove the parents or matants. Other factors involved include the number of cells from samples that are tested and the presence of markers within the parent and the matant cells which might affect growth rates.
Figure 3.33: An overall schematic of the in vivo model or oral colonisation. The ability to detect matants in vivo is dependent on several factors. Denoted here as $m_f$: frequency of mating, numbers of each parent $n_{P1}$ and $n_{P2}$ $S_f$: frequency of switching from white ($P_w$) to opaque ($P_o$) from, the proportion of white and opaque parents ($n_{Pw}: n_{Po}$). Other factors include sample size, growth rate of matants (M), marker loss and host factors.
3.8.1.1 Presence of a marker does not affect the growth of C. albicans in vivo

It was decided to determine whether the presence of the NAT\textsuperscript{r} marker adversely affected the growth and fitness of a C. albicans parental strain compared to the other marker MPA\textsuperscript{r}. This was done to eliminate the possibility of one marked parental strain having a competitive advantage over the other in vivo being due to the particular marker used. The two markers used in this study were CaNAT\textit{I} which is a gene conferring resistance to nourseothricin (Shen, Guo \textit{et al.} 2005), and the other is a mycophenolic acid resistance marker (MPA\textsuperscript{r}) (Beckerman, Chibana \textit{et al.} 2001). In this study it was seen that neither marker altered the fitness of the parent. It was observed that the presence of a marker did make the marked strain weaker when compared to its unmarked counterpart. Since this study did not implement the use of unmarked strains it is reasonable to exclude this as a factor. Additionally, as this effect of the marker conferring a disadvantage in vivo to the marked strains was seen uniformly across all marked vs. unmarked strains to the same degree, it is unlikely that there would be a difference in ability to colonise rats based purely on the marker difference. Thus we were able to select for matants using positive selection markers that did not affect growth in the absence of the selection drugs.

Another way of detecting mating is the use of auxotrophic markers. Auxotrophic markers were used in the study conducted by Magee and Magee (2000) in which they obtained prototrophic matants from parental strains with complementing auxotrophic markers. Thus only matants, and not the auxotrophic parents, grew on minimal medium. However auxotrophy affects yeast growth in nutritionally poor environments and lowers the virulence of the strains(Kirsch 1991). One could therefore argue that using auxotrophs would select for the formation of prototrophs and give them an advantage over their parents, thus masking the effects of sex on cell survival. In other words, the mating of two auxotrophs would be more a result of a forced survival mechanism rather than an option to asexual reproduction.

3.8.1.2 In vitro growth curves can be used to serve as an indicator of in vivo growth

In this study of in vivo mating three sets (6 combinations) of strains were used, selected on the basis of their ability to mate in vitro with: high, medium, or no/low frequency (Zhang 2012 ). In vitro growth curves were conducted to investigate whether the lack of mating seen in the in
The in vivo model with medium or low/no in vitro mating frequency pairs was due to a competitive disadvantage of one parent, i.e. if one parent shows a slower growth rate in vitro than the other parent, this may affect mating frequency. As expected, the growth rates of the strains tested varied, with the matants showing slower growth than the parents. However, what was of more interest was that, with one exception (C. albicans FJ11), the in vitro growth rates reflected the detection of strains in vivo. So much so that in all combinations tested, with the exception of the combinations using C. albicans FJ11, it was seen that strain with the higher in vitro growth rate outcompeted whichever strain it was paired with in vivo (Figure 3.14-3.24 and Appendix 2, Figure A2.8-A2.20). Excluding combinations where C. albicans FJ11 was used (as it is unable to mate), this study shows that it is possible to pick winners in the in vivo mating combinations based on their growth rates in vitro. However, as only two strain combinations resulted in matants from an in vivo experiment, this experiment needs to be taken further by testing matants from other in vivo strain combinations.

### 3.8.1.3 Ability to detect matants of C. albicans in vivo depends on the numbers of each parent present.

Firstly, in order for mating to occur, both parents have to be present in sufficient numbers (n\text{P1} and n\text{P2}, Figure 3.33) and at high concentration to be in close proximity. This requirement can be met by inoculating the oral cavity with the appropriate numbers of each parent. Ideally, an equal number (or proportion) of parents (n\text{P1}: n\text{P2} = 1:1) would ensure availability of both parents so that mating can occur.

In strain pair combinations with a high frequency of in vitro mating (two combinations were tested in vivo) it was seen that when rats were inoculated with an equal number of each strain (n\text{P1} \approx n\text{P2}) in the white form (P\text{w}), matants were detected in both strain combinations i.e. C. albicans OD8916 + C. albicans W43 (H1 pair) and C. albicans W17 + C. albicans W43 (H2 pair) [Table 3.9]. However, no matants were detected beyond 14 days post-inoculation in the H1 pair and none were detected beyond 7 days post-inoculation in the H2 pair (Appendix 4, Table A4.1 and Table A4.2). When the proportion of parents over the time course of the study was investigated (Figure 3.14 – 3.16), it was seen that in the oral samples, parents were present in similar numbers (n\text{P1} \approx n\text{P2}) up to 21 days after inoculation. Even though the proportion of the parents changed at day 28 to one parent being detected 10 times more frequently than the other, the current study showed that both parents were still available to undergo mating until the end of the experiment. Therefore, it can be concluded that parent
numbers were sufficient for mating to occur and matants were not detected due to reasons other than that of the parent numbers.

In the strain pair combinations with a medium frequency of in vitro mating, namely C. albicans YSU63 + C. albicans W43 (M1) and C. albicans Au90 + C. albicans HUN97 (M2), inoculation with equal numbers (n_p1 ≈ n_p2) of parents in white form (P_w) resulted in no detectable matants. On examining the growth and proportion of parents over the time course of the study it was seen there was a trend where in one parent outcompeted the other (C. albicans YSU63 in M1 and C. albicans Au90 in M2). It was therefore decided to change the initial inoculum to contain parents so that n_p1 × 2 = n_p2, where P2 was the parent that outcompeted the other parent (P1) in the first set of experiments. With the altered proportion of parents in the inoculum it was observed that both parents were detected at the end of the study in numbers sufficient for mating to occur in vivo. However no matants were detected in this experiment either. What we can infer from this is that there is another reason, other than parental numbers, why these strains did not mate in vivo.

With strains showing low/no frequency of mating in vitro, namely C. albicans FJ11 + C. albicans Au90 (L1) and C. albicans FJ11 + C. albicans W43 (L2), again it was seen that one parent outcompeted the other. However, no further experiments were done to alter the number of parents with these strain combinations as it was found that C. albicans FJ11 had a stop codon mutation in its MTL alpha 1 gene that leads to truncation of the gene product which is essential for mating (Zhang 2012).

3.8.1.4 Mating of C. albicans in vivo depends on the proportion of cells in the opaque phenotype

Once the parents are in close proximity, in order for mating to occur at high frequency they need to be in the less-common mating-competent opaque (P_o) form. The P_o cells, however, are highly unstable in the absence of carbon dioxide and at 37°C and switch to the mating-incompetent (P_w) form in vivo therefore rendering the cells unlikely to recombine. This finding is supported by the work of Rikkerink et al. (Rikkerink, Magee et al.1988), Srikantha (Srikantha and Soll 1993) and Soll (Soll, Lockhart et al.2003), who reported that the opaque phenotype converted to the white phenotype at 37°C. It is also known that at high CO₂ (as would be seen in the gut) concentrations white cells switch en masse to opaque (Slutsky, Staebell et al. 1987). The frequency of switching, from white (P_w) to opaque (P_o) and vice
versa thus plays an important role in the mating process and as a result affects the ability to detect matants in the in vivo model. In the high frequency mating combinations it was seen that when inoculated with an equal number of each parent ($n_{P1} \approx n_{P2}$) in the white form ($P_w$), matants were detected in both the strain combinations i.e. C. albicans OD8916 + C. albicans W43 (H1) and C. albicans W17 + C. albicans W43 (H2). However, no matants were detected beyond 14 days post-inoculation in H1 and none were detected beyond 7 days post-inoculation in H2 even though both parents were present in numbers sufficient for mating to occur (see Section 4.1.1). Another explanation for the lack of matants could be the white to opaque ratio ($nP_o:nP_w$). On examining the $nP_o:nP_w$ ratios, it was seen that in both the oral and the fecal samples, the opaque numbers declined to almost no opaque cells being detected after day 14 in the oral cavity. It was observed that although almost 50% of the total cells were opaque at day 7, by day 2, for both strain combinations opaque cells were present in far smaller numbers (<10%) than the white cells (Appendix 2, Figure 1, Figure 2, Figure 3 and Figure 4). This could be because of two factors. Firstly, it is known that white cells are more virulent than opaque cells and are able to establish themselves better in the host (Kvaal 1997; Kvaal 1999; Ramírez-Zavala 2008) thus providing an explanation why there is a decline in opaque cell numbers. Secondly, it is also known that the oral cavity, while it possesses some sites which might be anaerobic, is predominantly aerobic (Amerongen and Veerman 2002), which means a lowered CO$_2$ content which would cause the unstable opaque form to switch to the white form (Huang, Srikantha et al. 2009). Therefore it was decided to test whether an inoculum made up of predominantly opaque cells ($P_o$) would help overcome mating hurdles caused due to a low number of mating-competent opaque cells and, as a result, facilitate more mating events increasing the frequency of detection of matants. On inoculation of the H1 combination with opaque only cells it was observed that, in one rat, matants were detected up to 21 days post-inoculation (Appendix 4, Table 20). This could be due to the fact that in the rat where the matant was detected, opaque cells were still present until day 21 post-inoculation (Appendix 2, Figure 4A). However, no matants were detected after day 21 of the experiment, suggesting that even though matants were generated, they were not detected after this due to other reasons. In the strain pair combinations with a medium frequency of in vitro mating, no matants were seen even after adjusting the ratio of parents ($n_{P1}:2n_{P2}$) to ensure that there were sufficient parents in vivo to undergo mating (see Section 4.1). However, more striking was the fact that no $P_o$ cells were detected in either the oral cavity or the fecal pellet throughout the course of the study (an example of which is shown in Appendix 2, Figure
A2.5). It was thus decided to investigate whether an inoculum consisting predominantly of opaque cells (P_o) would result in a higher frequency of detection of matants. However, even when inoculated with P_o-only cells, no matants were detected and when checking the nP_o:nP_w ratio it was seen that no opaque cells were detected at 7 days post-inoculation (data not shown). What can be concluded from these data is that the opaque forms of these strains that undergo mating at low/no or medium frequency in vitro are highly unstable and switch rapidly from the white to opaque form at ~37°C in the rat oral cavity. Thus mating is probably precluded due to the fact that very few mating competent opaque cells are present. Another explanation for the inability to detect matants from the in vivo sample could be that matants are produced but are not detected due to one of many factors as described below.

3.8.2 Several factors affect the ability to detect matants in vivo

The factors that could be playing key roles in the inability to detect matants in this model are:

1. The sample size i.e. the number of cells analysed to detect matants.
2. The growth rates of the matants, i.e. the matants produced are quickly outcompeted by one or both parents, due to their comparatively slower growth rates.
3. Marker loss (so matants are not detected by PCR).
4. Host factors, namely immune status and other behavioural patterns of the host.

3.8.2.1 Sample size affects the ability to detect matants in vivo

An important feature of this in vivo model is that it permits several sampling points over a period of time. This enables us to observe the colonisation behaviour of C. albicans over a prolonged period of time. This however comes at the cost of the amount of sample that can be obtained at each time point (one oral swab). As several different parameters were required to be tested, namely total cell counts, white opaque counts and matant presence, it was therefore plate the sample on various media (in different dilutions to get accurate counts). This limited the amount of sample that could be used to identify matants. Additionally, obtaining a representative sample from the fecal pellet proved more difficult than anticipated. The consistency of the fecal pellet varied within and between rats at different time points. The size
of the fecal pellet varied depending on the feeding status of the individual rat. This made it very difficult to obtain a reproducible and consistent sample (see Section 4.7 “Future directions” to improve the sampling). Another limitation that could have affected the frequency at which matants were detected was that in DSM plates that showed no large colonies, only 20 small colonies were randomly selected to be screened for matants. All the colonies could not be tested for time, workload and cost reasons (DSM plates showed as many as 300 small colonies). This factor additionally reduced the ability to detect matants.

3.8.2.2 Matants grow at a slower rate than their respective parents

In the strain pair combinations with a medium frequency of in vitro mating it was found that adjusting the parent numbers and adjusting the inoculum to contain a suitable proportion of mating competent parents (P₀) still did not result in any matants being detected in the model. This could be because the progeny have growth deficiencies such that they are immediately outcompeted by their parents. In support of this explanation is a study comparing in vivo and in vitro passaging of strains (Forche, Magee et al. 2009). The authors compared genome-wide genetic and phenotypic evolution, using a genome wide array of single nucleotide polymorphisms (SNPs), in C. albicans by following passage through a mouse host (in vivo) and during propagation in liquid culture (in vitro). They found that growth in vivo resulted in a slower population growth and higher rates of chromosome-level genetic variation and concluded that the net population growth rates were 5-10 fold lower in vivo than in vitro. In this study, the fitness of the matants was compared with that of a single parent by inoculating mice with the matants (obtained form an in vivo mating event) and either of its corresponding parents. It was found that parents outcompeted the matants in all studies and no matants were detected by the end of all in vivo co-inoculation experiments (Figure 44, Figure 45, Figure 46 and figure 47), except for the study using C. albicans W43 vs. C. albicans W43XW17 in which matants made up about 10% of the total population at day 14, however, no matants were detectable by day 21 in any of the experiments. In conclusion, the results of the present study show that if mating does occur in vivo the matants appear to be less fit than their parents. In support of this is a recent study conducted by Zhang investigating the fitness of MTL homozygotes (resulting from mating in vitro) and their sexual offspring (Zhang 2012). She measured recombinants’ fitness relative to that of their parents, by comparing the in vitro growth rates of 9 fertile parents and 34 recombinants. It was found that when first isolated, the
recombinants’ median growth rate was 7% lower than that of the MTL homozygote parents. This provides further evidence that matants grow more slowly than their parents. It was also seen that matants obtained in this study retained their MTL heterozygosity and both their resistance markers, despite chromosome loss (recombinants had a DNA content < 4n). This suggests that there may be some selection to retain the markers. However it is known that matants, (now having 4 copies of each chromosome) randomly lose chromosomes in order to return to a diploid state in a parasexual process (Forche, Alby et al.2008). As this loss is a random, there is a possibility that this loss results in a matant that is not well adapted to growth in vivo thus providing another explanation for the slower growth rates of the matants in the in vivo rat model.

3.8.2.3 The frequency of the detection of matants is not affected by marker loss

Still another possibility for the low frequency of matants detected is that even though recombination occurred, loss of chromosome (s) post mating (resulting in loss of one or more marker [MTLa, MTLα, NATr, MPAr]) prevented possible matants from being detected when using PCR. Therefore an experiment was conducted to investigate whether marker loss occurred in vivo (see section 3.7.7). It was observed that marker loss in vivo was not significant (p=0.9; as 2 out of 229 lost marker) when the rats were inoculated with matants obtained from in vivo mating experiments.

3.8.2.4 The frequency of the detection of matants is affected by host-dependent factors.

As described in the general introduction (Table 1.2 and section 1.1.2.6) there are several ways in which the host and C. albicans interact that affect the ability of C. albicans to colonise the host. When it comes to the frequency with which matants are detected in the in vivo model, host factors could also play an important role. Firstly, the rat is a coprophagic animal, this eating habit could have two effects: a) it results in the re-inoculation of matants into the oral cavity increasing the frequency with which they are detected; b) it re-inoculates the oral cavity with more opaque parents (as they could have switched from white to opaque from in the GI tract) increasing the frequency of mating in the oral cavity and c) The re-inoculation of the
oral cavity with more parents could lead to the matants present in the oral cavity at that time being outcompeted. Secondly, the immune suppressant used in this study was administered via the drinking water. This means that the levels of immunosuppression depended on each individual rat’s drinking habits. There is therefore the possibility that the extent to which *C. albicans* (both parents and putative matant) is “cleared” from the host by host immune defences would vary depending on the drinking habits of the rat and affect the ability to detect matants in samples. Eating also increases the salivary flow in the host’s oral cavity promoting microbial clearance. The salivary flow delivers other salivary factors that inhibit yeast growth and as a result could affect the cell numbers in the oral cavity (Cannon, Holmes *et al.* 1995).

In conclusion *in vivo* mating in the rat oral colonisation model does occur and can be detected in some (but not all strains) of *C. albicans*. However, it is difficult to ascertain whether mating confers an advantage to (the fitness of) *C. albicans* in the host, by co-inoculating each parent in the rat colonisation model, with the provision of what could be described as close to ideal conditions for mating to occur. The fitness of the matants (obtained by an *in vivo* mating event) can be ascertained by co-inoculating the resultant matants in the model with their respective parents. This study indicates that matants are less fit than the parents and are not able to survive in the host for prolonged periods of time.
CHAPTER FOUR

GENERAL DISCUSSION
4.1 Introduction

"...Natural selection acts only by taking advantage of slight successive variations; she can never take a great and sudden leap, but must advance by short and sure, though slow steps."

Charles Darwin.

Although mating in fungi has been extensively studied in the past, much attention has been paid recently to the processes involved in the mating of *C. albicans* as it was formerly considered an asexual fungus. Population structure studies have indicated that sexual reproduction, if it occurs, is rare. Rather, reproduction occurs primarily through clonal means (Odds, Bougnoux *et al.* 2007; Bougnoux, Pujol *et al.* 2008; Jacobsen, Rattray *et al.* 2008). It has been suggested that the mating genes may play an alternative role such as in the formation of biofilms and signalling (Zhao, Daniels *et al.* 2006; Sahni, Yi *et al.* 2009; Srikantha, Daniels *et al.* 2012). As *C. albicans* is an important cause of disease, these findings have brought to the forefront the need to study the biological significance of mating in *C. albicans*. Throughout the thesis the candidate has tried to understand two aspects of the mating process, namely, can *C. albicans* undergo mating in the oral cavity and are the progeny of mating fitter for *in vivo* survival compared to the parental strains?

4.2 The mating process: overcoming hurdles

The conundrum that *C. albicans* presents too many mycologists is: First; why is a Mating Type Like (*MTL*) locus present despite population evidence of clonal reproduction? Second; what is the significance of its ability to mate both *in vitro* and *in vivo*?

Studies have shown that although meiosis has not been detected, *C. albicans* returns to its diploid state (or aneuploid state) from a tetraploid state via a non-meiotic parasexual mechanism (Forche, Abbey *et al.* 2011). Forche *et al.*, (Forche, Abbey *et al.* 2011) analysed mating progeny of *in vitro* matings using SNP analysis and comparative genome hybridisation and found that the matings resulted in a range of genetically related strains with altered phenotypes. Furthermore, it was also seen that a subset of these strains showed that recombination between homologous chromosomes had occurred. It has been suggested that the parasexual programme, without the formation of spores, has an advantage in a commensal
yeast such as *C. albicans* because spores are often highly antigenic and the parasexual cycle minimises detection of mating products by the hosts immune system (Sherwood and Bennett 2009).

Although population studies suggest that recombination resulting from mating is rare, this study shows that in the minority of strains that do mate, it is not an uncommon event *in vivo*. Additionally, strains that resulted from *in vivo* mating were observed in this study over a very short time scale in evolutionary terms. However, no matant was detected *in vivo* at times post-inoculation of greater than 3 weeks, suggesting that the resultant matants were less fit than their parents. The rat model was used to improve the chances of the introduced mating competent pair of strains to mate *in vivo*. Several inoculum parameters were adjusted to improve the frequency of mating. Initially, the frequency of opaque (mating competent) cells in the inoculum was low and so the inoculum was adjusted to contain only opaque cells and where one parent was seen to out-compete the other, the inoculum was adjusted to contain more of the less-fit parent. Although these adjustments did not increase the number of matants detected for pairs of strains that previously did not mate *in vivo*, it did increase the number of matants detected early in experiments where the strains used to inoculate rats had previously been shown to mate *in vivo*, i.e. strain pairs *C. albicans* OD8916 and *C. albicans* W43 and *C. albicans* W17 and *C. albicans* W43 (Chapter 3, Table 3.9). However, this increase in number of matants detected early in the experiment was not maintained with time, suggesting that even if matants are produced they are unable to compete with their parents.

Subsequently, when a separate experiment was conducted to investigate the ability of the matants to colonise the rats when co-inoculated with their respective parents, once again it was observed that the matants (that had been obtained *in vivo*) were less fit than the parents and were quickly outcompeted. This finding suggests that when mating does occur, the resultant matants are not as fit as the parents for *in vivo* growth under the conditions applied.

**4.3 Is sex *in vivo* a frequent occurrence in *C. albicans*?**

In order to answer this question one needs to understand the several stages involved in order for *C. albicans* have sex. The first stage, the parental strain has to achieve homozygosity, or loss of heterozygosity (LOH) at the *MTL* locus. This occurs, in the laboratory, by gene disruption or by a process involving selection for the loss of a whole chromosome, chromosome 5. The second stage of this event is switching from the white to opaque...
morphology which occurs at a frequency of about $10^{-4}$ and is regulated by \textit{WOR1} gene (Slutsky, Staebell \textit{et al.} 1987; Miller and Johnson 2002). Finally, the third stage requires another mate, of the opposite mating nucleus, to be close enough to send and receive pheromone signals.

In order to understand the frequency and evolutionary significance, if any, of sex, one has to look more closely at the probability of each of the three stages occurring in nature in a host organism, and the possible hurdles \textit{C. albicans} has to overcome in order to produce mutants (Berman and Hadany 2012). In order for a mating event to occur, \textit{C. albicans} has to become \textit{MTL}-homozygous. While this event can be induced in the laboratory under stress conditions (Forche, Abbey \textit{et al.} 2011) it occurs rarely in nature; a survey by Lockhart \textit{et al.} of more than 220 clinical isolates found only seven (3\%) that were homozygous or hemizygous for the \textit{MTL} locus (Lockhart, Pujol \textit{et al.} 2002). In addition to the low numbers of naturally detected homozygotes, it has been shown in this study (Appendix 2 Figure A2.1 and A2.2) and in previous studies (Huang, Srikantha \textit{et al.} 2009) that \textit{C. albicans} switches \textit{en masse} from opaque to white phenotype at 37°C, thus making the maintenance of the mating competent opaque state in the host a major hurdle in the way of sex for the commensal \textit{C. albicans} in the human host. Also as in cells that are heterozygous at the \textit{MTL} locus cannot switch as white-opaque switching is inhibited in a/a cells due to the repression of the \textit{WOR1} gene by the a1/a2 complex, hence locking cells in the white state (Sherwood and Bennett 2009). Moreover, as studies by Wu \textit{et al.}, (Wu, Lockhart \textit{et al.} 2007) have also found, LOH at the \textit{MTL} locus in \textit{C. albicans}, results in reduced virulence and competitiveness. So even if a strain of \textit{C. albicans} \textit{does} undergo LOH at the \textit{MTL} locus \textit{in vivo}, and white to opaque switching, it is likely that the \textit{MTL}-homozygote will be outcompeted by its heterozygous counterparts.

Another issue that could preclude a high frequency of mating is that once switching has occurred, two cells of the opposite mating type must be in close proximity so that they both can send and receive mating pheromone signals and make cell-cell contact. The likelihood of this happening in nature would probably be very rare, given the nature and diversity of most natural commensal biofilms (especially in the oral cavity). However, there have been cases in which mating competent biofilms have been analysed for the presence of \textit{C. albicans} of different mating types and 10\% have been found to constitute a/a or a/α cells. While a/α biofilms were found to be impermeable and impenetrable to white blood cells and low and high molecular weight substances, biofilms formed by a/a or a/α are permeable and penetrable making them susceptible to host defence systems and also antibiotic treatment.
(Daniels, Srikantha et al. 2006; Yi, Sahni et al. 2011). It has recently been suggested that one reason for formation of biofilms with homozygous MTL locus strains to support chemotropism and fusion of opaque cells (Yi, Sahni et al. 2011). This hypothesis can be taken a step further to suggest the possibility that earlier on during the evolution of C. albicans there was a more frequent occurrence of MT-homozygous biofilms that facilitated pheromone signalling and mating of C. albicans thus ultimately achieving what is now the more common conserved form, impenetrable and impermeable to host defences.

However, even if after overcoming all these hurdles and achieving mating, the degree to which these progeny are beneficial, in terms of virulence or fitness, is largely unknown. There have been suggestions that matants might incur a cost of fitness. Suggestions that ploidy plays a role in the fitness of C. albicans have been mentioned in a few studies. In a murine model of disseminated candidiasis it was seen that tetraploids are less virulent than diploids. It was seen that there was a rapid loss of tetraploids which could be attributed to early and rapid clearance of the larger tetraploid cells and also to changes in ploidy during infection. Additionally, when growth rates of progeny from parasexual cycle were compared it was seen that aneuploid strains grew at slower rates than the control diploid strains (Ibrahim, Magee et al. 2005). Furthermore it was shown that the growth rates got slower as the number of trisomic chromosomes increased (Forche, Alby et al. 2008). The results of this study have contributed to answering the question of fitness of matants. When matants obtained from an in vivo recombination event were co-inoculated with one of either parental strain in vivo, the matants were quickly outcompeted and in most cases no matants were recovered by the end of the study. This suggests that even though matants can be isolated from an in vivo mating event, the matants incur a very high fitness cost and are outcompeted.

**4.4 Why does C. albicans prefer clonal reproduction?**

Sex is a very expensive process for any organism. It firstly requires two sexes to participate and involves the meiotic reduction of the genome complement followed eventually by karyogamy. Sexual reproduction also requires organisms first to produce, and then maintain gametes. Asexual reproduction on the other hand does not require a mate and many offspring are produced quickly. Maintaining this type of asexual mainly clonal population is advantageous to a successful commensal like C. albicans because it enables it to rapidly divide and maintain sufficiently high numbers in its host which helps in establishing itself in
its host. *C. albicans* is also morphologically more diverse than most commensals and is able to produce true hyphae, pseudo-hyphae and blastospores, which enable it to survive and disseminate in its mammalian host. Additionally, it has also been shown that biofilms formed by clonal heterozygous a/α strains are harder for host immune molecules and antifungals to penetrate. The transition from white to opaque form of *C. albicans* requires G1 arrest (shmoo formation) and this inhibits the possibility of hyphal formation which is induced during the G1-S transition (Sudbery, Gow *et al.* 2004) thereby reducing the capability of *C. albicans* to invade host tissue impairing the establishment of the yeast in its host. Additionally, clonal reproduction does not produce spores, which are highly antigenic and thus susceptible to clearance via host defence mechanisms (Berman and Hadany 2012). Also it had been shown that a/α strains that are not mating competent are more virulent and have a competitive advantage over mating competent a/a or α/α strains which further drives the population towards a clonally reproducing one (Lockhart, Wu *et al.* 2005). All these factors contribute to the advantages of clonal reproduction over parasex in order for *C. albicans* to establish itself in its host.

### 4.5 So why does *C. albicans* have (para-)sex?

One answer could be the process wherein opaque cells signal white cells to form biofilms (Daniels, Srikantha *et al.* 2006). These authors suggest that that *C. albicans* has maintained only part of its mating process, essentially the switching from white to opaque, *in order to aid biofilm formation and as a result, its survival as in the commensal state*, an argument also supported by others (Rikkerink, Magee *et al.* 1988; Magee and Magee 2004; Daniels, Srikantha *et al.* 2006).

In a recent review Berman and Hadany suggest that perhaps stress plays a role in the induction of the parasexual cycle in *C. albicans* (Berman and Hadany 2012). This is indeed a possibility once again from a commensal prospective, because stress incudes the signalling to form biofilms which leads to an increase in number of cells retained on a host surface and thus an increase in the chances of survival.

However, the loss of fitness of the matant strains observed in this study does not make sense from a purely evolutionary perspective.
4.6 Sexual reproduction from an evolutionary perspective

According to the theory of evolution, sex confers an advantage when it allows the shuffling of genes leading to an introduction of genetic variation and along with the removal of deleterious mutations. However flying in the face of this theory are several, highly “fit” and successful ancient organisms that do not undergo sex e.g. ferns and rotifers (Judson and Normark 1996). C. albicans is also one such ancient and successful organism that reproduces primarily by asexual means. C. albicans is able to evolve very rapidly, especially in cases where it is exposed to antifungal drugs (Cowen, Sanglard et al. 2000; Coste, Selmecki et al. 2007). This evolution in C. albicans occurs via a parasexual cycle while maintaining a primarily clonal population structure (Pujol, Reynes et al. 1993; Nebavi, Ayala et al. 2006; Odds, Bougnoux et al. 2007). Is it thus fair to say that C. albicans is like most of its ancient counterparts (Sanders 1999) and is now able to evolve and introduce variation without sexual recombination? Thus what is coined as a “parasexual” cycle is not really sex; because “recombination” in the true sense does not occur (no meiotic cycle is observed). However, it is suggested that a parasexual cycle could provide some advantages for C. albicans under stress conditions. The imprecise nature of parasex generates many aneuploid strains as well as euploid strains. These euploids are commonly trisomic diploids therefore introducing greater genetic diversity in the progeny population. These variations in chromosome number have been found to be associated with drug resistance in clinical isolates (Coste, Selmecki et al. 2007). An example of this is the presence of extra copies of chromosome 5 containing ERG11, a gene involved in ergosterol biosynthesis and TAC1 a transcriptional activator of efflux pump genes. The combined effect of the presence of gene dosage confers an increased amount of antifungal drug resistance. However, these aneuploidies do not seem to confer any fitness advantage when grown under drug free conditions (Selmekki, Dulmage et al. 2009) and as a matter of fact, as mentioned earlier aneuploids grow at slower rates than its diploid their parents, which in the commensal stage of C. albicans could prove to be disadvantageous (Forche, Alby et al. 2008).

The rare occurrence of a sexual event, the hurdles presented by the several stages discussed earlier, and the results presented here that show that the fitness of matants produced in vivo are severely compromised and would suggest that sex in C. albicans does not confer any evolutionary advantage unless under unusual stress conditions and that C. albicans is clearly able to survive without sex.
4.7 Future Directions

1. Flow cytometry analysis of the matants indicates a niche-dependent chromosome loss: possible future considerations in understanding the mating process.

Flow cytometry carried out on the matants obtained in vivo indicated that all the matants had higher DNA content than the average DNA content of both parents. Interesting to note, however, is the fact that the matants showing the highest DNA content were from the oral samples, suggesting that the matants from the fecal sample are closer to the 2n (diploid) state and have lost more chromosomes than oral matants in their return to the diploid state. This could be because the GI tract of the model is a more stressful environment (low pH, low oxygen, and water content) than the oral cavity inducing a more efficient stress-induced chromosome loss to enable the matants to survive better in the host, as described by Forche et al. (Forche 2011). Additionally, this difference in the rate of loss of chromosomes in different environments is consistent with a study conducted by Bennet and Johnson. In their experiment they observed that different patterns of random chromosome were induced in tetraploid matants following growth different media (Bennett and Johnson 2003). We can infer from this that the host environment possibly plays an important role in the manner by which C. albicans tetraploids lose chromosomes to return to the diploid state. It would be useful to test this hypothesis in the future by attempting to obtain more in vivo matants using other strain combinations and specifically looking at the type of chromosomes lost and also whether the chromosome loss is dependent on the time spent in a particular niche in vivo.

2. Immune response of rats to the parents and matants (Antigenicity of matants).

Nothing is known as yet about the role of the immune system and possible strain-specific responses to C. albicans in the in vivo model. Therefore a study investigating the immune response of the in vivo model to each parent and also the matant would provide us with further evidence as to why matants are not detected in the host. Are the matants (now possibly possessing antigenic characteristics of both parents) more antigenic than each of its parents? And if not, then does the host produce matant-specific antibodies? To provide a more accurate model for the measurement of host immune response, the immunosuppression levels of the rats can be controlled by placing rats in individual cages and standardising their diet (both water and food) in an attempt to achieve consistent immunosuppression.
References:


142

NCBI.


146


Appendix 1: FORMS AND ETHICAL APPROVALS

Otago University
Animal Ethics Committee

Date: 19/11/07

Prof R Cannon
Oral Sciences
School of Dentistry

Dear Prof. Cannon,

Thank you for your reply to the provisos requested for protocol 66/07. These were considered at the most recent meeting of the Otago University Animal Ethics Committee and I am pleased to advise that your protocol has now been approved in full.

Yours sincerely,

Barbara Lee
Secretary
Otago University Animal Ethics Committee
Otago University Animal Ethics Committee

Faculty Office
Medical School
18 September 2007

Professor R Cannon
Oral Sciences
Discipline of Molecular Microbiology
Dental School

Dear Professor Cannon

Application No. 60/07

I have pleasure in advising you that the Animal Ethics Committee at its meeting on 13 September 2007 has given approval for your application for use of animals in a programme/project entitled “Optimisation of an oral colonisation competition model in the rat”. This is approved with the following proviso:

a) in #5.2.2: please complete the tick boxes for the entire range of possible adverse effects, even if the answer is “No”.

In any correspondence on this subject, including the ordering of animals for your research, would you please quote the application number given above.

Approval is given on the basis that the Code of Ethical Conduct for the Manipulation of Animals will be adhered to and accurate records of animal usage will be maintained.

Please be aware that there is a requirement to submit a brief report on the outcome of this project. Information regarding the format of this report, plus a request for the standard animal use statistics will be mailed to you just prior to the expiry of this protocol from the Animal Welfare Office.

 Permit holders are asked to advise the Secretary of the Animal Ethics Committee when an approval will be activated, when a project has been completed or when an approval will not be activated due to lack of funding.

Please confirm in your response to this letter that all personnel listed on the original application and your Head of Department have been made aware of any changes to the original protocol as a result of these provisos. Responses should take the form of a letter providing commentary or answers to the questions asked. Please do not resubmit a new corrected protocol unless specifically requested.

Yours sincerely

Barbara Lee
Secretary
Otago University Animal Ethics Committee

cc Professor J Kieser

Please Note: this protocol is only approved subject to further information being received which addresses the specified provisos. Failure to provide information as requested within six weeks of receipt of this letter will result in this approval being withdrawn.
Otago University
Animal Ethics Committee

Date: 15/2/11

Prof. R Cannon
Oral Sciences
Dentistry

Dear Prof. Cannon

Thank you for your reply to the provisos requested for protocol 97/10. These were considered at the most recent meeting of the Otago University Animal Ethics Committee and I am pleased to advise that your protocol has now been approved in full.

Yours sincerely

Barbara Lee
Secretary
Otago University Animal Ethics Committee

Final Proviso Approval
Otago University Animal Ethics Committee

Hunter Centre
C/o Faculty Office
Medical School

14 December 2010

Prof R Cannon
Oral Sciences
School of Dentistry

Dear Prof Cannon

Application No. 97/10

I have pleasure in advising you that the Animal Ethics Committee at its meeting on 8 December 2010 has given approval for your application for use of animals in a programme/project entitled ‘Use of rat model of oral colonisation to study Candida albicans biology’. This is approved with the following provisos:

a) in # 3.1: if the experiments need to be repeated with a higher dose of dexamethasone will the same rats be used, or new ones? Please clarify.

b) in # 5.2.1: please revisit and answer.

c) in # 6.1: i) the loading dose of doxycycline (1.25 mg/ml) should be included here as well as the already noted maintenance dose (0.125 mg/ml).

ii) the dexamethasone dose rate should read 0.5 μg/ml to mirror the information in # 3.1.

We will change these figures on your behalf, but please confirm these modifications are correct.

In any correspondence on this subject, including the ordering of animals for your research, would you please quote the application number given above.

Approval is given on the basis that the Code of Ethical Conduct for the Manipulation of Animals will be adhered to and accurate records of animal usage will be maintained.

Please be aware that there is now a requirement to submit a brief report on the outcome of this project. Information regarding the format of this report, plus a request for the standard animal use statistics will be mailed to you just prior to the expiry of this protocol.

Permit holders are asked to advise the Secretary of the Animal Ethics Committee when an approval will be activated, when a project has been completed or when an approval will not be activated due to lack of funding.
Please confirm in your response to this letter that all personnel listed on the original application and your Head of Department have been made aware of any changes to the original protocol as a result of these provisos. Responses should take the form of a letter providing commentary or answers to the questions asked. Please do not resubmit a new corrected protocol unless specifically requested. As the Committee members will not necessarily have access to this letter when evaluating your response to these provisos, please include a brief summary of the original questions you are responding to.

Yours sincerely

Barbara Lee
Secretary
Otago University Animal Ethics Committee

Please Note: this protocol is only approved subject to further information being received which addresses the specified provisos. Failure to provide information as requested within six weeks of receipt of this letter will result in this approval being withdrawn. Notwithstanding the above provisos, for the purposes of the release of research funds, this letter may be considered as formal approval.
Institutional Biological Safety Committee decision form to develop genetically modified organisms in containment

**Institutional Biological Safety Committee:** University of Otago

**IBSC Institution Code:** GMO004/U0003

**Application category:** To develop in containment a genetically modified organism under section 40(1)(b) of the Hazardous Substances and New Organisms (HSNO) Act.

**Purpose:** The objective of this project is to determine whether clinical *Candida albicans* isolates, and laboratory strains, can undergo sexual recombination under *in vitro* laboratory conditions, and *in vivo*, in a rat model of oral colonisation.

**Applicant:** University of Otago

**Date application received by IBSC:** 19 March 2004

**Considered by what members:**

**Date of consideration:** 23 March 2004

### 1 Summary of the decision:

The application to develop the following organism(s) is **approved**, with controls having been considered in accordance with the relevant provisions of the Hazardous Substances and New Organisms (HSNO) Act 1996, the Hazardous Substances and New Organisms (Low-Risk Genetic Modification Regulations) 2003, and the HSNO (Methodology) Order 1998.

The application was considered by the IBSC under delegation from the Authority as provided for under section 19(2)(a) of the HSNO Act.

The organism(s) for development by **genetic modification** are:

<table>
<thead>
<tr>
<th>Name of the organism(s):</th>
<th>Specify the category of host organism*, e.g. Category 1 or 2</th>
<th>What the organism is modified with: Please specify vector and donor DNA</th>
<th>Please specify the category of genetic modification*, e.g. Category A or B</th>
<th>Containment level* e.g. PC1/PC2.</th>
<th>Approved /Declined</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Candida albicans</em> Berkhout (1923), clinical isolates and laboratory strains</td>
<td>2</td>
<td>Integrative vectors; DNA fragments containing, characterised reporter genes, a site-specific recombinese, a transcriptional activator, and a gene conferring mycophenolic acid resistance. These genes will be sourced</td>
<td>B</td>
<td>PC2</td>
<td>Approved</td>
</tr>
</tbody>
</table>

---

*This decision form should be used in conjunction with the checklist.*

*According to the HSNO (Low-Risk Genetic Modification) Regulations 2002.*

*According to the HSNO (Low-Risk Genetic Modification) Regulations 2003.*

*As in the Australian/New Zealand Standard AS/NZS 2243.3 2002 with modifications referred to in the MAF Biosecurity Authority Biosecurity Authority Containment Standards.*
from *Escherichia coli* and *Candida albicans*, genes originally from the yeast *Saccharomyces cerevisiae*, *Aequorea victoria*, and *Renilla reniformis* will be sub-cloned from *E. coli*. Expression of these genes will be controlled by promoters from *E. coli* and *C. albicans*.

<table>
<thead>
<tr>
<th>Description</th>
<th>Code</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Escherichia coli Migula (1895) strain K12 and B derivatives that are non-pathogenic</td>
<td>A</td>
<td>PC1 Approved</td>
</tr>
</tbody>
</table>

Please note: the organism description can be specific to individual GMOs or it can encompass a project description. HOWEVER, the organism description needs to CLEARLY delineate the

---

3 As described in our “Policy relating to the rapid assessment of low-risk new organisms, including medicines” (ER-PO-01-2) or for more guidance refer to “ERMA New Zealand User Guide to making an application for approval to import into containment low risk genetically modified organisms under section 40 of the Hazardous Substances and New Organisms Act 1990”.

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boundaries so ERMA New Zealand can be satisfied that it conforms with the HSN0 (Low-Risk Genetic Modification) Regulations 2003. For example: “not low-risk” modifications need to be clearly excluded from the vectors and donor nucleic acids if you are using uncharacterised nucleic acid sequences from pathogenic organisms OR, for example, if using Escherichia coli as a host, specify it is the non-pathogenic strains or strains K, 12 or 13.

<table>
<thead>
<tr>
<th>Human Genes or Native flora and fauna:</th>
<th>YES</th>
<th>NO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Does the proposed development use genetic material from native flora and/or fauna?</td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>Does the proposed development involve human genes?</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

PLEASE MAKE SURE YOU COMPLETE SECTIONS 5 AND 6 OF THE CHECKLIST AND PROVIDE EVIDENCE OF ANY CONSULTATION OR ETHICS COMMITTEE APPROVAL.

2  Containment

Work with Escherichia coli to be done under a minimum of Containment Level PC1 conditions as described in AS/NZS 2243.3:2002.

Work with Candida albicans to be done under Containment Level PC2 conditions as described in AS/NZS 2243.3:2002 and with additional controls listed in section 5.

Work to be done in a Containment Facility registered under MAF/ERMA Regulatory Standard 154:03:02, such as University of Otago Dunedin Campus Containment and Transitional Facility for Microorganisms and Uncleared Biological Products.

Exposure of vertebrate animals to genetically modified C. albicans cells to be carried out under PC2 conditions in a Containment Facility registered under MAF/ERMA Regulatory Standard 154:03:03.

All operating requirements for that Facility to be complied with, as detailed in the Containment and Quarantine Manual for the Facility.

3  Identification of the significant risks and costs of the organism

In accordance with section 42 and 42A of the Act (rapid assessment), the approach adopted by the IBSC was to identify the type and the circumstances of the genetic modification(s), to evaluate these against the criteria specified in section 41, and to consider whether there are any residual risks of significance that require further consideration. Refer to Annex A for guidance on identifying and assessing significant risks and costs.

Candida albicans was identified as an opportunistic pathogen that can cause infections in immunocompromised individuals.

This risk was addressed by imposing an additional control, listed in section 5, that persons known to be immunocompromised shall not work with genetically modified C. albicans.

4  Controls

In considering all the matters to be addressed detailed in the Third Schedule Part I Containment Controls for Importing, Developing or Field Testing of Genetically Modified Organisms of the HSN0 Act, the IBSC approval of the organism(s) is subject to the following controls:

1) The operation, management and construction of the facility shall be in accordance with the:
a) MAF Biosecurity Authority/ERMA New Zealand Standard 154.03.02 Containment Facilities for Microorganisms and the MAF Biosecurity Authority/ERMA New Zealand Standard 154.03.03 Containment Facilities for Vertebrate Laboratory Animals.


2) The facilities shall be approved and registered by MAF Biosecurity Authority as containment facilities under section 39 of the Biosecurity Act, in accordance with the MAF Biosecurity Authority/ERMA New Zealand Standards 154.03.02 and 154.03.03 and additional controls as listed below.

3) All approved organism culture products and associated materials shall be autoclaved or incinerated before being disposed of.

4) If for any reason a breach of containment occurs the applicant shall notify the facility Supervisor and ERMA New Zealand immediately the event is noticed (and at least within 24 hours of the breach being detected) and shall immediately implement a contingency plan for the recovery and eradication of any organisms or viable material that has escaped.

5) The Authority or its authorised agent or properly authorised enforcement officers, may inspect the facilities at any reasonable time.

5 Additional controls

Persons known to be immunocompromised shall not work with genetically modified C. albicans.

Vertebrate animals will only be exposed to genetically modified C. albicans cells after ethical approval for the exposure has been obtained from an accredited animal ethics committee and the IBSC has sighted this ethical approval.

Signed: ........
(on behalf of the institution)

Date

Nat

Position: IBSC Member (deputising for ) has a conflict of interest on this application

Send a copy of the decision form, the checklist and the application to:
- ERMA New Zealand, PO Box 131, Wellington
  Attention: Libby Harrison

Send a copy of the decision form to:
- Applicant
Checklist

NB: this checklist should be completed by the IBSC, and signed and dated by the Chair of the IBSC and returned to ERMA New Zealand with the decision form.

- Sections referenced indicate sections of the Hazardous Substances and New Organisms Act 1996
- Clauses referenced indicate clauses of the Hazardous Substances and New Organisms (Methodology) Order 1998

<table>
<thead>
<tr>
<th></th>
<th>Legislative criteria for the application</th>
<th>Yes</th>
<th>No</th>
<th>N/A</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>The application was lodged pursuant to section 49(1)(b) of the HSNO Act. The decision was determined in accordance with section 42 and 42A (rapid assessment) and matters relevant to the purpose of the Act, as specified under Part II of the HSNO Act</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.2</td>
<td>Consideration of the application followed the relevant provisions of the Hazardous Substances and New Organisms (Methodology) Order 1998 (the Methodology).</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.3</td>
<td>Was any expert advice sought under clause 17?</td>
<td>✓</td>
<td></td>
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</tr>
<tr>
<td>1.5</td>
<td>If YES – name of the expert</td>
<td></td>
<td></td>
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<tr>
<td>1.6</td>
<td>If YES – was the applicant informed under clause 18?</td>
<td>✓</td>
<td></td>
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</tr>
</tbody>
</table>

2 | Consideration of the application | |
| 2.1 | The IBSC holds delegation from the Authority as provided under section 192(2)(a) of the HSNO Act. | ✓   |    |     |
| 2.2 | The purpose is appropriate under section 39(1)(a) of the Act: The development of any genetically modified organism. | ✓   |    |     |
| 2.3 | Does the IBSC consider the information provided by the applicant relevant and appropriate to the scale and significance of the risks, costs, and benefits associated with the application (as required by clause 8 of the Methodology)? | ✓   |    |     |
| 2.4 | If NO – discuss | ✓   |    |     |

3 | Sequence of the consideration | |
| 3.1 | In accordance with sections 42 and 42A of the Act (rapid assessment), the approach adopted by the IBSC was to identify the circumstances of the genetic modification(s), to evaluate these against the Regulations established under section 41 of the Act, and to consider whether there are any residual risks of significance that require further consideration. | ✓   |    |     |

4 | Identification of significant risks | |
<p>| 4.1 | Are there any significant risks or costs to the environment? | ✓   |    |     |
| 4.2 | Are there any significant risks or costs to human health? | ✓   |    |     |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th>Yes</th>
<th>No</th>
<th>N/A</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.4</td>
<td>Are there any significant risks to Maori and their taonga?</td>
<td></td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>4.5</td>
<td>Are there any significant economic risks or costs?</td>
<td>✓</td>
<td></td>
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</tr>
<tr>
<td>4.6</td>
<td>Are there any risks to New Zealand’s international obligations, including DNA derived from CITES species or use of CITES species as host organisms?</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>If YES is checked in any of 4.1-4.6, please list the risks identified on the decision form and discuss how they were assessed in terms of likelihood and consequence, and what controls were imposed to manage them. Refer to clauses 12 and 13.</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cordyceps australis was identified as an opportunistic pathogen that can cause infections in immunocompromised individuals. This risk was addressed by imposing the additional control that persons known to be immunocompromised shall not work with genetically modified C. australis.</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Applications involving native flora and fauna</td>
<td></td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>5.1</td>
<td>Does the application use genetic material from native flora and/or fauna?</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.2</td>
<td>Does the application use native flora and/or fauna as host organisms?</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.3</td>
<td>In accordance with section 8 of the Act, was consultation with Maori carried out?</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>If YES, please provide a discussion below about who was consulted, their status and the results of the consultation.</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Applications involving human DNA</td>
<td></td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>6.1</td>
<td>Does the application use any genetic material obtained either directly or indirectly from human beings?</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.2</td>
<td>If YES is answered to 6.1, has approval from an Ethics Committee been obtained for any DNA sourced directly from human beings?</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.3</td>
<td>If YES, please provide a discussion below about who was consulted, their status and the results of the consultation.</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Assessment against the criteria for low risk genetic modifications</td>
<td></td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>7.1</td>
<td>Does the IBSC consider that the development of each of the genetically modified organisms described in the application meet the criteria for a low-risk genetic modification specified in the regulations made under section 41 of the Act, being the HSNO (Low-Risk Genetic Modification) Regulations 2003?</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Containment of the organisms</td>
<td></td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>8.1</td>
<td>In carrying out its consideration did the IBSC consider the adequacy of containment in accordance with section 42(2) NB The IBSC should include details of the modifications and state which Category of the low risk regulations that they fall within. The IBSC should also specify the level of containment relevant to that category (the controls relevant to the level of containment are detailed at the end of the decision form). Note that the IBSC may add additional controls where it considers these are necessary to ensure containment, but that controls relevant to the physical containment level set in the Regulations cannot be removed.</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.2</td>
<td>Will the containment facility be operated and constructed in accordance with the: (a) the Australian/New Zealand Standard AS/NZS 2243.1 Safety in Laboratories: Part 3: Microbiological aspects and containment facilities at Physical Containment Level 2 (PC2); and (b) the MAF Biosecurity Authority/ERMA ERMA New Zealand Standards 154.03.02 and 154.03.03</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.3</td>
<td>Are any additional measures proposed because of the particular nature of the organism(s) or the proposed procedures?</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>---</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>If YES, these are: Persons known to be immunocompromised shall not work with genetically modified C. albicans. Vertebrate animals will only be exposed to genetically modified C. albicans cells after ethical approval for the exposure has been obtained from an accredited animal ethics committee and the IBSC has sighted this ethical approval.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.5 Are there any other matters that may affect the adequacy of containment such as the expected time frame for the project, and external matters such as the potential for sabotage?</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>If YES, please discuss</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Decision</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In this section YES confirms approval – if any of the answers to 9.1-9.4 are NO, then the application is declined.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.1 The IBSC is satisfied that the application is for one of the purposes specified in section 39(1) of the Act, being section 39(1)(a): The development of any genetically modified organism?</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.2 Based on an analysis of the information provided, and having considered the characteristics of the organisms and the modifications and the criteria for low-risk genetic modification detailed in the HSN (Low-Risk Genetic Modification) Regulations 2003, it is the view of the IBSC that the organism(s) meet the criteria for rapid assessment (as per section 42(2)).</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.3 The IBSC is satisfied that the proposed containment regime together with any additional controls imposed will adequately contain the organism(s) as required by section 42(2) of the Act?</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.4 In accordance with clause 36(b) of the Methodology the IBSC records that, in reaching this conclusion, it has applied the following criteria from the Methodology: Where relevant briefly discuss relevant clauses of the Methodology • clause 9 • clause 10 - minimum standards criteria (sections 36 and 37) • clause 12 - evaluation of assessment of risks (to meet requirements of section 41) • clause 21 - the decision accords with the requirements of the Act and regulations</td>
<td>✓</td>
<td></td>
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Date 16th April 2004

University of Otago Institutional Biological Safety Committee
Appendix 2: MATING IN VIVO – WHITE OPAQUE PROPORTIONS AND MAT ANT COUNTS

In the measurement of White to opaque cells in the oral cavity (Figure A2.1 A) and fecal pellet (Figure A2.1 B), it was seen that the proportion of white to opaque is nearly equal till up to 14 days past the inoculation, however almost all cells switched to white isoform by day 21 post inoculation in both the oral cavity and in the fecal sample.

![Graph A2.1](image1)

**Figure A2.1:** The proportion of white to opaque cells measured (by growth on medium containing the dye Phloxine B) in both the oral swab (A) and the also the fecal pellet (B) following co-inoculation with white cells of *C. albicans* W43 and *C. albicans* OD8916. The R in the figure is the rat number, triangles represent rat 1, squares rat 2 and circles rat 3.

![Graph A2.2](image2)

**Figure A2.2:** The proportion of white to opaque cells measured (by growth on medium containing the dye Phloxine B) in both the oral swab (A) and the also the fecal pellet (B) following co-inoculation with *C. albicans* W43 and *C. albicans* W17. The R in the figure is the rat number, triangles represent rat 1, squares rat 2 and circles rat 3.
**Figure A2.3:** The proportion of white to opaque cells measured (by growth on medium containing the dye Phloxine B) in both the oral swab (A) and the also the fecal pellet (B) following co-inoculation with opaque cells of *C. albicans* W43 and *C. albicans* OD8916. The R in the figure is the rat number, triangles represent rat 1, squares rat 2 and circles rat 3.

**Figure A2.4:** The proportion of white to opaque cells measured (by growth on medium containing the dye Phloxine B) in both the oral swab (A) and the also the fecal pellet (B) following co-inoculation with opaque cells of *C. albicans* W43 and *C. albicans* W17. The R in the figure is the rat number, triangles represent rat 1, squares rat 2 and circles rat 3.
**Figure A2.5:** The proportion of white to opaque cells measured (by growth on medium containing the dye Phloxine B) in both the oral swab (A) and the also the fecal pellet (B) following co-inoculation with co-inoculation with opaque cells of *C. albicans* YSU63 and *C. albicans* W43. The R in the figure is the rat number, triangles represent rat 1, squares rat 2 and circles rat 3.

**Figure A2.6:** Mutants obtained from oral (A) and fecal (B) samples after inoculating the oral cavity with opaque only cells with *C. albicans* W43 and *C. albicans* OD8916. The R in the figure is the rat number, triangles represent rat 1, squares rat 2 and circles rat 3.
**Figure A2.7**: Isolation of yeast from oral sample following co-inoculation with *C. albicans* HUN97 and *C. albicans* Au90. Graph of yeast present in oral samples co-inoculated with white cells of *C. albicans* HUN97 and *C. albicans* Au90. The R in the figure is the rat number, triangles represent rat 1, squares rat 2 and circles rat 3.

**Figure A2.8**: Isolation of yeast from fecal sample following co-inoculation with *C. albicans* HUN97 and *C. albicans* Au90. Graph of yeast present in fecal samples co-inoculated with white cells of *C. albicans* HUN97 and *C. albicans* Au90. The R in the figure is the rat number, triangles represent rat 1, squares rat 2 and circles rat 3.
Figure A2.9: Isolation of yeast from oral sample following co-inoculation with *C. albicans* HUN97 and *C. albicans* Au90. Graph of yeast present in for oral samples for rats co-inoculated with opaque cells of *C. albicans* HUN97 and *C. albicans* Au90. The R in the figure is the rat number, triangles represent rat 1, squares rat 2 and circles rat 3.

Figure A2.10: Isolation of yeast from fecal sample following co-inoculation with *C. albicans* HUN97 and *C. albicans* Au90. Graph of yeast present in for fecal samples for rats co-inoculated with opaque cells of *C. albicans* HUN97 and *C. albicans* Au90. The R in the figure is the rat number, triangles represent rat 1, squares rat 2 and circles rat 3.
Figure A2.11: Isolation of yeast from oral sample following co-inoculation with *C. albicans* HUN97 and *C. albicans* Au90. Graph of yeast present in for oral samples for rats co-inoculated with uneven number of opaque cells of *C. albicans* HUN97 and *C. albicans* Au90. The R in the figure is the rat number, triangles represent rat 1, squares rat 2 and circles rat 3.

Figure A2.12: Isolation of yeast from fecal sample following co-inoculation with *C. albicans* HUN97 and *C. albicans* Au90. Graph of yeast present in for fecal samples for rats co-inoculated with uneven number of opaque cells of *C. albicans* HUN97 and *C. albicans* Au90. The R in the figure is the rat number, triangles represent rat 1, squares rat 2 and circles rat 3.
**Figure A2.13:** Isolation of yeast from oral sample following co-inoculation with *C. albicans* FJ11 and *C. albicans* Au90. Graph of yeast present in oral samples for rats co-inoculated with white cells of *C. albicans* FJ11 and *C. albicans* Au90. The R in the figure is the rat number, triangles represent rat 1, squares rat 2 and circles rat 3.

**Figure A2.14:** Isolation of yeast from fecal sample following co-inoculation with *C. albicans* FJ11 and *C. albicans* Au90. Graph of yeast present in fecal samples for rats co-inoculated with white cells of *C. albicans* FJ11 and *C. albicans* Au90. The R in the figure is the rat number, triangles represent rat 1, squares rat 2 and circles rat 3.
Figure A2.15: Isolation of yeast from oral sample following co-inoculation with *C. albicans* FJ11 and *C. albicans* Au90. Graph of yeast present in for oral samples for rats co-inoculated with opaque cells of *C. albicans* FJ11 and *C. albicans* Au90. The R in the figure is the rat number, triangles represent rat 1, squares rat 2 and circles rat 3.

Figure A2.16: Isolation of yeast from fecal sample following co-inoculation with *C. albicans* FJ11 and *C. albicans* Au90. Graph of yeast present in for fecal samples for rats co-inoculated with opaque cells of *C. albicans* FJ11 and *C. albicans* Au90. The R in the figure is the rat number, triangles represent rat 1, squares rat 2 and circles rat 3.
Figure A2.17: Isolation of yeast from oral sample following co-inoculation with *C. albicans* FJ11 and *C. albicans* W43 (Oral sample). Graph of yeast present in for oral samples for rats co-inoculated with white cells of *C. albicans* FJ11 and *C. albicans* W43. The R in the figure is the rat number, triangles represent rat 1, squares rat 2 and circles rat 3.

Figure A2.18: Isolation of yeast from fecal sample following co-inoculation with *C. albicans* FJ11 and *C. albicans* W43 (Fecal sample). Graph of yeast present in for fecal samples for rats co-inoculated with white cells of *C. albicans* FJ11 and *C. albicans* W43. The R in the figure is the rat number, triangles represent rat 1, squares rat 2 and circles rat 3.
Figure A2.19: Isolation of yeast from oral sample following co-inoculation with *C. albicans* FJ11 and *C. albicans* W43 (Oral sample). Graph of yeast present in for oral samples for rats co-inoculated with opaque cells of *C. albicans* FJ11 and *C. albicans* W43. The R in the figure is the rat number, triangles represent rat 1, squares rat 2 and circles rat 3.

Figure A2.20: Isolation of yeast from fecal sample following co-inoculation with *C. albicans* FJ11 and *C. albicans* W43 (Fecal sample). Graph of yeast present in for fecal samples for rats co-inoculated with opaque cells of *C. albicans* FJ11 and *C. albicans* W43. The R in the figure is the rat number, triangles represent rat 1, squares rat 2 and circles rat 3.
Appendix 3: *IN VITRO GROWTH CURVES*

**Figure A3.1:** *In vitro* growth curves for A: *C. albicans* W17; B: *C. albicans* OD8916; C: *C. albicans* W43; D: *C. albicans* OD8916 X W43; and E: *C. albicans* W17 X W43.
# Appendix 4: COLONY COUNTS FOR ALL MATING COMBINATIONS

Table A4.1: Investigation of mating *in vivo* between *C. albicans* OD8916 and *C. albicans* W43.

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**Table A4.2:** Investigation of mating *in vivo* between *C. albicans* W17 and *C. albicans* W43.

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Table A4.3: Colony counts for test crosses involving the use of *C. albicans* YSU63 and *C. albicans* W43.
Table A4.4: Colony counts for test crosses involving the use of *C. albicans* HUN97 and *C. albicans* Au90.

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Table A4.5: Colony counts for test crosses involving the use of *C. albicans* FJ11 and *C. albicans* Au90.

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Table A4.6: Colony counts for test crosses involving the use of *C. albicans* FJ11 and *C. albicans* W43.

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Appendix 5 : MARKER DESIGN AND CONSTRUCTION

Figure A5.1: Principle of the two marker system for marking parental strains and the generation of recombinant strains (Zhang 2012). Parent 1 is made MPA-resistant by integrative transformation with a MPA resistance cassette \((MPA^R)\) into chromosome 7(chr7). Parent 2 is made NAT-resistant by integrative transformation with NAT resistance cassette \((NAT^R)\) into chromosome 1(chr1) and mutants can be selected on the basis of resistance to both drugs.
Plasmid pNZ11 was constructed from plasmid pJK850, containing a URA3 ORF with a URA3 promoter (pURA3) and a pCaACT1+CaNAT+tCaACT (a NAT resistance gene) created for C. albicans under control of a C. albicans ACT1 promoter plus an ACT1 terminator) cassette, in a pBSKS(+) backbone. The pCaACT1+CaNAT+tCaACT cassette was amplified using primers M13pr/Sacpf1, digested pBlueScript KS (+) vector. Locations of primers (arrows) and the restriction sites used in cloning are labelled (Zhang 2012 ).
Figure A5.3: Construction of MPA resistance cassette-containing plasmid pNZ4. Plasmid pNZ4 was constructed from pNZ1, pNZ2, and pNZ3 as described in the text. TS1 and TS2 are targeting sequences from the *C. albicans* genome. The promoter pTR (promoter of tetracycline-responsive gene) was amplified from the plasmid p99RLU, while the promoter pENO1 (promoter of the enolase 1 gene) and the tet-ScHAP4-WH11 (tetracycline activator ORF) were amplified from the plasmid pCAITHE5. The MPA resistance gene *IMH3* was amplified from plasmid p3408. Locations of primers (arrows) and restriction sites used in the cloning are labelled (Zhang 2012).