Differences In The Pathogenic Potential Of *Candida* species Especially *C. albicans*.

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Rajab

DECLARATION

I declare that, with the exception of the assistance acknowledged, no portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or institution of learning.

SUMMARY

In 1977, Winner stated that "*Candida* has turned out to be a key organism in modern medicine, both as an opportunist aggressor and as an indicator of multiple opportunistic infection". During the intervening period, this statement has proved to be true and the prevalence of candidosis has increased dramatically.

To date, most of the studies which have been performed on the differences in the pathogenic potential of *Candida* species have focused on either the virulence attributes of the organism or the host defence parameters. Only a few workers have investigated in depth, in a single comprehensive study, the relationships between different potential pathogenic attributes of a considerable number of *Candida* species and/or isolates, and putative host defence mechanisms. Most studies have investigated only one factor (e.g. adhesion), with the result that there is very little information about the range of pathogenic factors produced by a single yeast isolate.

In the current study, the adhesion of *Candida* species to buccal epithelial cells (BEC's) and acrylic surfaces; the production of phospholipase and proteinase, and the susceptibility to bronchoalveolar lavage fluid (BLF) and lysozyme of 49 *Candida* isolates comprising *C. albicans* (22), *C. tropicalis* (7), *C. krusei* (7), *C. parapsilosis* (5), *C. guilliermondii* (5). and *C. glabrata* (3), were investigated. The results of this thesis reveal that variations in adhesion between isolates of the same or different species to either BEC's or acrylic surfaces occur. For example, variations in adhesion, between *C. albicans* isolates to BEC's ranged from 542 to 1569 yeasts/100 BEC's and 180 to 577 yeast/mm^2 acrylic with mean

values of 988 and 395 respectively. Moreover, variations in adhesion of *C. albicans* isolates to BEC's collected from the same donor were also observed. The order of mean adhesion values for the yeast cells to 100 BEC's was as follows: *C. albicans* (988), *C. tropicalis* (464) *C. parapsilosis* (163), *C. glabrata* (183), *C. guilliermondii* (171) and *C. krusei* (89). However, *C. tropicalis* (669 yeast/mm2) headed the rank order of adhesion to acrylic strips, followed by: *C. albicans* (395) *C. parapsilosis*, (375), *C. glabrata* (340), *C. guilliermondii* (222) and *C. krusei* (131).

Only *C. albicans* isolates produced phospholipase extracellularly whereas the other *Candida* species investigated failed to release this enzyme into the growth medium. The phospholipase activity of *C. krusei* and *C. guilliermondii* were investigated for the first time, although they were found to be uniformly negative. The variations in phospholipase activity of the 22 *C. albicans* isolates tended to follow the adhesion values to BEC's, i.e., those with high adhesion potential released more phospholipase. Production of phospholipase also appeared to be related to the clinical sources of the isolates; strains isolated from candidosis cases produced more than 30 units of phospholipase activity, whereas those isolated from asymptomatic carriers produced lesser quantities of the enzyme.

Proteolytic enzymes were produced *in vitro* by strains of *C. albicans* (68.2%), *C. tropicalis* (71.4%) and *C.parapsilosis* (100%) but not by the other *Candida* species studied. No obvious relationships could be demonstrated in the current study, between the proteinase activity of *Candida* species and either adhesion to BEC's, acrylic surfaces or phospholipase activity.

The susceptibility of *Candida* species to the effect of non-specific host defences was investigated using lysozyme and BLF. The results of the lysozyme study suggest that due to its anti-candida activity the enzyme which is presented could act as a potent inhibitor of candidal colonization of the oral cavity. Also the lysozyme present in the lysosomes of phagocytes may contribute to the anti-candida activity of these cells. A significant dose response relationship between lysozyme concentration and fungicidal activity was also found. Overall the results showed variations in the susceptibility of different *C. albicans* isolates to lysozyme and indeed, however, other *Candida* species also demonstrated wide variations in their susceptibility to the enzyme. The most susceptible *Candida* species was *C. glabrata* followed by *C. albicans C. guilliermondii, C. tropicalis, C. parapsilosis* and *C. krusei*.

The other non-specific host defence factor studied in this thesis was bronchoalveolar lavage fluid, which was collected from 12 Sprague Dawley rats. The results suggest that BLF may well play an important role in non-specific host defences of the pulmonary tree combating infection with *Candida* species. Bronchoalveolar lavage fluid had a significant growth inhibition and killing effect on *C. albicans* isolates. When incubated for 120 minutes the percentage survival rate of *Candida* species investigated were as follows: *C. albicans* (5.27%) *C. parapsilosis* (38.3%) *C. tropicalis*.(45.9%). *C. krusei* (59.1%), *C. glabrata* (63.3%) and *C. guilliermondii* (89.9%). Inactivation of BLF by heat (56°C for 30 minutes) produced slight reduction in anti-candida activity when compared to neat fluid. This may indicate the presence of both heat labile and heat stable anti-candidal factors in BLF. Inter-

estingly, the susceptibility of most of the *C. albicans* isolates to BLF was generally related to that of lysozyme.

In conclusion the results of this work enhances and complements the present concepts and relationships between potential pathogenic parameters of *Candida*. Further the factors responsible for the differences in mucosal response to candidal invasion, such as body fluids (e.g., BLF) and their contents (e.g., Lysozyme) would appear to play a critical role in the pathogenesis of the disease. Therefore, further work is required to clarify the role of BLF, lysozyme and other cytolytic candidal enzymes in initiating or inhibiting candidosis,.and much work remains to be done before the mechanisms of *Candida* pathogenicity is fully established.

PREFACE

The present work described in this study was undertaken in the Department of Oral Medicine, University of Glasgow.

Parts of investigations reported in this thesis have been published or submitted for publication in scientific Journals or have been presented at scientific conferences as follows:

A- Publications :

Referreed paper

Tobgi, RS., Samaranayake LP., MacFarlane TW., (1988). *In vitro* susceptibility of *Candida* species to lysozyme. *Oral Microbiology and Immunology,* **3,** 35-39.

Reprinted abstracts

Tobgi, RS., Samaranayake, LP., and MacFarlane, TW., (1986). The distribution of factors related to pathogenicity of *Candida albicans Journal of Dental Research,* **65,** 523.

Samaranayake, LP., Tobgi, RS., Anderson, J. and MacFarlane, TW., (1988). Studies on the susceptibility of *Candida* species to lysozyme. *Journal of Dental Research,* **68,** 592.

Tobgi, RS., Samaranayake, LP., MacFarlane, TW., and Rennie, JS., (1989). The Fungicidal effect of murine bronchial lavage fluid. *Journal of Dental Research,* **68,** 595.

B- Presentation at scientific meetings

An investigation into the factors involved in the pathogenicity of *Candida albicans* (with Samaranayake LP., and MacFarlane, TW.). British Society for Dental Research, April, 1986, Dundee.

The fungicidal effect of murine bronchial lavage fluid on *Candida* species. (with Samaranayake L.P., and MacFarlane, TW.).

British Society for Mycopathology, March (1988), Glasgow, Scotland.

The *in vitro* susceptibility of *Candida* species to lysozyme. (with Anderson, J., Samaranayake L.P., and MacFarlane, T.W.). British Society for Mycopathology, March (1988), Glasgow, Scotland.

The oral carriage of 'virulent' and 'avirulent' *Candida albicans* in rats. (with Samaranayake L.P., Rennie, J.S. and MacFarlane, T,W.). British Society for Mycopathology, March (1988), Glasgow, Scotland.

Studies on the susceptibility of *Candida* species to lysozyme. (with Samaranayake L.P., Anderson, J. and MacFarlane, T.W.). British Society for Dental Research, April (1989), Liverpool, England.

I. ABBREVIATIONS

ANOVA = Analysis Of Variance
BEC = Buccal Epithelial Cells
BLF = Bronchoalveolar Lavage Fluid
CAC = Chronic Atrophic Candidosis
CFU = Colony Forming Units
cm = centimeter
CMI = cell-mediated immunity
EDTA = Ethylenediaminetetraacetate
g = gram(s), gravity
GDH = Glasgow Dental Hospital & School
GRI = Glasgow Royal Infirmary
Hg = Constant Pressure (Latin, *hydrargyrum*)
ml = millilitre(s)
mm = millimeter(s)
MP = Mycological peptone
MRL = Mycological Reference Laboratory, London School of Hygiene and Tropical Medicine, London WCIE 7HT UK
NCPF = National Collection of Pathogenic Fungi
NCYC = National collection of Yeast Culture
NS = Not Significant
p = probability
PBS = Phosphate Buffer Saline
PHLS = Public Health Laboratory Service
PMN = Polymorphonuclear
rpm = revelution per minute
SDA = Sabouraud's Dextrose Agar.
SEM = Standard Error of Mean.
YNB = Yeast Nitrogen Base.
Mon = Monday
Wed = Wednesday
Fri = Friday
UV = Ultra-violet

II. Abbreviations Uesd In *Candida* Isolates Code

a	=	*C. albicans*
t	=	*C. tropicalis*
k	=	*C. krusei*
p	=	*C. parapsilosis*
gu	=	*C. guilliermondii*
g	=	*C. glabrata*

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1. A Brief Historical Review.

It is known that *Candida* infection occurred in ancient times; Veron for instance, in 1835 postulated that *Candida* was acquired during birth and, he also described the first cases of oesophageal candidosis(cited in Rippon 1988). Bennett in 1844 conclusively characterized the fungal aetiology of thrush, and the relationship between the thrush fungus and mouth lesions was defined subsequently by Berg in 1846 (cited in Odds 1988).

The causative organism was classified in the genus *Sporotrichum* by Gruby (1842) but in 1853 Robin made extensive observations on the occurrence of the organism and reclassified it in the genus *Oidium* with the name *Oidium albicans*. The name *Monilia albicans* used for several decades, was first introduced by Zopf in 1890 and gave rise to the name moniliasis. In 1923 Berkhout proposed the generic name *Candida* to include this fungus (cited in Odds 1988). A clear preference has been made by a number of reviewers, for *Candida* over *Monilia* (Lodder 1970; Skinner and Fletcher, 1960; Winner and Hurley 1964; Van Uden and Buckley 1970; Hurley *et al.*, 1986), with the result that candidosis is the currently accepted nomenclature for infections caused by *Candida* species, although, candidiasis is still used by other workers. Candidosis will be used in this thesis for the reasons listed by Odds (1988).

1.2. Epidemiology and Prevalence

1.2.1. Distribution Of *Candida* species

Prevalence Of *Candida*

Yeasts belonging to the genus *Candida* are unicellular, dimorphic fungi which reproduce mainly by budding and have been detected in humans as well as in many warm-blooded animals (Hurley *et al.*, 1986). *Candida albicans, C. stellatoidea,* and *C. glabrata* are obligatory animal saprophytes, while other species such as *C. guilliermondii, C. krusei, C. parapsilosis* and *C. tropicalis* may be found as facultative saprophytes, i.e., they may be recovered from sources other than animals in terrestrial and aquatic habitats. Other species of *Candida* that have been rarely demonstrated in human infections include *C. viswanathii* (Sandhu and Randhawa 1962), *C. lusitaniae* (Holzschu *et al.*, 1979), *C. claussenii, C. intermedia, C. lambica, C. macedoniensis, C. robusta* (Hantschke and Zobel 1979), *C. norvegensis, C. zeylanoides, C. catenula* (Ström *et al.*, 1978), *C. ravautii* (Crozier and Coats 1977) and *C. lipolytica* (Nitzulescu and Nitzulescu 1976).

It is well known that the prevalence of *Candida* is relatively high in normal subjects especially so during old age, pregnancy, and infancy. Reports of isolation of pathogenic *Candida* species from humans and many animals have been reviewed by Gentles and La Touche (1969); Hurley (1980); Hurley *et al.* (1986). Despite it s wide prevalence in these animal reservoirs, the source of human infection by *C. albicans* is mainly,

endogenous, arising from carriage sites on the patient himself. It has been found that the prevalence of *C. albicans* in the human body ranges from 6-50 percent in the mouth, 20-40 percent in the stomach and small intestine, 15-30 percent in the large intestine, and 30-50 percent in the genital-urinary tract. On the skin of unselected healthy individuals, *Candida* is not commonly present, and it seems that skin carriage varies dramatically depending on the geographical location and occupation of the study group (Odds 1988). The highest carriage rates of *Candida* on the skin were obtained from the fingers of medical students (Clayton and Noble 1963).

Many investigators have suggested other exogenous sources of candidal colonization such as the mother's birth canal, the skin of both nurses and mothers (Winner and Hurley 1964; Jennison 1966), and infected feeding bottles (Robson and Anderson 1964). It is also frequently transmitted by venereal infection (Winner 1977; Horowitz *et al.*, 1987). *Candida albicans* has also been implicated in cross infection in a hospital where it was associated with spread of infection from the hands of staff to patients (Russell and Lay 1973). Furthermore, the mouth and perineum of hospital staff members may act as secondary reservoirs of infection (Clayton and Noble 1963; Burnie *et al.*, 1985 a,b).

Pathogenic Potential of *Candida* species:

In 1912 Castellani recognized that moniliasis (Candidosis) is not caused by a single yeast species and suggested that species other than *Monilia albicans* (*Candida albicans*) might be

involved. He described many other strains, some of which are currently known i.e., *C. guilliermondii, C. krusei, C. pseudotropicalis* and *C. tropicalis.* However, more recently, ten species have been reported with varying degrees of virulence (Hurley, de Louvois and Mulhall 1986). They are in order of decreasing virulence: *C. albicans, C. tropicalis, C. stellatoidea, C. glabrata, C. krusei, C. parapsilosis, C. pseudotropicalis, C. guilliermondii, C. viswanathii,* and *C. lusitaniae.* Despite such groupings, *C. albicans* is usually encountered in most of the clinical forms of candidosis (85-90 %), and appears to be the only member of the genus regularly able to cause fatal disease in man and animals (Male 1977; Hurley *et al,* 1986). In some of the less common clinical conditions such as *Candida* endocarditis, other species are more frequently isolated (Table 1.1).

Candida albicans, C. tropicalis, C. stellatoidea, C. pseudotropicalis and *C. viswanathii* can all be shown to produce the typical lesions of systemic candidosis on injection into mice or other laboratory animals (Sandhu, Randhawa and Gupta 1965). *Candida parapsilosis* produces visceral lesions in cortisone-treated mice (Goldstein *et al.,* 1965) and typical lesions have been produced by *C. glabrata* in pregnant or cortisone-treated mice (Hasenclever and Mitchell 1963; Knudtson *et al.,* 1973). The variation in the pathogenicity of different species of *Candida* have been studied by Hurley and her colleagues (Stanley and Hurley 1967) and they categorized this genus into three

Table 1.1. Percentage of distribution of *Candida* species from different sources, (data from several studies).

Source of isolation	*C. albicans*	*C. glabrata*	*C. guilliermondii*	*C. krusei*	*C. parapsilosis*	*C. tropicalis*
Oral Cavity	69.6	6.6	0.4	1.7	1.9	6.9
Vagina with vaginitis	84.2	5.5	0.5	1.7	1.2	5.3
Vagina without vaginitis	69.7	11.7	0.5	2.6	1.9	4.7
Anorectal Tract	50.9	9.1	0.7	2.9	5.4	2.3
Gastointestinal Tract	56.5	16.1	0.5	2.6	6.1	9.7
Blood Culture	50.4	9.7	0.9	1.1	12	18.5
Urine	55.4	20.2	1.9	2.1	4.1	8.9

Adapted from Odds 1988.

groups depending on the destructive effect on renal epithelial cells of five-day-old mice. Group I: consisted of *C. albicans*, *C. tropicalis*, and *C. stellatoidea*. Group II: of *C. krusei*, *C. parapsilosis*, *C. pseudotropicalis* and *C. guilliermondii*.was the only yeast in Group III: The pathogenesis of *C. glabrata* which is the second commonest isolate from oral lesions, is placed between Groups I and II. Hurley, de Louvois and Mulhall (1986) showed that *Candida* species could be ranked in order of virulence for man, based partly on the results of their cytopathic effect in tissue-culture systems and partly on clinical experience. Accordingly *C. albicans* was the most pathogenic, followed in order by *C. tropicalis*, *C. stellatoidea*, *C. glabrata*, *C. krusei*, *C. parapsilosis*, *C. pseudotropicalis*, *C. guilliermondii*, *C. viswanathii* and *C. lusitaniae*. Although the hierarchy of *Candida* species with respect to their virulence has been established to a certain degree it is likely that most candidal virulence factors still remain undiscovered.

1.2.2 The Spectrum OF Candidosis:

Candida species produce a diverse spectrum of diseases in humans provided that the appropriate predisposing conditions exist (Table 1.2). *Candida* primarily attacks the mucous membranes, but the yeast may invade and damage deep seated tissues if the host defences are impaired. As seen in Table 1.2 there are many variations of deep seated candidosis although they are relatively rare. Nevertheless, superficial candidosis (i.e., oral and vaginal candidosis) are fairly common and localized

Table 1.2 The spectrum of candidosis

Mucosal and cutaneous candidosis:

-Oral candidosis.

-Vaginitis and vulvovaginitis.

-Intrauterine and neonatal cutaneous candidosis.

-Candida intertrigo.

-Onychia and paronychia

-Perianal candidosis

-Keratoconjunctivitis and other ophthalmic manifestations

-Chronic mucocutaneous candidosis.

Candidosis of the alimentary tract:

-Oesophageal candidosis.

-Enteric candidosis.

Renal candidosis:

Cardiac candidosis:

-Candida endocarditis

-Candida myocarditis and pericarditis

Candidosis of central nervous system :

-Candida meningitis.

-Cerebral candidosis.

Candida septicaemia and disseminated candidosis:

Adapted from Seelig and Kozinn (1982).

oral candidosis seen in humans can be divided into 5 major categories (MacFarlane and Samaranayake 1989), as described below.

1.2.3.Superficial Candidosis.

A- Oral Candidosis

Although oral thrush is the best known form of mouth infection with *Candida*, other types of oral candidosis also occur. Localized oral candidosis have been grouped into the following descriptive categories by MacFarlane and Samaranayake (1989):

1.Acute pseudomembranous candidosis (thrush)
2.Acute atrophic candidosis
3.Chronic atrophic candidosis (denture stomatitis)
4.Chronic hyperplastic candidosis (candidal leukoplakia)
5.Angular cheilitis (perlèche).

It should be noted that, systemic infections of *Candida* species may manifest orally in rare disorders such as chronic mucocutaneous candidosis, and candida endocrinopathy syndrome (Valdimarsson *et al.*, 1973; MacFarlane and Samaranayake 1989). These will not be discussed further in this review.

i.Acute pseudomembranous candidosis (thrush)

Oral thrush, is a not uncommon disease of new born infants who are probably susceptible to candida infection because their antimicrobial defences are immature, and they do not possess a normal bacterial flora in the upper respiratory tract. Thrush may occur also in older children and adults who have impaired

natural defences, (Lehner 1967; Seelig and Kozinn 1982). The disease is generally associated with some prior local disturbance or systemic illness, e.g., recent antibiotics, steroid, and immunosuppressive drug therapy, diabetes mellitus, anaemia, blood dyscrasias such as leukaemia, and advanced malignant states. The condition is characterized by the presence of loosely adherent, whitish membranes, particularly on the tongue and cheek, which can be easily rubbed off, leaving an area of erythema or bleeding.

ii. Acute atrophic candidosis.

This type of candidosis may follow thrush after the disappearance of the overlying pseudomembrane. It may also appear following the administration of broad spectrum antibiotics such as tetracycline. These drugs tend to disrupt the balance of the normal flora which is no longer able to control yeast growth, perhaps due to its inability to inhibit candidal adhesion and germination (Samaranayake and MacFarlane 1982b; Seelig and Kozinn 1982). This condition is sometimes referred to as antibiotic sore mouth, and it presents as a red and often painful area of oral mucosa (most commonly the tongue) which resembles thrush, without the overlying pseudomembrane (Soames and Southam 1985).

Recently, this condition has assumed a renewed importance, as atrophic candidosis is commonly seen in HIV-infected individuals. A new term, erythematous candidosis has been proposed for this condition (Pindborg 1989).

iii.Chronic atrophic-candidosis (denture stomatitis)

Chronic atrophic candidosis is the commonest form of oral candidoses (Odds 1988) and effects about 60 percent of denture wearers aged 65 or more (Budtz-Jörgensen 1977). The condition is characterized by chronic inflammation and oedema of the portion of the palate covered by the upper dentures. If dentures are removed for a part of the day, for example at night, the condition is seen much less frequently (Nyquist 1952; Love *et al* 1967). The disease has also been initiated experimentally by giving repeated oral sucrose rinses (Olsen and Bonbevik 1978), such treatment being thought to enhance the multiplication of yeasts and so diet may be a further initiating factor (Samaranayake 1986).

iv.Chronic hyperplastic candidosis (Candida leukoplakia)

The lesions in this condition are white, firmly adherent and often raised. They are usually present on the cheek or tongue giving a picture difficult to differentiate from true leukoplakia. The white patches cannot be removed by scraping, but fragments may be detached by firm scraping with a sharp instrument and identification of hyphae in smears of such material assists in the diagnosis of the disease (Soames and Southam 1985). Hyphae may be seen in the keratinized epithelium in cases of oral leukoplakia, more particularly when the leukoplakia is of the speckled form and it has been suggested that *C. albicans* is a secondary invader in these lesions, rather than the causative

agent (Cawson and Lehner 1968). The heavy infiltration of lesions by macrophages, lymphocytes and neutrophil leucocytes suggests that the host is able to mobilize the immune and phagocytic cellular defences to the sites of candidal invasion although they appear to be unable to eliminate the fungus (Axelsen,Kirkpatrick and Buckley 1974;Shepherd 1986).

v.Angular cheilitis

This condition is most frequently seen as a complication of chronic atrophic candidosis. The disease has a multifactorial aetiology, one factor being infection by *Candida* and/or *Staphylococcus aureus* (MacFarlane and Helnarska 1976).

1.2.4.Deep-Seated Candidosis

Systemic Candidosis

Candidosis exemplifies the whole range of states that may exist between the host and an indigenous parasite, ranging from the colonization of the gastrointestinal tract or oropharynx by *Candida* species to fulminating systemic illness which has an extremely poor prognosis if untreated. Unfortunately, there is no characteristic group of clinical signs and symptoms to indicate the diagnosis of disseminated candidosis. The condition has been divided into three major categories by Bodey and Fainstein (1985): I.Disseminated infection, II.Fungemia, and III.Organ infection.

In the last two decades there has been an increased prevalence of candidosis (both superficial and invasive) and changes

in the categories of patients who become infected. This increase has been attributed to the widespread use of antibiotics and immunosuppressive agents (Seelig 1966; Hurley, de Louvois and Mulhall 1986). Therefore, candidosis can be considered as a disease of medical progress. For instance, in 1937, Bogen and Kessel reported the first adult case of disseminated candidosis, while in 1959, Braude and Rock were able to find only 4 reported cases of the disease. However, six years later, Hurley (1964) listed 48 published cases. Systemic infection with *C. albicans* or related species presents clinically in different ways depending on the site of invasion, the effectiveness of the host's immune response, and the presence of underlying disease (Myerowitz *et al.*, 1977). Recent reports have postulated an apparently new systemic candidal syndrome in heroin addicts (Hay 1986).

1.2.5 Predisposing Factors To Infection.

Although yeasts are commonly present as commensals in man, the incidence of *Candida* infection is relatively low, which suggests that these organisms have low intrinsic pathogenicity, and that the presence of predisposing factors are necessary for establishment of the infection. Ordinarily, *C. albicans* lives in balance with the other microorganisms in the body but various factors can upset this relationship and can lead to the development of active progressive symptomatic disease (Table, 1.3). These factors lead, either directly or indirectly, to modification of the immune responses which are important components in the elaborate defence mechanisms of the body (Seelig and Kozinn

1982). When host immunity is reduced, the integrity of the complicated protective barrier systems can be weakened or breached, and infection may be established or enhanced. Hence, the equilibrium state between the organism and the body is affected so as to allow invasion of host tissues by the organism.

Malignant and endocrine disorders, the use of antibiotics and immuno-suppressive agents and abnormal nutrition (Table, 1.3), may all predispose the host to candidal infection. Multiple factors are mainly responsible for opportunistic fungal infections in patients with malignancies. The host immune system may be impaired by the malignant process *per se* or the attendant chemotherapeutic procedures. The latter may in turn reduce the number of polymorphonuclear (PMN) leukocytes and macrophage phagocytes (Kurrle *et al.*, 1981; Peterson 1984).

The reduction in T-helper lymphocytes observed in acquired immunodeficiency syndrome (AIDS), lead to opportunistic infection, particularly oral and oesophageal candidosis (Klein *et al*, 1984; Tavitian, Raufman and Rosenthal 1986). Indeed, Torssander *et al.*, (1987), found that the oral candidal carriage among human immunodeficiency virus (HIV) sero-positive homosexual men was 77.8 percent indicating that oral candidal carriage is also affected by the defects in the immune system.

Corticosteroids, cytostatics, anti-inflammatory and immuno-suppressive agents are other factors which predispose the host to candidal infection (Bistoni *et al.*, 1984; Waldorf 1986), while

Table 1.3 .FACTORS WHICH PREDISPOSE THE HOST TO CANDIDOSIS:

PHYSIOLOGICAL STATES.	-Infancy -Pregnancy -Old age.
PATHOLOGICAL	
-Severe microbial or viral infection	-HIV infection, AIDS -Bacterial endocarditis
-Endocrine disorders.	-Diabetes -Hypothyroidism
-Defects in cell-mediated immunity	-Progressive septic granulomatosis.
-Myeloperoxidase deficiency	
-Local occlusion or maceration of tissue	-Dentures -Thumb sucking
MECHANICAL.	
-Trauma, maceration	-Burns -Eye injury -Dentures
IATAROGENIC	
A Treatment with drugs	
1 Suppressing endogenous microflora -Aminoglycosides	-Tetracycline, -Metronidazole
2 immunosuppressive and other agents.	-Corticosteroids -Cytostatics -Heroin
B Surgical treatment.	-Prostheses. -Catheters -Heart-valve replacement.
NUTRITIONAL.	
Vitamin deficiencies;	-Carbohydrate rich diets.

Modified after Hurley(1980); Odds (1988).

broad-spectrum antibiotics suppress host-microbial flora interactions and permit *Candida* proliferation and overgrowth (Seelig and Kozinn 1982).

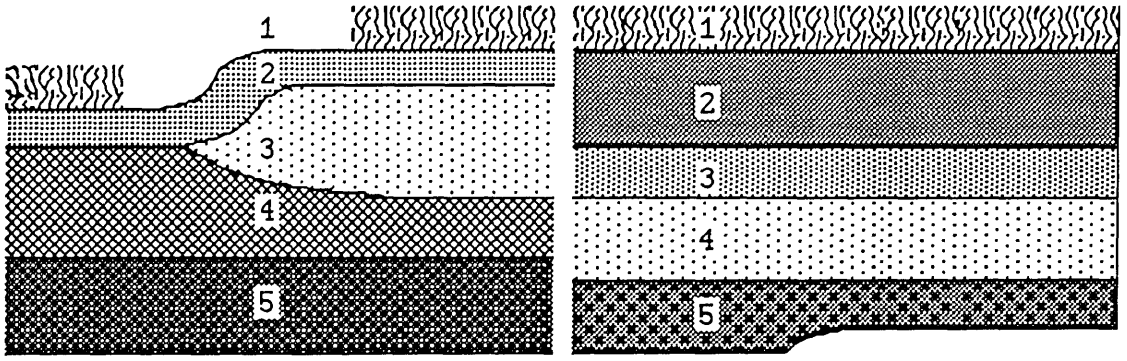
1.3. Microbial Factors In Pathogenicity

1.3.1. CELL WALL

When *Candida albicans* enters the human mouth, the first site of contact with host environment is, the outer layer of the organism i.e., the cell wall. The outcome of this relationship is of importance and eventually leads either to a sustained or transient saprophytic association with the host or to localized infection of mucosal surfaces depending upon its ability to overcome the defence mechanisms of the host. Therefore, the external components of both, the microbe, and the human mucosa are very important in the pathogenesis of oral candidosis.

The cell wall of yeasts was thought to consists of an outer electron-dense layer composed of mannan-protein complexes and enzymes, and an inner electron-transparent layer containing complexes of mannan-protein-glucan. Farkas, in 1979 suggested that the outer layer is composed of manno-protein, and the inner electron-transparent layer, glucan and chitin. However, ultrastructural studies of the cell wall of *C. albicans* have shown that it possesses a multi-layred wall, with at least five or more layers (Djaczenko and Cassone 1971; Cassone *et al.*, 1973; 1979; Chattaway *et al.*, 1976; Poulain *et al.*, 1978; Scherwitz *et al.*,

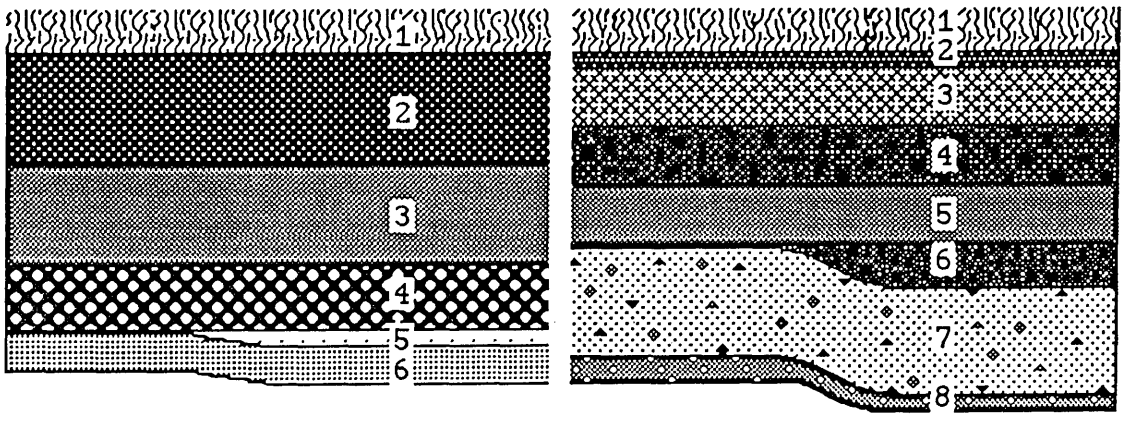
Cell Surface*



Plasmalemma

Scherwitz, et al., (1978) Djaczenko and Cassone (1971)

Cell Surface



Plasmalemma

Persi & Burnham, (1981) Poulain et al., (1978)

Figure 1.1: Ultrastructure of *Candida albicans* cell wall as proposed by different workers.

*Numbers represent different layers.

1978; Persi and Burnham 1981;) as shown in Figure, 1.1. Extracts of the cell walls from both the yeast and mycelial phases of *C. albicans* have been analysed recently, demonstrating that the outer cell wall layers of *Candida* blastospores and germ-tubes contain a complex array of polysaccharides, glycoproteins, and proteins (Ponton and Jones 1986)

Yu *et al.*, (1967) showed that the major structural feature of *C. albicans* cell wall was a chain of 1-6 linked D-gluco-pyranose units (72 %) with a smaller, but a significant number of 1-3 linkages (27 %) present in the linear portion of the polysaccharides. However, significant amounts of material were released with a chitinase preparation containing some protease activity but 1,3-ß-glucanase was required for major changes. These results may indicate that the cell wall of *C. albicans* possess an overall glyco-protein architecture (Phaff 1971). Djaczenko and Cassone (1971) suggested that the first and second layers contain mainly mannan-protein and perhaps chitin, whereas the third and fourth layers contain glucan-protein predominantly.

Some investigators have described the presence of a cell wall coat (extracellular material) consisting of mucoid material surrounding the outermost protein of *C. albicans*. It is thought that this coat may represent an external capsular or slime layer which mediates adhesion of the yeast to epithelial cells and is formed particularly in the presence of excess nutrients (Joshi *et al.*, 1975; Pugh, and Cawson 1978a; Tronchin *et al.*, 1981; Samaranayake and MacFarlane 1981; McCourtie and Douglas 1981;) (For more details please see 3..4.5).

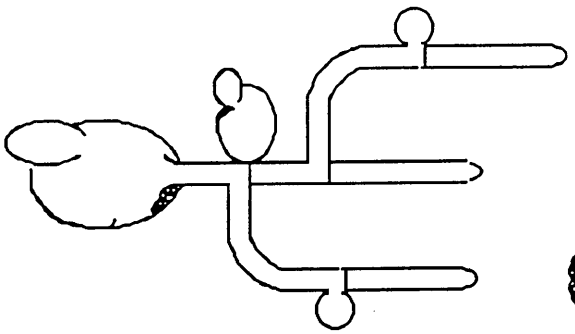
1.3.2 Morphogenesis

Candida Dimorphism

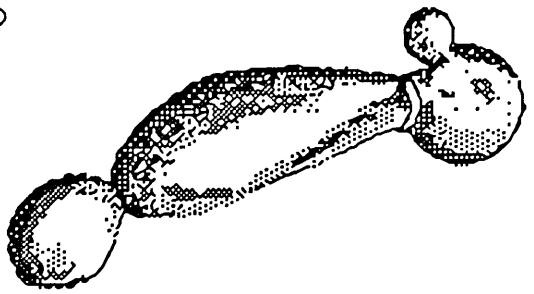
Candida albicans cells have the ability to germinate in the presence of serum and produce active apically growing hyphae within two to three hours. This phenomenon is called germ-tube development and is one of a number of morphogenic features of *C. albicans*. However, it should be noted that *C. albicans* is polymorphic and reproduces in four different forms, blastospores, pseudohyphae, hyphae and chlamydospores (Figure 1.2). In any one culture system it is not unusual to find these forms co-existing at a particular time, under the same conditions, even though one form or another tends to predominate (Odds 1985[a]). Members of the genus *Candida* characteristically develop both as yeast cells and pseudohyphae, but chlamydospores and truly mycelial filamentous cells are virtually unique to *C. albicans*. A number of reports have described the formation of germ-tubes (Martin 1979; Martin *et al.*, 1981; 1984; Tierno and Milstoc 1977; Tani, Yamada and Kamihara 1979; Joshi *et al.*, 1983) and chlamydospores (Duncan and Floeder 1963; Hasenclever 1971) by *C. tropicalis*.

Significance Of Dimorphism

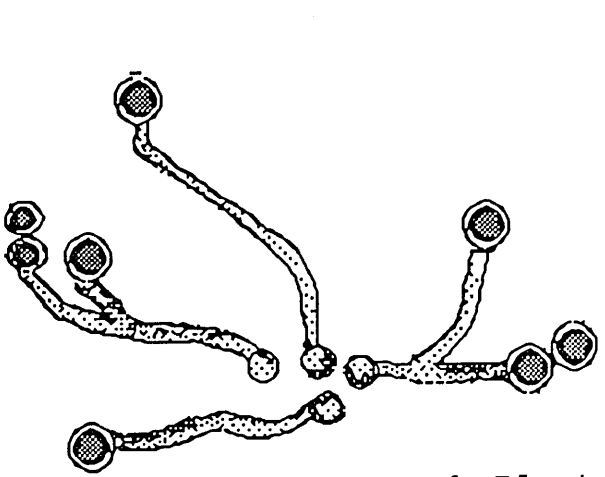
Dimorphism in *Candida albicans* has stimulated extensive research by many workers, mainly because of the long-held view that dimorphism is somehow related to the virulence of the



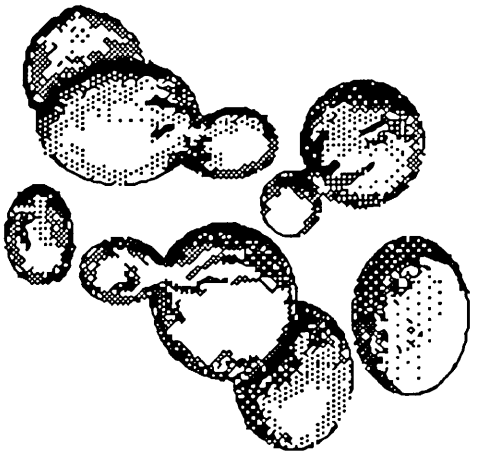
Hyphal Formation



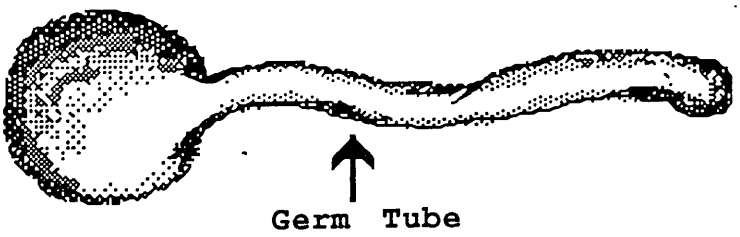
Pseudohypha



Chlamydospores and Blastospores



Blastospores



Germ Tube

Figure 1.2: Polymorphism of *Candida albicans*.

isolates, and it may play a crucial role in the pathogenicity of *C. albicans* (Odds 1985a) Since *C. albicans* is usually seen in the mycelial form in samples from human infection, it has been assumed that the yeast form is saprophytic, whereas the mycelial form is invasive (Montes and Wilborn 1968; Borgers *et al.*, 1983). Hence several studies have concentrated on investigating the infective potential of the different morphogenic forms of *C. albicans.* Conflicting evidence implicates the blastospore (Winsten and Murray 1956; Rebora, Marples and Kligman, 1973) or the mycelial form (Hill and Gebhardt 1956; Gresham and Whittle 1961) or both (MacKenzie 1964; Winblad 1975; Parker *et al.*, 1976), as the main causative factors in infection. For instance, Simonetti and Strippoli (1973) reported that death rates in mice varied irrespective of whether the *Candida* were in mycelial or yeast phase. Mardon *et al.*, (1975), reported that when a strain of *C. albicans* in yeast form was injected into mice, a death rate of 80 percent resulted, whereas the mycelial form did not kill. When another strain was used, 60 percent of the mice were killed by the mycelial phase and 100 percent by the yeast form.

Kimura and Pearsall (1980) found a strong correlation between germination and increased adhesion of *C. albicans* to human buccal epithelial cells. In addition enhancement of adhesion by 2- to 50-fold over that of yeast-phase organisms has been reported by others (Kimura and Pearsall 1978; 1980; Samaranayake and MacFarlane 1981). These workers agreed that germination or other changes in the fungi accompanying germination, were responsible for enhanced adhesion.

Samaranayake and MacFarlane (1982b) demonstrated that the adhesion of hyphal phase cells of *C. albicans* to HeLa cells, was significantly greater than when the cells were in the blastospore phase.

Studies by Winsten and Murray (1956) showed that a strain of *C. albicans* which was unable to convert to the yeast phase was non-pathogenic for mice, and this led to the supposition that mycelial forms of *C. albicans* could not successfully initiate infection and that the yeast was pathogenic by virtue of its ability to transform into mycelia *in vivo*. More recently, Martin, Craig and Lamb (1984) have investigated experimentally the pathogenic potential of a serum-pseudohyphal and a germ tube-negative *C. albicans* variants and found that both variants failed to induce palatal candidosis in rats, in contrast to a germ tube-positive *C. albicans* (serotype A) control strain, which produced histological evidence consistent with palatal candidosis after two weeks in the rat model. Martin *et al.*, (1984) strongly suggested that both the pseudohyphal-positive and germ tube-negative variants were non-pathogenic under the experimental conditions used.

It has been found that most strains of *C. albicans* switch frequently and reversibly between a limited number of phenotypes which can be related to changes in colony morphology (Soll *et al.*, 1987). The yeast cell switch spontaneously and reversibly between a white and a grey, or 'opaque', colony forming phase (Morrow 1989) characterized by differences in (1) colony phenotype (Slutsky *et al.* 1987a); (2) cell shape, volume and mass

(Slutsky *et al*, 1987b); (3) surface topography and wall morphology (Anderson and Soll 1987); (4) cytoplasmic ultrastructure (Slutsky *et al*, 1987a);(5) antigenicity (Anderson and Soll 1987);(6) sugar assimilation pattern constraints on the bud-hypha transition (Anderson *et al*, 1989). The white-opaque transition includes many of the traits of the original switching system characterized in *C. albicans* (Soll and Kraft 1988), including (1) a relatively high frequency and reversibility of switching; and (2) a limited number of alternative phenotypes. They found that high frequency UV irradiation not only stimulated switching at the time, or very soon after exposure, but also effected a heritable state of high frequency switching in *Candida* (Slutsky *et al.*, 1985).

1.3.3 Adhesion Of *Candida* species:

The attachment of *Candida* species to the susceptible host mucosal tissues (or to a closely associated surface such as dentures) is now recognized as an important preliminary step in the colonization and establishment of infection or mucosal surfaces (Douglas 1985, 1987; Hurley *et al.*, 1986; Kennedy 1987). This process may be considered as occurring in three phases: firstly, adhesion and colonization of the tissue surface (Liljemark and Gibbons 1973) secondly, invasion of the superficial epithelial cells to establish an intracellular habitat (Montes and Wilborn 1968), and finally the initiation of an inflammatory response in the underlying tissues (Calderone *et al.*, 1984).

i.Mechanisms involved in *Candida* adherence.

Adhesion is usually mediated by a number of environmental and host factors, and is believed to involve specific interactions. There is evidence that receptor-ligand binding is important, with microbial adhesins (ligands), binding to specific receptors on the surface of epithelial cells. A wide range of factors may affect their specific interactions and so influence the early stages in the colonization and infection by *Candida* species (Freter 1981; Samaranayake and MacFarlane 1982b; Critchley and Douglas 1987b; Mehentee and May 1989). Therefore, attempts to determine the effect of factors such as dietary carbohydrates on adhesion, have been investigated by several workers (Lee and King 1983; Douglas 1987). Samaranayake and MacFarlane (1985) have also hypothesised the role played by dietary carbohydrates in candidal adhesion and invasion of the oral mucosa.

There is evidence that cell wall coat glycoproteins (notably manno-proteins) play an important role in yeast adhesion. McCourtie and Douglas (1981), and Douglas, Houston and McCourtie (1981) demonstrated that adhesion of *C. albicans* to human epithelial cells or denture acrylic surfaces was proportional to the concentration and type of sugar in the growth medium. This enhanced adhesion appeared to be related to the production of an additional cell wall surface layer. Samaranayake and MacFarlane (1981) have also suggested that this phenomenon could imply an extracellular metabolic product of the yeast. The attachment of

yeasts to epithelial cells appear to be mediated by fibrillar material consisting of polysaccharidec like granules distributed on the cell wall coat. Indeed, McCourtie and Douglas (1981) detected this outermost fibrillar, floccular layer in blastospores harvested from medium containing high concentrations of certain sugars (e.g., galactose, and sucrose). It was suggested that this layer may be responsible for the enhanced adhesion of yeast to acrylic surfaces. Later, (1984), the same workers reported that strains of *C. albicans* isolated from active infection were able to modify their surface composition in response to high concentrations of certain carbohydrates (e.g., galactose) in the growth medium. It appeared that these changes enhanced both, the ability to adhere to surfaces and the virulence of one strain isolated from an oral infection, whereas strains isolated originally from asymptomatic carriers lacked this capability or possessed it to a lower degree (Houston and Douglas 1989).

Recently, Critchley and Douglas (1987a) have investigated the effect of various lectins and sugars on the adhesion of five *C. albicans* strains to buccal and vaginal cells *in vitro*. Lectin-like proteins with affinities for L-fucose, *N*-acetyl-D-glucosamine and D-mannose were detected in extracellular polymeric material, from *C. albicans* strains in different amounts. However they suggested that there are at least two types of adhesion mechanisms and that glycosides containing L-fucose, *N*-acetyl-D-glucosamine can function as epithelial cell receptors for *C. albicans*. Furthermore, they have reported that all five *C. albicans* strains contained a protein capable of binding to L-

fucose, *N*-acetyl-D-glucosamine and D-mannose, but the proportion of each type varied from one strain to another. Hence, different strains of *C. albicans* had different attachment mechanisms depending on such variations (Critchley and Douglas 1987b).

The chemical nature of candidal ligands has been studied by testing their analogues for their ability to inhibit adhesion *in vitro*. Various lectins and sugars such as concanavalin A, have been used to study the possible role of saccharide-containing moieties on the surface of both *C. albicans* and human buccal epithelial cells, in yeast adhesion to mucosal surfaces. These studies have revealed, so far, that saccharides, particularly mannose or mannose-containing compounds are effective in the attachment process (Collins-Lech *et al.*, 1984; Ray *et al.*, 1984; Kennedy and Sandin 1988). Thus, Sandin *et al.*, (1982) demonstrated that mannose-containing moieties on the surface of *C. albicans* and buccal cells could mediate *in vitro* adhesion. While, Maisch and Calderone (1981), have also pointed out a role for yeast-surface mannan in the adhesion of *C. albicans* to fibrin-platelet clots.

ii. Factors affecting fungal adhesion to epithelial cells

A- *Candida* cells

Kimura and Pearsall (1978) showed that the *in vitro* adhesion of viable *Candida albicans* to human buccal epithelial cells in clarified human saliva was better than when non-viable fungi were used. Furthermore it has been found that *C. albicans* cells

killed by heat, or formaldehyde (Maisch and Calderone 1980) or ultra violet-light (Rotrosen *et al.*, 1985) failed to adhere *in vitro* as well as viable cells to a fibrin-platelet matrix or human endothelial cells (Sandin *et al.*, 1982).

Samaranayake and MacFarlane (1982b) have reported that *C. albicans*, in the hyphal phase showed significantly greater adhesion to cultured HeLa cells than those in blastospore phase. This feature of candidal yeast to hyphal transition leads to the suggestion that the attachment, invading power and thereafter, the pathogenicity of *C. albicans* may be linked to the formation of germ-tubes and to the development of pseudo and true mycelium. Kimura and Pearsall (1978) indicated that the greater adhesion of germinated forms of *Candida* to epithelial cells may explain why those species which more easily form germ-tubes (e.g. *C. albicans*) colonize mucosal epithelial cells to a greater extent than other species (King *et al.*, 1980; Hurley *et al.*, 1986; Douglas 1987). Similar results by Sandin *et al.*, (1982) have indicated that germinated yeasts adhered to buccal epithelial cells more effectively than non-germinated cells. Martin, Craig and Lamb (1984) have also reported that atypical clones of *C. albicans* (germ-tube negative and variants that formed only pseudohyphae in serum) failed to induce palatal candidosis in rats, which indicate that filamentation is necessary for the induction of oral candidosis in this experimental model.

Although *C. albicans* is undoubtedly the most pathogenic *Candida* species, it has been reported that, within the species, clinical isolates of *C. albicans* demonstrate greater overall

enhancement in adhesion when compared with a reference laboratory strain of *C. albicans* (Samaranayake and MacFarlane 1982a). *Candida albicans* also adheres in significantly higher numbers to both vaginal and buccal epithelial cells, compared to other species of *Candida* (King *et al.*, 1980). However, the genus contains several other established human pathogens of which *C. tropicalis, C. stellatoidea, C. glabrata* and *C. parapsilosis* (in that order) are probably the most important clinically isolated species (Hurley, de Louvois and Mulhall 1986). King *et al.*, (1980) demonstrated a correlation between the ability of the different species to adhere to host surfaces and their ability to cause infection. For example, *C. albicans* adhered in high numbers to exfoliated buccal or vaginal epithelial cells, but the adhesion of *C. tropicalis* or *C. stellatoidea* was moderate and that of *C. parapsilosis* relatively slight; whereas, *C. guilliermondii, C. krusei,* and *C. pseudotropicalis,* showed little or no adhesion (King, Lee and Morris 1980). Ray, Digre and Panye (1984) found that *C. albicans* and *C. stellatoidea* demonstrated marked adhesion to buccal epithelial cells, whereas, *C. tropicalis, C. parapsilosis, C. guilliermondii,* and *C. krusei,* showed little or no adhesion. Similar variations among *Candida* species have been reported for *in vitro* adhesion to epidermal corneocytes (Ray *et al.*, 1984), fibrin-platelet matrices, (Maisch and Calderone 1980; Samaranayake, Mclaughlin and MacFarlane 1988) and vascular endothelium (Klotz *et al.*, 1985).

B-Epithelial cells and inert surfaces

In addition to the fungal related receptors involved in adhesion there have been a number of studies examining the host surface related factors which mediate adhesion. Initially, human mucosal cells were chosen as a substrate because they are a natural host surface for yeast attachment.(Kimura and Pearsall 1978). However, a number of difficulties were associated with these assays e.g., heterogenicity of the epithelial cells which are a mixture of viable and non-viable cells, with various degrees of keratinization. King *et al.*, (1980) reported that the source of the mucosal epithelial cells may have a marked influence on adhesion, and significant variation was observed in adhesion assays when vaginal epithelial cells were obtained from different donors. Further, they also found adhesion to vaginal epithelial cells to be significantly higher than adhesion to buccal epithelial cells, a finding confirming the work of Douglas, Houston and McCourtie (1981). The difference between adhesion of *C. albicans* to buccal epithelial cells and to vaginal epithelial cells was studied by Sobel *et al.*, (1981) and they found that four isolates of *C. albicans* adhered equally well, irrespective of the type of epithelial cell used. On the other hand, Kearns *et al.*, (1983) found that adhesion of *C. albicans* to human buccal epithelial cells varied from day-to-day even with the same *Candida* isolate and cell donor. Therefore, Samaranayake and MacFarlane (1982b) have used two systems, buccal epithelial and HeLa cell monolayers to determine the effects of dietary carbohydrates on candidal adhesion. Although both methods produced

similar results, they found that the HeLa-cell system gave significantly lower percentage coefficient of variation between replicate experiments than the BEC method. They suggested that as BEC were coated *in vivo* with bacteria, as well as saliva and serum components this may lead to variations in candidal adhesion in subsequent assays. The age of the individual may also affect yeast adhesion to buccal epithelial cells. For instance, Tobgi *et al.*, (1987), found that adhesion of *C. albicans* to BEC's isolated from healthy twin female children is higher than to an adult healthy individual. However, Cox (1983) reported that yeast adhesion was the same in normal adults and in children and he suggested that cell receptor system, is not age dependent.

Oral candidosis is very prevalent among patients with dentures. Usually, *C. albicans* is recovered from the fitting surface of the denture, suggesting that denture acrylic acts as a reservoir of infection (Budtz-Jörgensen and Löe 1972). Therefore, adhesion of *C. albicans* to denture acrylic has been studied *in vitro*. Samaranayake and MacFarlane (1980), have described an *in vitro* assay to measure quantitatively the adhesion of *C. albicans* to acrylic surfaces. Samaranayake, McCourtie and MacFarlane (1980) have investigated various factors affecting *C. albicans* adhesion to the acrylic surfaces, and they suggested that an extracellular metabolic product of the `sucrose grown' *Candida*, could be responsible for the enhanced adhesion associated with sucrose. When a comparison was made between the adhesion of *Candida* species to epithelial cells and acrylic surfaces, the adhesion to either surface was found to be similar,

(McCourtie and Douglas 1984). Critchley and Douglas (1985) investigated the adhesion of six *Candida* species (*C. albicans, C. tropicalis, C. stellatoidea, C. guilliermondii, C. pseudotropicalis,* and *C. parapsilosis*) to acrylic strips, and BEC's and reported that a strain of *C. tropicalis* grown in 500 mM galactose adhered in substantially increased numbers to buccal cells but not to acrylic, when compared with controls. On the other hand, other species (including a second strain of *C. tropicalis*) showed little or no increased adhesion to either surface. Furthermore, when *C. tropicalis* and *C. parapsilosis* species were grown in 50 mM glucose they were significantly more adherent to acrylic than other species. These studies imply that the substrate with which the *Candida* interacts may play a significant role in the adhesion process.

The relationship between cell surface hydrophobicity and adhesion capacities to acrylic surfaces was studied with *Candida* species (Minagi *et al.*, 1985; Miyake *et al.*, 1986; Klotz 1989) and a positive correlation was observed between the adhesion to acrylic surfaces and the degree of hydrophobicity of *Candida* species.

C-Saliva

The influence of saliva as a nutritional and environmental factor, on candidal adhesion, has been studied. The *in vitro* adhesion of yeast cells to epithelial cells was greater when tests were done in clarified saliva, (i.e., the supernatant collected after centrifugation at 10 g.) within two to three hours after collection, than when carried out in phosphate buffered

saline (Kimura and Pearsall 1978). On the other hand, Samaranayake, McCourtie and MacFarlane (1980) found that pretreatment of acrylic strips with clarified whole saliva for 18 hours significantly reduced the number of attached *Candida* compared to control strips exposed to phosphate-buffered saline. No significant difference in adhesion was found when acrylic strips pre-treated with parotid saliva were compared with untreated strips. The contrast between these two studies, may be due to the use of different substrates (epithelial cells in the former and acrylic strips in the latter), and the fact that in the first study the saliva was free of anti-candida antibodies. The literature is further confused by the report of Minagi *et al.*, (1986) who found that saliva-coating of the resin (heat-curing polymethyl methacrylate) plate caused increased adhesion of *C. albicans*, and decreased adhesion of *C. tropicalis*.

D-Carbohydrates

The effect of dietary carbohydrates on the adhesion of *Candida* species has been studied extensively. Samaranayake and MacFarlane (1981), have indicated that sucrose, even at low concentrations, facilitates the *in vitro* adhesion of yeasts to HeLa cells in a dose dependent manner. Furthermore, the same investigators, (1982a) also demonstrated that preincubation of *Candida* in either 0.5 M glucose, sucrose, galactose, xylitol, or maltose medium produced a significant enhancement in adhesion to buccal epithelial cells. McCourtie and Douglas (1981), reported that *C. albicans* isolates grown to stationary phase in media contain-

ing high concentrations of certain sugars, notably galactose, maltose and sucrose, elicit up to ten times more adhesion than control yeasts grown in medium with a relatively low concentration of glucose. They observed a change in cell surface composition and an additional fibrillar-floccular layer on the yeast cell surface, which also confers increased resistance to spheroplast formation with the lytic enzyme Zymolase. Centeno *et al.*, (1983) found that pre-incubation in dextrose had no effect on yeast attachment to epithelial cells whereas mannose significantly decreased attachment. Therefore, they suggested that inhibition of yeast attachment by mannose was probably based on a competitive inhibition between the mannose and yeast surface mannose for receptor sites on the epithelial cell surface.

Nevertheless, a number of adhesion studies with acrylic surfaces support the overall view of enhancement of candidal adhesion by sugars. Samaranayake, McCourtie and MacFarlane (1980) have shown that *C. albicans* cultured in glucose- and sucrose-supplemented media adhere to acrylic surfaces in significantly greater numbers (4-fold) than organisms grown in peptone alone. These findings have been confirmed by McCourtie and Douglas (1981), who observed that adhesion after growth in galactose was more than 10-fold that of cells grown in medium with glucose, whereas fructose grown organisms were least adherent to acrylic. Thus, dietary carbohydrates appear to have a significant affect on the pathogenesis of oral candidosis by promoting yeast adhesion to epithelial and denture surfaces.

E-Antifungal and Chemical agents

The alteration of the surface components of microbial cells by chemotherapeutic agents, such as antibiotics, and antifungal agents and the effect of such changes on microbial adhesion have received much attention. The adhesion of *C. albicans* to buccal epithelial cells, exposed to amphotericin B, was studied by Brenciaglia *et al.*, (1986) who reported a reduction in the adhesion of *C. albicans*. It has been shown previously that pre-treatment of either denture acrylic or *Candida* cells with chlorhexidine, results in a reduction in their adhesion to acrylic (Samaranayake and MacFarlane 1980, McCourtie, MacFarlane and Samaranayake 1985; 1986). The chlorhexidine also decreases the adhesion of *Candida* cells to buccal epithelial cells *in vitro* (Tobgi, Samaranayake and MacFarlane 1987). Concanavalin A (1-100 mg/ml) was found to enhance candidal adhesion whereas polyspartic acid (10 mM), polyglutamic acid (10 mM) and poly-arginine (10 mM) did not effect the adhesion (Rotrosen *et al* 1985). Formaldehyde (0.5 %), trypsin (100-250 mg), and chymotrypsin (100-250 mg) were all found to reduce adhesion (Maisch and Calderone 1980).

Elimination of bacteria, which may compete with *Candida* for the available colonization sites, due to broad spectrum antibiotics, may lead to *Candida* over growth and invasion. Many workers have studied this phenomenon and a number of reviews have been published, (Winner and Hurley 1964; Seelig 1966; Seelig and Kozinn 1982).

F-pH

Mehentee and Hay (1989) reported that the adhesion of strains of *C. albicans* grown in Dulbecco's Modified Eagle's Medium, to gastro-intestinal mucosal cells *in vitro*, was affected by pH. Binding between the *C. albicans* strains and stomach mucosal cells fluctuated as the pH was raised from pH 1.2 to pH 3.4. Optimal adhesion by both strains to jejunal mucosal surfaces occurred at neutral pH.

G-Commensal oral bacteria

The indigenous flora coats the oral mucosal surfaces, teeth, and denture acrylic, and other microorganisms may initially adhere to the indigenous bacteria themselves, rather than to the host surface directly (Freter 1981). It has been suggested by Helstrom and Balish (1979) that the indigenous bacterial flora may suppress the colonization of a mucosal surface by *Candida* by competing for the same receptor sites on mucosal epithelial cells. Furthermore Kennedy and Volz (1985) found that the indigenous microflora reduced candidal adhesion in the gastro-intestinal mucosal gel, both by competitive adhesion, and by producing inhibitory substances. On the other hand, the mucosa of germ-free animals are colonized by *C. albicans*, in higher numbers than those of control normal animals (Balish and Phillips 1966; Liljemark and Gibbons, 1973). Liljemark and Gibbons (1973) and Samaranayake and MacFarlane (1982b) have observed that *Streptococcus salivarius* and *Streptococcus mitior* significantly reduced the adhesion of *C. albicans* to epithelial cells.

However, Makrides and MacFarlane (1982), while supporting the above results, reported that cell suspensions and cell free supernatants of *Staphylococcus aureus*, *Streptococcus mitior* and a strain of *Streptococcus sanguis* significantly reduced candidal adhesion, whereas those of *Escherichia coli* and *Klebsiella aerogenes* significantly enhanced candidal adhesion to HeLa cells. It is generally accepted that indigenous oral flora can interfere with the adhesion and colonization of oral surfaces by *C. albicans*.

1.3.4 Enzymes and toxins produced by *Candida albicans*:

There are a number of reports on the various enzymes and toxins produced by *Candida* species. More than 40 enzymes for *C. albicans*, have been reported (Hasenclever and Mitchell 1962; Louria *et al.* 1963; Chattaway *et al.*, 1971; Iwata 1972; Odds 1988), and it has been suggested some of these contribute to the initiation or development of candidosis, although, no direct evidence has been presented as yet. It is known that if a large number of *Candida* blastospores are injected intravenously, a high percentage of the mice often die in the first 24 hours, although there is no significant increase in the numbers of yeasts within the tissues during this period (Louria *et al.*, 1963). This observation suggests that these early deaths might be due ,in large part, to the effects of preformed candidal toxins. Furthermore, experiments with formalin-killed *Candida* blastospores and supernatants of sonically disrupted *Candida* cells were lethal

for mice. As a result it has been suggested that *Candida* species possess endotoxin-like substances (Salvin 1952).

Iwata *et al.* (1972) and his co-workers have claimed the isolation of a so called `canditoxin´ produced by a *C. albicans* strains from the spinal fluid of a patient suffering from candidal meningitis. Studies by Cutler, Friedman and Milner (1972) reported that these toxic substances were clearly different from microbial endotoxins. Although, Chattaway, Odds and Barlow (1971) were unable to demonstrate canditoxin in four strains of *C. albicans*, this led to the suggestion, that the organisms may produce enzymes, instead of canditoxin, that enhance the penetration of the mucous membranes by the yeast cells. Indeed, these workers (Chattaway, Odds and Barlow 1971) have found that *C. albicans* produce acid phosphatase, peptidases, and ß-glucosidase. Braun and Calderone (1978) isolated a chitin synthetase from sonically treated protoplasts of both pseudohyphae and blastospores and obtained evidence that chitin synthetase is located on the inner portion of the plasma membrane. This enzyme is believed to exist as a zymogen because its activity can be increased by incubation with a protease obtained from the vacuole of *S. cerevisiae* (Braun and Calderone 1979). Orlean (1982) found (1,3)-ß-D-glucan synthetase as a particulate fraction from cell free extracts prepared after mechanical breakage of *C. albicans* cells. The role of these intracellular enzymes in the pathogenesis of candidosis remains to be determined.

i. PHOSPHOLIPASE

Phospholipase has an obvious role in cellular metabolism as a catabolic enzyme that hydrolyse phospholipids, which are important structural components of cell membranes. There are two forms of phospholipase A in nature, water-soluble and membrane-bound. The activity of phospholipase was first demonstrated in *C. albicans* by growing the fungus on media containing egg yolk or lecithin (Costa *et al.*, 1967). Later, phospholipase activity was found in many pathogenic strains of *C. albicans* using media containing blood, serum and sheep red cells (Costa *et al* , 1968). The latter group using chemical and chromatographic techniques, identified two enzymes, phospholipase A and C; but not phospholipase B or D in *C. albicans*. Pugh and Cawson, (1978b) demonstrated the presence of the enzyme, lysophospholipase in *C. albicans*, that acts on lysolecithin, and further by using cytochemical techniques they also showed that phospholipase activity in *C. albicans* was concentrated at the growing tip of hyphal cells. The presence of enzymes capable of hydrolysing phospholipids at the site of bud formation (Pugh and Cawson, 1975; 1977) suggests that the increase in size and change in shape of the cell wall during budding is a complex process involving the controlled growth and remodeling of the cell membrane. However, in older cultures, phospholipase appears at the periphery of the cell and is secreted into the medium (Pugh and Cawson 1975). In

addition, Price and Cawson (1977) detected the phospholipase activity in the cell wall extracts of *C. albicans* .

Odds, Hall and Abbott in 1978 described a biochemical assay to measure intracellular phospholipase A and lysophospholipase.and later Price, Wilkinson and Gentry (1982) demonstrated a simple plate method for the detection of phospholipase activity. The latter workers, quantitatively estimated the phospholipase activity of 23 isolates of *C. albicans* from urine, blood and wound infections and showed large variations among strains. The phospholipase activity of 41 isolates of oral *Candida* species was assessed by Samaranayake, Raeside and MacFarlane (1984) using a plate assay method. The results showed that 79 percent of the *C. albicans* isolates were phospholipase producers whereas none of the *C. tropicalis, C. glabrata* or *C. parapsilosis* isolates produced the enzyme. In addition, the degree of phospholipase activity of individual isolates was remarkably constant despite the large variation in activity among different isolates.

Recently, Banno, Yamada and Nozawa (1985), used radioisotopes, ^{3}H lecithin and ^{3}H lysolecithin, as substrates to measure phospholipase activities at various stages of growth of yeast cells, and demonstrated that several phospholipases were secreted into the culture medium and that the culture filtrates were able to hydrolyse lecithin and lysolecithin. Analysis of products of hydrolysis showed that the enzyme activities were lysophospholipase, lysophospholipase-transacylase and a phospholipase B. Moreover, they indicated that these enzymes accumulated in the culture filtrate as the age of the yeast culture increased..

Although, phospholipases have been demonstrated in *C. albicans*, as yet the precise role played by the enzymes in the pathogenesis of candidosis is unknown, although there is some evidence to suggest that they may have a role in pathogenicity (Costa *et al.*, 1968; Louria, Brayton and Finkel 1963).

ii. PROTEOLYTIC POTENTIAL OF *Candida albicans*

Candida albicans is known to produce an extracellular acid protease (pH optimum of 3.5 to 4) and an intracellular neutral protease (pH optimum 6.6), (Remold *et al.*, 1968; Chattaway *et al.* 1971). Subsequently, extracellular proteolytic activity was also detected among the majority of isolates of *C. tropicalis* and *C. parapsilosis* (Rüchel and Böning 1983; MacDonald 1984). However, the extracellular proteinase, which was purified extensively by Rüchel (1981), has been shown to consist of three separate enzymes, depending on the strain used as the source. Indeed, MacDonald and Odds (1980a, b) have reported that antibodies to purified enzymes are found commonly in serum samples from patents with systemic candidosis but not in samples from normal individuals. The same workers (MacDonald and Odds 1983) also described a mutant, produced by nitrosoguanidine mutagenesis, of a proteinase-producing, *C. albicans* strain, phenotypically identical to its parent in nearly all biochemical and morphological characteristics except proteinase production. The mutant was considerably less lethal than the parent when inoculated intravenously into mice and lower counts of *C. albicans* were recovered from the organs of the infected animals. When this mutant and its parent grew in a medium that did not induce

proteinase production both were phagocytosed and killed to a similar extent by human and murine polymorphonuclear leukocytes.

Kwon-Chung *et al.*, (1985) reported the relationship between extracellular proteinase-production and the virulence of *C. albicans* for mice. Three strains, of *C. albicans* were investigated, including a proteinase-producing parent from which, a proteinase-deficient mutant was derived by nitrous acid treatment. The parent isolate produced a high level of proteinase *in vitro* and caused a fatal infection in all the mice within 21 days. The mutant produced no detectable enzymes *in vitro* and all mice survived until day 22, while 30 percent of the mice infected with the mutant died between day 23 and 30. The isolates recovered from the dead mice were found to contain adequate amounts of proteinase in order to kill the animals. This shows that the animals died after the mutant had reverted to an enzyme producing state.

The genus *Candida* comprise three species which are proteolytic *in vitro* (Budtz-Jörgensen 1971) namely *C. albicans* and *C. tropicalis,* which are of foremost medical importance, and *C. parapsilosis* which is of less importance. However, Rüchel, Böning and Borg (1986) have reported that the latter organism was unable to produce the enzyme during infection. Schreiber *et al.*, (1985) employed a quantitative approach to evaluate the amount of proteinase produced by 95, clinical isolates of *Candida* species (75 isolates of *C. albicans,* 6 isolates of *C. tropicalis,* 6 isolates of *C. parapsilosis* and 8 isolates of *C. glabrata*). They were categorized into four groups, according to the infectious

process present in the patient (group 1: isolates from patients with invasive disease, group 2: isolates from patients with possible invasive disease, group 3: isolates from superficially infected patients and group 4: isolates from colonized individuals with no apparent infection). The majority (97 %) of *C. albicans* isolates produced a detectable proteinase, when incubated for 7 days. Also some of the non-*C. albicans* isolates produced the enzyme, (67 %, 17 % and 13 % of *C. tropicalis, C. parapsilosis* and *C. glabrata* respectively), although, the amount of activity was generally less than that for *C. albicans*. No significant differences in proteolysis was found when the isolates from different clinical groups were compared. Thus, it would appear that *C. albicans* and *C. tropicalis* are the most proteolytic species while others demonstrate little or no enzyme activity *in vivo* .

It has been suggested that a number of factors may regulate proteinases activity. There include the low pH and Eh which exist in the space between the palatal mucosa and the fitting surface of the denture, due to poor salivary flow with associated weak buffering activity and a probable reduction in oxygen levels. Such a milieu would favour an enzymatic 'destruction' of the epithelium, due to proliferation of the yeast and penetration of the mucosa if such an environmental situation persists (Staib, 1965; Budtz-Jörgensen, 1971; Chattaway *et al.*, 1971). Samaranayake (1984)* have investigated the production of proteases by *C. albicans* in batch cultures of human saliva supplemented with glucose. Salivary proteolysis was detected by biochemical

* Samaranayake, Hughes and MacFarlane (1984)

and isoelectric focusing techniques. Results showed a highly significant positive correlation between acid production and salivary proteolysis. However in glucose-free control samples, neither candidal growth nor proteolytic activity was detected. Rüchel *et al.*, (1986) reported similar results and found that the proteolytic activity of *C. parapsilosis* (a clinical isolate) was dependent on the supply of glucose in the medium.

Several investigators have suggested a relationship between pathogenicity and the proteinase activity of *Candida* species (Remold *et al.* 1968; MacDonald and Odds 1980). However, the role of such enzymes in the pathogenesis of candidal disease has been the subject of some controversy. Reports of non-proteolytic highly virulent strains (Saltarelli *et al.*, 1975) conflict with reports of infection in mice caused solely by proteolytic strains (Remold *et al.*, 1968; Staib 1969). Therefore, Germaine *et al.*, (1978, 1981); and Budtz-Jörgensen, (1971) argue against the importance of this enzyme, at least in strains of *C. albicans* causing oral disease. On the other hand, Kwon-Chung *et al.*, (1985); MacDonald and Odds (1983), have concluded that the extracellular proteinase produced by *C. albicans* is one of the virulence factors associated with this organism and that the degree of virulence is correlated with level of proteinase produced. Such a correlation may also exist among isolates of *C. tropicalis*, but not in the case of *C. parapsilosis*. Isolates of the latter species are mostly proteolytic *in vitro* (Rüchel *et al.*, 1982; MacDonald 1984) but they possess only low virulence *in vivo* (Borg *et al.*, 1984 and Vecchiarelli *et al.*, 1985).

Recently, Borg and Rüchel (1988) demonstrated proteinase antigens by immuno-scanning electron microscopy on the surface of adhering blastoconidia and invading filamentous cells of *C. albicans* serotype A in an infection of the human buccal mucosa, and proteinase positive isolate of *C. tropicalis*. Proteinase antigens were also present on blastoconidia of *C. albicans* serotype B, but these antigens were not found on filamentous cells of this serotype.

Finally, it seems that the *Candida* species have a number of pathogenic factors which are interrelated and play a crucial role in the pathogenic process. Cell wall alterations during germ-tube formation may assist the organism in adhesion and penetration of the mucosal surfaces. Furthermore, the thinning of the external cell wall of the germ tube tip may also assist in releasing fungal enzymes into the environment. Extracellular proteinase and phospholipases produced by *C. albicans* may eventually help the organism to establish the infection and overcome the host defences. However, little is known about the interactions occurring between the host and the *Candida* and much work remains to be done to clarify this area.

1.4 Host Factors In Pathogenicity

1.4.1 Host Susceptibility And Defence Mechanisms

Candidosis either oral or systemic, is the eventual outcome of an altered balance between the pathogenicity of the organism and the activity of the host defences. Generally, a very minor and subtle defect in host defences may allow these organisms to invade, and penetrate the epithelial tissues thereby cause illness (Hurley *et al.*, 1986).

The mucosal surfaces are the initial site of infection for most pathogenic *Candida* species. Adhesion to and invasion of these tissues by *Candida* species are important steps in the process of colonization and infection (Howlett and Squier 1980). However, the eventual outcome of the infection process, depends on the strength of the non-specific and specific host defence mechanisms as described below.

i NONSPECIFIC HOST DEFENCE

The nonspecific host defence mechanisms, include factors that may affect microbial adhesion and colonization of superficial mucous membranes, together with other mechanisms such as phagocytosis, and the inflammatory response. A number of nonspecific host defences factors operate within the oral cavity. In the human mouth the epithelium acts as a physical barrier, and epithelial turnover contributes to the host defences.by the continuous shedding of epithelial cells with their attached microorganisms. Saliva functions by its diluting and washing effects as

well as containing antimicrobial factors, e.g., lysozyme, lactoferrin, and lactoperoxidase (MacFarlane and Mason 1975 and Jenkins 1978). The indigenous bacterial flora can restrict candidal colonization by their physical presence and by the production of antagonistic substances as described previously (see section, 1.3.3).

Body fluids such as saliva, serum and bronchoalveolar fluids may play a role in modulating candidal pathogenicity. Saliva has a dominant role in microbial ecology, and the antibacterial factors in saliva, may be important in the host defence mechanisms in the mouth (MacFarlane 1977).

Some studies have shown higher levels of anti-candida IgA in saliva from oral *C. albicans* carriers and patients with candidosis than in uninfected controls (Lehner 1967; Epstein *et.al.*, 1984). It has also been shown that the average salivary levels of anti-*Candida* IgA, in children between the ages of 3 and 6, years are lower than in older children (Palacios 1977). This may be one reason why infants are more susceptible to colonization with the yeast than older children (Russell and Lay 1973; Cox 1983).

Few studies have concentrated on the antifungal activity of bronchoalveolar fluids (La Force, Sharrar and Arai 1979; Nugent and Fick 1987). Despite the use of antibiotics, cytotoxic therapy and immuno-suppressive drugs, pneumonia due to *Candida albicans* is very rare. This may suggest that this body fluid has antifungal activity against *Candida*. Indeed, it has

been reported that growth and germ-tube formation of *C. albicans* was inhibited by rabbit bronchoalveolar fluid (Peterson and Calderone 1977).

Recent studies have shown that phagocytosis of unicellular eukaryotes proceeds by complement receptor type 3 (CR3), which is found on the surface of neutrophils and monocytes (Ross *et.al.*, 1985). The importance of C3 in phagocytosis of *C. albicans* was demonstrated by Morrison and Cutler (1981) who found that serum depleted of C3 would not opsonize *C. albicans* for phagocytosis.

Lysozyme (muramidase) is a low molecular-weight protein present in relatively high concentration in the oral cavity. In the oral environment, lysozyme originates from saliva, gingival crevice fluid, and polymorphonuclear luekocyes. The anti-candidal activity of the lysozyme has been investigated by a few investigators (Collins and Pappagianis 1974; Marquis *et al.*, 1982). Marquis *et al.*, (1982), found that *C. albicans* was sensitive to microgram amounts of hen egg-white lysozyme and they reported cell wall damage in *C. albicans* when the yeast was exposed to the enzyme. They suggested that enzymatic hydrolysis of the *N*-glycosidic bonds that link the polysaccharides and structural proteins of the cell wall, as a mechanism for this phenomenon. Lysozyme together with other salivary factors referred to above may play an important role in preventing candidal colonization of the oral cavity.

ii Specific Host Factors

In addition to the non-specific host factors discussed above, there are a number of specific host factors which may control the colonization of *Candida* in the oral cavity, for example, specific serum and salivary antibodies against *Candida* (IgG, IgA, IgM). Bergendal and Holmberg (1982) reported a correlation between such IgG levels in serum and the presence of *Candida* in the oral cavity, yeast numbers on the fitting surface of the denture and signs of oral candidosis. Others have detected increased titres of IgA, the predominant immunoglobulin produced at mucosal surfaces in patients with oral candidosis (Lehner 1966; Epstein *et.al.*, 1982).

Taschdjian *et al.*, 1973 found that up to 80 percent of the population without evidence of infection, possess cellular hypersensitivity to *C. albicans*. Other investigators (Budtz-Jörgensen 1974; Jenkins *et al.*, 1977) have also suggested that immediate or delayed hypersensitivity may have a role in the pathogenesis of oral candidosis. Evidence for the importance of cell-mediated immunity (CMI) in host defence against *Candida* infections is provided by animal studies and by the recognition of specific defects of CMI in patients with chronic mucocutaneous candidosis or receiving immunosuppressive drugs. A number of workers (Jenkins *et al.*,1977; Arendorf and Walker 1979; Holmberg 1980) have found evidence of cell mediated hypersensitivity to *Candida* in the pathogenesis of candidosis and suggest that enzymes and toxins may aggravate immune-related lesions. Several

reports also indicate that specific immune imbalance in response to *C. albicans* plays a role in the pathogenesis of oral candidosis (Sahay, Chatterjee and Stanbridge 1979; Mackie, Parratt and Jenkins 1978).

Animal studies generally indicate protection following immunization with *C. albicans* used as either live (Kagaya, Shinoda and Fukazawa 1981), or heat-killed organisms (Hurtrel, Lagrange and Michel 1981). Most authors agree that protection against infection correlated better with development of CMI than with humoral immunity (Kagaya, Shinoda Fukazawa, 1981; Hector, Lyon and Domer 1981). Miyake *et al.*, (1977), found that immunity was associated with passive transfer of immune lymphocytes, but not with immune serum. However, not all investigators are in agreement in this area; Pearsall, Adams and Bunni (1978) failed to transfer protection using lymphocytes from immunized mice, and Hurtrel, Lagrange and Michel (1981) felt that the protective effect of immunization was due to an induced granulocytosis rather than increased CMI.

1.5.Nutrition

Undoubtedly, information on morphological regulations are very important in understanding the pathogenicity of the *Candida*. Nickerson and Mankowski (1953) indicated that nutritional and environmental factors influence the yeast to mycelial transition of *Candida in vitro*. Thus, the dimorphism of *Candida* and the factors which control it, are very relevant to an understanding of its overall physiology and pathogenic mechanisms. Indeed, Samaranayake and MacFarlane (1985), have proposed a scheme that hypothesized how nutritional factors such as carbohydrates can play a role in oral candidosis, and several others have categorized carbohydrate-rich diets as one of the eminently receptive states conducive for oral candidal infections (Gentles and La Touche 1969; Douglas 1987; Kennedy 1987).

A number of nutritional factors and cultural conditions have been investigated in relation to oral candidosis. These factors include iron, folic acid, vitamins A, B, C and K, and zinc, all of Which have been reported to affect the morphology of *C. albicans* (Evans, Odds and Holland 1975; Manning and Mitchell 1980). The addition of glucose to the growth medium of a yeast culture can lead to a variety of effects on metabolic enzymes, commonly called glucose effects (Manning *et al.*, 1980). Thus on the basis of the degree of the repressive effect on cellular respiratory activity, fungi are generally divided into two groups, glucose-sensitive and glucose-insensitive types. With the exception of *C. glabrata* the medically important species

of the genus *Candida*, have been reported to belong to the glucose-insensitive category because respiration of these organisms shows only weak, if any, sensitivity to glucose (Fietcher, Fuhrmann and Kappeli 1981). However, Nimi, Kamiyama and Tokunaga (1988) indicated that the oxygen consumption of glucose grown cells of *C. glabrata*, was reduced without any change in the cytochrome pattern, compared to acetate-grown cells, while no such decrease was detected in any of the other strains of *Candida* species tested (*C. albicans*, *C. tropicalis* and *C. parapsilosis*).

In addition to the metabolic effects of dietary sugars they may also promote the carriage and persistence of *Candida* in the oral cavity (Samaranayake *et al.*, 1986). Thus, Kearns *et al.* (1983) found that the adhesion of this fungus to human epithelial cells, varied with the glucose composition of the culture medium used. Recent data have revealed that candidal adhesion to either epithelial cells (Samaranayake and MacFarlane 1981; Samaranayake and MacFarlane 1982b), or denture acrylic surfaces (Samaranayake and MacFarlane 1980; Samaranayake, McCourtie and MacFarlane, 1980), is markedly enhanced in the presence of dietary carbohydrates including glucose and sucrose. This enhanced adhesion is thought to be due to sticky, extracellular fibrillar layer (McCourtie and Douglas 1984) analogous to bacterial fimbriae whose importance in adhesion is widely recognized, (for more details please see Chapter Three, section 3.4.1).

1.6 Aims Of This Study

Knowledge of the pathogenic factors implicated in candidosis is at a comparatively early stage; nevertheless, a rapidly expanding literature attests to the potential importance of these factors in candidal pathogenicity. It is also clear that these factors, in addition to the characteristics of the host defences and other environmental factors, are coordinated in the process of infection.

Clinical studies of human populations and animal experiments, in recent years, have yielded much of the information related to candidosis and the most comment aetiologic agent, *C. albicans*. However, relatively, few studies on candidal pathogenicity have used different species of *Candida*, as well as a large number of different strains of the same species. The available data, is unfortunately often difficult to correlate due to lack of standardization in laboratory methods. In addition most studies investigate only one factor e.g., adhesion or phospholipase activity with the result there is very little information about the range of pathogenic factors produced by a single yeast isolate.

Therefore, the aims of this study are to investigate the pathogenic attributes of a large number of *Candida* species firstly by studying their putative virulence factors and secondly, by examining the susceptibility of the yeasts to either components of body fluids or body fluids *per se*.

The yeasts, 49 in total were isolated from symptomatic or asymptomatic carriers and comprised; *C. albicans* (22), *C. tropicalis* (7), *C. parapsilosis* (5), *C. krusei* (7), *C. guilliermondii* (5), and *C. glabrata* (3).

The organisms were obtained from a variety of sources although they were predominantly oral in origin, with a few vaginal isolates and strains from type culture collections. The sources of the 49 *Candida* isolates and their maintenance is discussed in Chapter Two.

The ability of *Candida* species to adhere to host surfaces is considered to be an important virulence attribute of the organism. Therefore, candidal adhesion to both buccal epithelial cells and denture acrylic surfaces were studied and the relative adhesion of the various *Candida* species to these surfaces were compared, as described in Chapter Three.

The ability of the 49 *Candida* isolates to produce both phospholipase and proteinase was compared in Chapter Four. These are other virulence attributes of *Candida* species which have previously had some attention although comprehensive data with large numbers of isolates are not available.

The susceptibility of the yeasts to lysozyme, a basic component of a number of body fluids including saliva, was studied in Chapter Five. Finally in Chapter Six the ability of the yeasts to withstand exposure to BLF, was studied. The concluding Chapter Seven is an attempt at collating the data from the complete study in order to hypothesise as to the importance of yeast virulence factors and host body fluids in the pathogenesis of human candidosis.

CHAPTER TWO

MAINTENANCE OF *Candida* species

2.1 Origin of *Candida* isolates.

2.1.1. *Candida albicans* isolates.

Twenty-two oral and vaginal isolates of *Candida albicans* were collected from the Routine Microbiology Diagnostic Services of both Glasgow Dental Hospital and School (10, isolates), and Glasgow Royal Infirmary (5 isolates) (Tables 2.1 and 2.2). In addition one isolate was obtained from the National Collection of Yeast Pathogenic Fungi (NCPF), Public Health Laboratory Service (PHLS).Mycological Reference Laboratory, Colindale, UK. And six isolates from the National Collection of Yeast Cultures (NCYN), Norwich.

Of the 13 oral isolates 10 were collected from patients with active oral candidosis and three from, apparently asymptomatic individuals. Of the vaginal isolates four were from patients with vaginal candidosis, whereas the other four were from apparently asymptomatic women. Only one skin isolate was studied, isolated from a cutaneous candidosis lesion.

Tables 2.1 to 2.7 show a summary of the code numbers and source of the cultures used in this study.

2.1.2: *Candida* species:

In addition to the 22 *C. albicans* isolates discussed above, 27 isolates of species other than *C. albicans* were used in this study (Tables 2.3-2.7). Twenty two of these were from oral samples investigated by the Routine Microbiology Diagnostic Service, Glasgow Dental Hospital and School, and four were from The National collection of Pathogenic Fungi (NCPF), Public Health Laboratory Service (PHLS), Mycological Reference Laboratory, Colindale, UK. The number and the identity of the isolates were as follows; *C. tropicalis*, 7, *C. krusei*, 7, *C parapsilosis*, 5, *C. guilliermondii*, 5, and *C. glabrata*, 3.

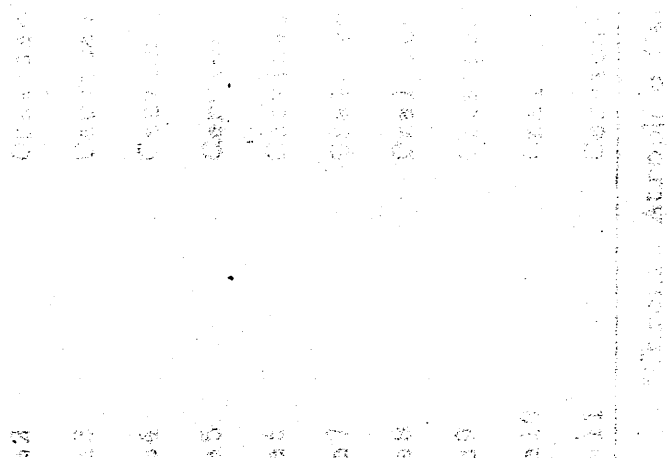


Table 2.1: *Candida albicans* code used in this study and clinical sources of strains.

Assigned Code No.	Source of isolates
a1	Oral isolate from a patient with CAC*
a2	Oral isolate from a patient with CAC
a3	Cervical isolate from a patient with vaginal candidosis
a4	Cervical isolate from a patient with vaginal candidosis
a5	Cervical isolate from an apparently asymptomatic woman
a6	Cervical isolate from a patient with vaginal candidosis
a7	Oral isolate from a patient with CAC
a8	Oral isolate from a patient with CAC
a9	Obtained from the Mycological Reference Laboratory, London, UK.
a10	Oral isolated from a patient with CAC
a11	Cervical isolate from a patient with vaginal candidosis

*Chronic Atrophic Candidosis

Table 2.1:(Continued).

Assigned Code No.	Source of isolates
a12	Skin isolate from a cutaneous candidosis lesion
a13	Cervical isolate from an apparently asymptomatic woman
a14	Oral isolate from a patient with CAC*
a15	Oral isolate from a patient with CAC
a16	Cervical isolate from an apparently asymptomatic woman
a17	Cervical isolate from an apparently asymptomatic woman
a18	Oral isolate from an apparently asymptomatic individual
a19	Oral isolate from a patient under going radiotherapy
a20	Oral isolate from a patient under going radiotherapy
a21	Oral isolate from a patient under going radiotherapy
a22	Oral isolate from a patient with CAC

*Chronic Atrophic Candidosis

Table 2.2. The code number, API identification profile and laboratory source of the *Candida albicans* isolates studied

Code Number	API Code	Source of isolates
a1	2566174	GDH* 1957
a2	2576074	NCYC* 1469 (GDH 2036)
a3	2566174	GRI* 941
a4	2574174	GRI 207199
a5	2566174	NCYC 1472 (GRI 681)
a6	2576174	GRI 42543
a7	2576170	NCYC 1467 (GDH 2346)
a8	2566170	GDH 850074
a9	2566170	NCPF* 3153
a10	2576170	GDH 1261
a11	2576174	NCYC 1470 (GRI 2773)

*GDH=Glasgow Dental Hospital and School, Glasgow, UK.

GRI=Glasgow Royal Infirmary, Glasgow, UK.

NCYC=National Collection of Yeast Cultures, Norwich, UK.

NLPF=National Collection of Pathogenic Fungi, London, UK.

Table 2.2:(Continued).

Code Number	API Code	Source of isolates
a12	2576174	NCYC 1466 (GDH 3968)
a13	2576174	GRI 651
a14	2576174	GDH 1786
a15	2576174	GDH 1878
a16	2576174	NCYC 1473 (GRI 682)
a17	2576174	GRI 2632
a18	2576174	GDH 2468
a19	2576174	GDH 2483
a20	2576174	GDH 2481
a21	2576174	GDH 2484
a22	2576174	GDH 2542

*GDH=Glasgow Dental Hospital and School, Glasgow, UK.

GRI=Glasgow Royal Infirmary, Glasgow, UK.

NCYC=National Collection of Yeast Cultures, Norwich, UK.

Table 2.3 The original code number, API identification profile and source of the *Candida tropicalis* isolates studied

Study Code	Source Code number
t1	NCPF 3111
t2	GDH 1647
t3	GDH 1783
t4	GDH 262
t5	GDH 1107
t6	NCPF 3111
t7	GDH 1166

*GDH=Glasgow Dental Hospital and School, Glasgow, UK.
GRI=Glasgow Royal Infirmary, Glasgow, UK.
NCPF=National Collection of Pathogenic Fungi, London, UK.

Table 2.4 The original code number, API identification profile and source of the *Candida krusei* isolates studied

Study Code	Source Code number
k1	GDH 731
k2	GDH 1558
k3	GDH 2119
k4	GDH 1713
k5	GDH 3164
k6	GDH 901
k7	GDH 11151

*GDH=Glasgow Dental Hospital and School, Glasgow, UK.

Table 2.5 The original code number, API identification profile and source of the *Candida parapsilosis* isolates studied

Study Code	Source Code number
p1	NCPF 2104
P2	GDH 2118
P3	GDH 1051
P4	GDH 109
P5	GDH 273

*GDH=Glasgow Dental Hospital and School, Glasgow, UK.
NCPF=National Collection of Pathogenic Fungi, London, UK.

Table 2.6 The original code number, API identification profile and source of the *Candida guilliermondii* isolates studied

Study Code	Source Code number
gu1	GDH 511
gu2	GDH 3109
gu3	GDH 1967
gu4	GDH 3099
gu5	GDH 1763

*GDH=Glasgow Dental Hospital and School, Glasgow, UK.

Table 2.7 The original code number, API identification profile and source of the *Candida glabrata*‡ isolates studied

Study Code	Source Code number
g1	NCPF 3240
g2	GDH 1647
g3	GDH 820

‡Lysozyme is the only occasion in this thesis when 20 strains of *C. glabrata* were used

*GDH=Glasgow Dental Hospital and School, Glasgow, UK.

NCPF=National Collection of Pathogenic Fungi, London, UK.

2.2. Identification procedure:

The identification of *Candida* isolates was performed by testing their ability to produce germ-tubes, and their biochemical profile using API 20 C strips (API Laboratory Products Ltd, Grafton Way. Basingstoke, Hants, RG22 6H9).

2.2.1: Germ-tube test.

The test procedure for the *Candida* germ-tube test was carried out as described by MacKenzie (1962). This involved mixing 0.2 ml of undiluted sterile horse serum (Gibco Bio-cult; Glasgow), with an equal volume of candidal suspension prepared as follows; a loopful of an overnight colony of *Candida* species grown on Sabouraud's dextrose agar (SDA) at 37°C was removed and emulsified in 1 ml of distilled water, mixed thoroughly, further diluted in distilled water and adjusted to give $1.0-1.5 \times 10^6$ colony forming units per ml by means of haemocytometer counting method (see Microbial Counts, Section 2.5). After incubation at 37°C for 3 hours, a drop of the germ-tube suspension was placed on a haemocytometer and kept at room temperature on the bench for a few minutes to allow the cells to settle and examined under the light microscope at a magnification of X400, (Olympus, Japan). Projections which at the end of the incubation time, were more than twice the diameter of a normal yeast cell in length, were considered as germ-tubes.

2.2.2: API 20 C *Candida* identification system.

The API 20 C, *Candida* identification system was used to check the identity of the yeast isolates used in this study. The preparation of the inoculum used was as recommended by the manu-

facturer. The required number of ampoules of the API 20 C growth medium were placed in a boiling water bath before the experiment was started, and then maintained in a water bath at 45°C, for about 10 minutes, when the top of the ampoule was snapped off. A loopful of an overnight colony of *Candida* grown on SDA at 37°C was removed and emulsified in 1 ml of distilled water, and mixed thoroughly. Two drops of the emulsion were added to each ampoule of the API 20 C medium,which was held at 37°C. Then all 20 cupules in the biochemical strip were inoculated with the labelled isolate, and then placed in a labelled tray (Figure 2.1). Each tray was moistened with 5 ml of tap water, incubated at 30°C (as recommended by the manufacturer). The tests were read normally after 24 hours but if the results were not clear, the API strips were incubated for up to 48 and 72 hours. The tests were grouped in threes, and each positive reaction recorded was given a determined numerical profile, 1, for the first test of each group, 2, for the second test of each group, and 4, for the third test of each group. For a negative reaction the value 0 was recorded (Figure, 2.1). By adding up the values in each group, a seven-figure number was obtained which corresponded to a numerical profile. The coded numbers were entered into an Apple IIE computer (Apple Computer, Inc., London) supplied with API identification software (API) which printed out the identity of the yeast with confidence limits.

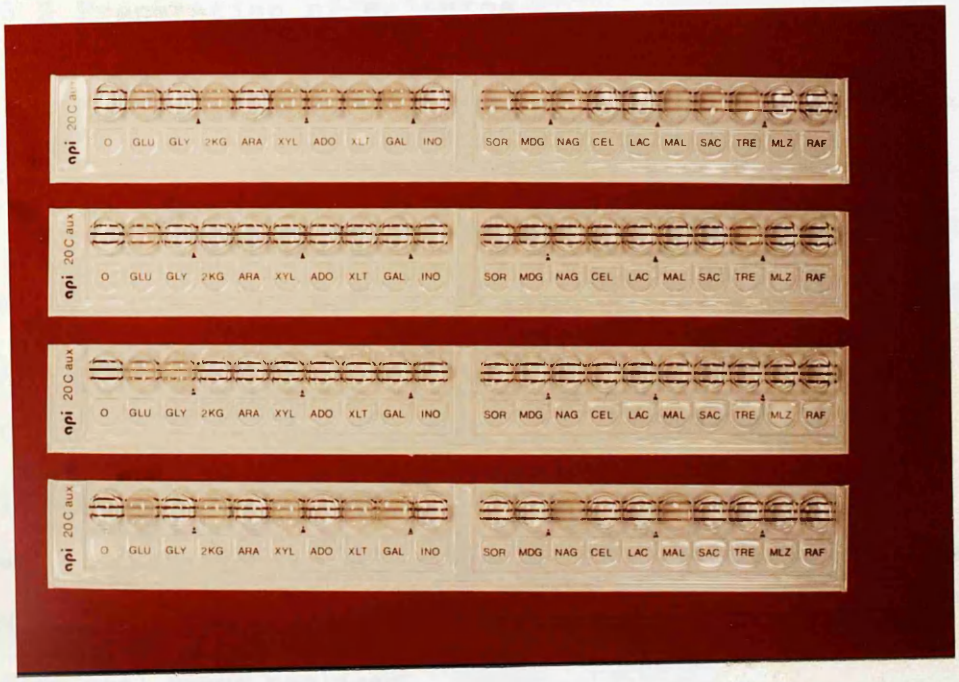


Figure 2.1. API biochemical strips inoculated with four different *Candida* species.

2.3 Freeze-drying Procedure

2.3.1 Preparation of ampoules.

The method described by Kirsop (1984), was used in this study. Glass ampoules (Woods, Paisly, Glasgow, UK.) were washed, dried and labelled by inserting a small printed card with the name of the *Candida* isolate and number. After plugging the ampoules with non-absorbent cotton wool, they were autoclaved at 121°C for 15 minutes.

2.3.2 Preparation of cultures.

Cultures to be freeze-dried were grown on SDA, and incubated aerobically for 18 hours at 37°C. Horse serum containing glucose to a final concentration of 7.5 percent (Gibco Biocult Glasgow), was inactivated at 56°C for 30 minutes. After sterilization by filtration the serum was aliquoted in 5 ml volumes in sterile glass bottles. Each bottle was coded and inoculated with the required organism to a concentration of approximately 10^6 yeast/ml. Using a sterile Pasteur pipette 6 drops (0.5 ml) of the yeast suspension was then transferred into the previously labelled ampoules and the cotton wool plugs replaced.

2.3.3 Primary freeze-drying.

The inoculated ampoules were placed immediately in the centrifuge head of an Edwards High Vacuum, freeze-dryer (Manor Royal, Crawley, west Sussex, RH10 2LW, UK) with the writing on the labels facing towards the centre of the centrifuge, as otherwise the liquid would cover the label and hide the strain identity. The

centrifuge motor and the vacuum pump were switched on and the primary freeze drying process carried out for 3 hours. The cotton wool plugs were gently pushed halfway down the ampoules The ampoules were then, constricted by an ampoule constrictor (Edwards High Vacuum)

2.3.4 Secondary drying.

In this process where the constricted ampoules were subjected to drying without freezing, they were placed on a manifold, comprising a central chamber with 48 peripheral rubber openings (Edwards High Vacuum). An ampoule was fitted onto each opening and secondary drying was continued for 2 hours. Next the ampoules were sealed under vacuum using a flame blow torch (Edwards High Vacuum), and stored at room temperature in the dark (Kirsop, 1984).

2.3.5 Viability test.

A viability test was carried out on the second day after the completion of freeze drying by opening a random ampoule from each freeze dried batch of isolates as described in the revival section below.

2.3.6 Revival.

An ampoule of the yeast strain to be revived, was marked with a file, just above the level of the cotton-wool plug. A molten glass rod was applied to crack the glass and the tip of the ampoule was removed, and safely discarded. To re-constitute the dried organisms in the ampoule, 5-6 drops of sterile yeast nitrogen base (YNB), were added using a Pasteur pipette. Two drops of the re-

constituted organisms in suspension were then plated onto Sabouraud's agar and the remainder inoculated into 10 ml of YNB broth. Both the agar plate and the broth cultures were incubated aerobically at 37°C for up to four days. The yeast reidentified using the same procedure above (section, 2.2).

2.4 Microbial counts.

2.4.1 Total cell count (Microscopical).

The total cell count, (living and dead) was performed using a haemocytometer counting chamber, Figure, 2.2. The chamber seen from one side in Figure, 2.3, consists of a rectangular glass block in which the central plateau lies precisely 0.1 mm below the level of the shoulders on either side. The central plateau is separated from each shoulder by a trough, and is itself divided into two parts by a shallow trough, Figures 2.2, and 2.3 On the surface of each part of the central plateau is an etched grid Figure, 2.4 consisting of a large square which is divided into 400 small squares, each 1/400 mm^2 (in blocks of 25 x 16). A thin glass cover slip is positioned as shown at Figure, 2.2 and pressed firmly onto the shoulders of the chamber. In order to achieve proper contact it is necessary, while pressing, to move the cover slip slightly against the surface of the shoulders. Proper contact is indicated by the appearance of a pattern of coloured lines (Newton's) , shown in Figure, 2.2.

Using a Pasteur pipette, small volume of a cell suspension (yeast or buccal epithelial cells) was introduced into the space bounded by the cover slip and one-half of the central plateau Figure, 2.2, care being taken to ensure that the liquid did not overflow into the trough. A second sample can be examined, if required, in the other half of the counting chamber. The chamber was left for 5 minutes to allow the cells to settle, and counting was then carried out at (400X) magnification using a light microscope (Olympus, Japan). Since the volume between grid and cover slip was accurately known, the count of cells per unit volume was calculated. If the sample has been diluted before examination in the counting chamber, the count obtained was multiplied by the dilution factor; e.g., if diluted 1 in 10, the count is multiplied by 10.

2.4.2 Viable microbial counts.

Sabouraud's agar plates were used for total viable counts of candidal cells. Sabouraud's agar was, prepared as recommended by the manufacturer in 500 ml quantities and stored at room temperature in screw-capped bottles. Within a month of preparation, plates were poured in 20 ml quantities on a carefully leveled surface and allowed to set completely before handling. Plates were dried for 3-4 hours at 37°C with the lids in place.

The spiral plate method (Spiral Plater Model D, Spiral Systems Inc. 6740, Clough Pike, Cincinnati, Ohio 45244, U.S.A.), was included in these experiments to assess candidal counts in a liquid

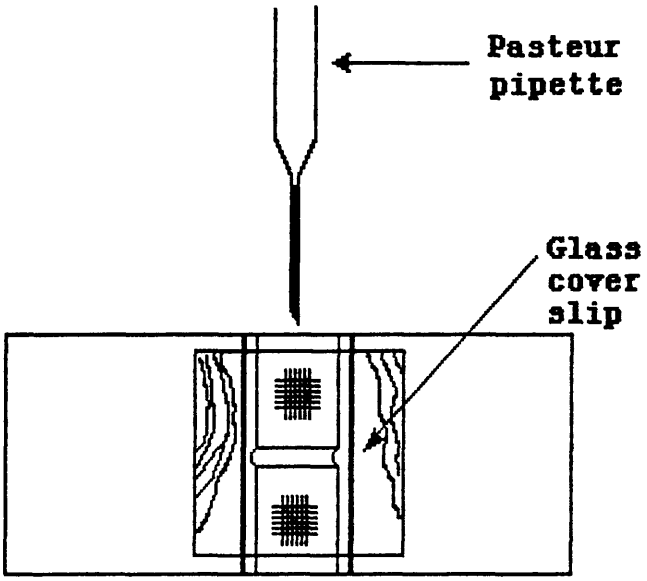


Figure 2.2 A counting haemocytometer chamber shows the central plateau and etched grids.

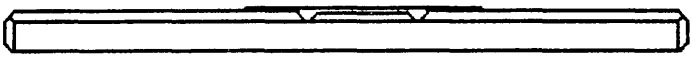


Figure 2.3. A lateral view of the counting chamber

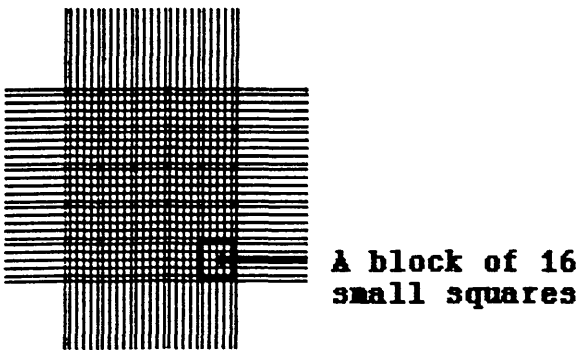


Figure 2.4. A grid consisting of a square which is divided into 400 small squares in 25 blocks of 16 small squares.

sample. No serial dilutions were required using this method in contrast to other counting techniques. The model D spiral plater (Figure, 2.5) is a specialized dispenser which distributes 50 µl of the sample on to the surface of a rotating 10-cm, Sabouraud's agar plate. The inoculating tip moves from the centre of the plate to the edge in an Archimedean spiral resulting in a gradual dilution of the sample. After incubation, colonies appear on the lines of the spiral inoculation and the microbial numbers were determined by counting the colonies on a suitable portion of the plate with the help of counting grid. The major divisions on this grid are 4 concentric circles and 8 pie-shaped wedges or sectors, which result in a number of annular segments. Areas typically counted for 10-cm Sabouraud's plates are shown in Figure, 2.6. A suitable sector was chosen and counted starting from the outer edge toward the centre. The number obtained was recorded along with the number of the segment that included the counted colonies (Figure, 2.7). Usually, the same segments in the opposite sector were counted also, in order to balance any irregularities in sample deposition. If the total number of colonies on the whole plate could be easily counted then this was recorded.

Figure 2.5. Spiral plater, Model D.

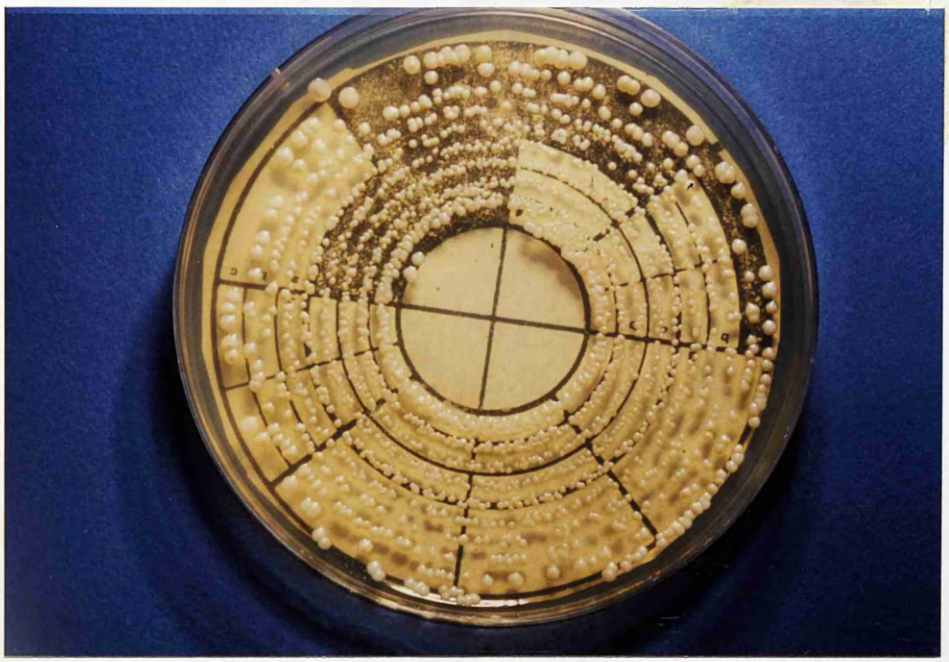
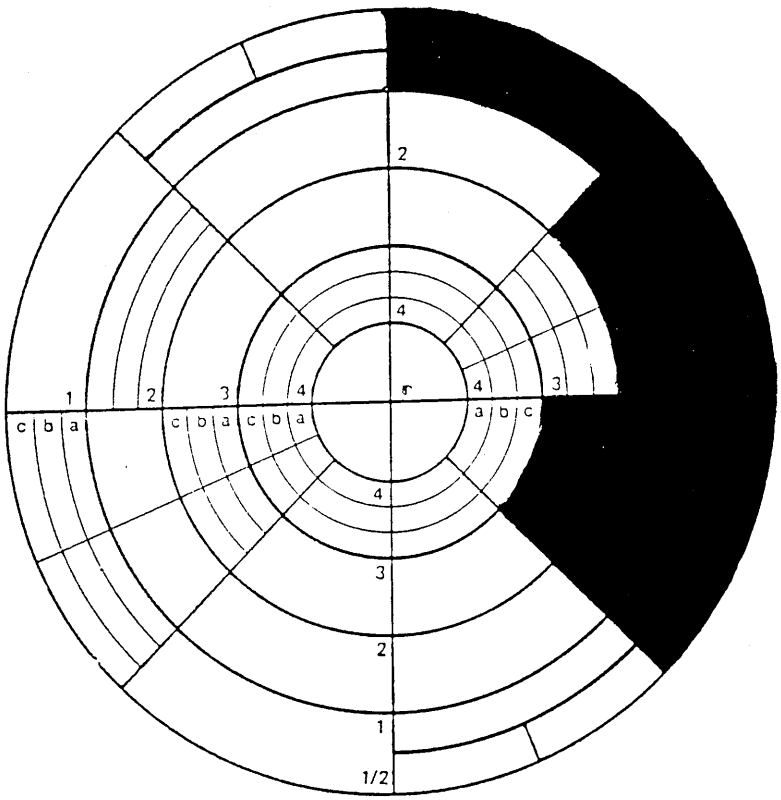


Figure 2.6. 50µl of *Candida* suspension distri-buted on 10-cm Sabouraud's agar plate.



Figure, 2.7. Figure 2.7. Spiral system counting grid of 10-cm Petri dish

CHAPTER THREE

ADHESION OF *Candida* SPECIES TO BUCCAL EPITHELIAL CELLS AND ACRYLIC SURFACES

3.1 INTRODUCTION:

Candida albicans is an opportunistic yeast which is present in the gastrointestinal tract and vaginal cavity of many normal individuals (Winner 1975; Hurley *et al.*, 1986). Since infections of the urogenital tract and mouth are the most common forms of superficial candidosis, epithelial cells from these sources have been most frequently used in candidal adhesion studies. Although the transition of *C. albicans* from saprophytism to pathogenic parasitism remains largely unexplained, it is generally accepted that the ability of *Candida* species to adhere to susceptible host mucosal tissues (or solid surfaces such as dentures), is an essential prerequisite for successful colonization, which may lead either to a sustained or transient saprophytic association with the host or to localized infections of mucosal surfaces (Odds 1988).

At the present however, the role of adhesion of *Candida* in pathogenesis is only partially defined, although it is known that adhesion may be affected by a number of environmental and host factors, (Douglas 1987; Kennedy 1987). For instance, some of the factors which affect candidal adhesion to epithelial cells and acrylic surfaces include, the viability, cell concentration and the ability to produce germ-tubes by the *Candida* species used (Odds 1988). Sugars have also been found

to enhance candidal adhesion both to mucosal epithelial cells (Douglas, Houston and McCourtie 1981; McCourtie and Douglas 1981; Samaranayake and MacFarlane 1981; 1982a) and acrylic surfaces (Samaranayake and MacFarlane 1982b).

The adhesion of *C. albicans* to epithelial cells has been demonstrated *in vitro* (Kimura and Pearsall 1978; Liljemark and Gibbons 1973; Samaranayake and MacFarlane 1982 a, b) and *in vivo*, (Calderone, Lehrer and Segal 1984). It has been suggested that this species adheres to human vaginal and buccal epithelial cells in considerably higher numbers than the other *Candida* species tested (Kennedy 1987). The genus *Candida* contains several other established human pathogens of which *C. tropicalis*, *C. stellatoidea* and *C. parapsilosis*, are probably the most important clinically (Odds 1988) followed by *C. guilliermondii*, *C. krusei* and *C. pseudotropicalis* (Ray, Digre and Payne 1984; Critchley and Douglas 1985; Klotz *et al.*, 1985). There is, however, scant data in the literature on the relative adhesion of a large number of *Candida* isolates conducted under same experimental conditions by the same worker.

Therefore, the purpose of this study was to investigate the relative adhesion of *C. albicans* (22 isolates), *C. tropicalis* (7 isolates), *C. parapsilosis* (5 isolates), *C. guilliermondii* (5 isolates), *C. krusei* (7 isolates), and *C. pseudotropicalis* (1 isolate), to buccal epithelial cells and acrylic surfaces.

3.2 Materials and Methods.

3.2.1 Candidal isolates and growth conditions.

Forty nine clinical isolates of *Candida* species, 22 *C. albicans*, 3 *C. glabrata*, 7 *C. tropicalis*, 5 *C. parapsilosis*, 7 *C. krusei*, and 5 *C. guilliermondii*, (See Maintenance of *Candida* species Chapter Two), were investigated. Yeast cells were grown on Sabouraud's dextrose agar plates (Oxoid) which were incubated aerobically at 37°C for 24 hour. A loopful of the confluent growth was transferred to 10 ml of yeast nitrogen base medium (Difco) supplemented with 500 mM sucrose (British Drug House) and incubated at 37°C on an orbital incubator 100 rpm for 18 hours. The growth was harvested by centrifuging the culture at 1500 g for 10 minutes. The cells were washed twice in 20 ml of sterile phosphate buffered saline (PBS 0.1 M, pH 7.2) and after resuspension in the same buffer were left to stand on the bench for 5 minutes to separate yeast clumps which usually formed when 500 mM sucrose was present in the medium. The supernatant was collected and adjusted with PBS to give a final suspension of $4.0\text{-}4.5 \times 10^7$ yeast cells per ml. The number of yeast cells per ml was regulated, in all the experiments, by means of a haemocytometer counting chamber (Hawksley England), (Collins and Lyne 1984).

3.2.2 Buccal epithelial cells (BEC).

Buccal epithelial cells for the assay were collected from a healthy adult male, by gently rubbing the inside of both cheeks with a sterile cotton wool swab then agitating the swab in 20 ml of phosphate buffered saline (PBS), pH 7.2. Subsequently, the epithelial cells were left to stand on the bench for 5 minutes to allow debris and clumps of BEC to separate by precipitation. Approximately 10 ml of the supernatant was added to a sterile bottle, then washed twice in PBS to remove loosely attached microorganisms by centrifugation at 300 *g*, for 5 minutes. The BEC's were resuspended in PBS at a concentration of $1.0-1.5 \times 10^5$ epithelial cells/ml as described in Chapter Two, section 2.5.1. Since it is known that the adhesion of *Candida* species to BEC's collected from the same donor can vary from day to day (Kearns Davies and Smith 1983). BEC's were collected from the same donor (RST) at the same time of the day (early morning and before breakfast), to minimize this variations.

3.2.3 Preparation of acrylic strips.

The adhesion of candida cells to acrylic strips was measured using the *in vitro* assay system described by Samaranayake and MacFarlane (1980). The strips were prepared by adding 1.5 g of transparent selfpolymerising acrylic powder (Simplex Rapid; Howmedica International Ltd, 622 Western Avenue, London) on to the surface of a cleaned glass microscopic slide (25 x 75 mm) followed by 1 ml of monomer liquid. After 45-50 seconds another glass microscopic slide was placed on top of the

polymerising mixture and the slides were secured at either end with two crocodile clips. The acrylic was then polymerised at room temperature for two hours. The room temperature polymerisation reduces bubbling within the acrylic and increases clarity. Subsequently, the acrylic sheet formed was stripped from the slides and cut into 4 x 4 mm square strips, the average thickness of the sheet being 0.3-0.5 mm. The strips were immersed for up to 24 hours in running tap water to leach excess monomer. The acrylic strips were stored in sterile distilled water usually for about a week at room temperature until required for adhesion assays.

3.2.4 Adhesion assay.

Adhesion to buccal epithelial cells

Adhesion of *Candida* species to buccal epithelial cells was studied using a modification of a previously described adhesion assay (Kimura and Pearsall 1978). Briefly, 0.5 ml samples of buccal epithelial cell suspension containing 10^5 cells/ml were pipetted into small plastic bottles (bijou), and incubated with an equal volume of a yeast cell suspension on an orbital incubator at 100 rpm for 60 minute at 37°C. After incubation, each mixed suspension was diluted in 4.5 ml PBS then divided into two equal aliquots. The epithelial cells of each aliquots were collected, under a constant negative pressure (5 mm Hg), on to the surface of a polycarbonate filters (25 mm diameter, 12 µm pore size; Millipore (UK) Harrow, Middx) using a manifold filter-technique (Micro measurements Ltd., Saffron Walden, Essex,

England). The cells were then washed with 50 ml of PBS under negative pressure, to remove any yeast cells unattached to the epithelial cells. The filters were placed on numbered glass microscopic slides, care being taken to ensure that the epithelial cells faced upwards. After drying in air and fixing with 95 percent methanol, the filters were stained with Gram stain, washed with water and allowed to dry. After drying, the filters were mounted on cleaned, marked glass slides with Harleco synthetic resin medium (Kodak Ltd., Kirby, Liverpool) For each strain studied, the growth conditions and donor BEC's were standardized as far as possible. The assay for each strain was carried out in duplicate on 6 separate occasions.

Adhesion to acrylic strips.

Adhesion of yeast cells to acrylic strips was evaluated with the aid of the *in vitro* assay system previously described by Samaranayake and MacFarlane (1980). The method is as follows: small 4 x 4 mm square transparent acrylic strips were placed vertically in flat-bottomed sterile serology plate wells of internal diameter 5 mm (Gibco Biocult Ltd. Middlesex) to which were added 0.4 ml of the yeast suspensions. The samples were set up in duplicate for each experiment. The serology plate was placed in a sterile square Petri dish (Sterilin, Middlesex., England) and incubated at 37°C for 1 hour on an orbital incubator (Lukham Ltd, England) at a speed of 100 rpm. The strips were removed from the wells and sequentially washed for a total of 75 seconds by gentle manual agitation in 5 jars of sterile PBS. This technique dislodged all the loosely

adherent yeasts and debris. The strips were dried in air, fixed in 95 percent methanol for 10 minutes and rehydrated after 5 minutes in 50 percent methanol. The strips were then stained in multiwell Disposo trays (Sterilin, Middlesex., England) using a modified Gram stain (Clin-Tech-Ltd, Laboratory reagents,London) omitting the counter stain. Thus, they were stained with the crystal violet solution for 1 minute, then washed with tap water, stained with iodine solution for 1 minute, decolorized with 95 percent ethanol for 1 minute and finally, washed with tap water, and left to dry. After drying the strips over night at 37 °C they were mounted on cleaned, marked glass slides with Harleco synthetic resin medium (Kodak) and the yeasts counted as described below. Each experiment was repeated on 6 separate occasions.

3.2.5 Microscopy

The majority of yeasts attached either to BEC's or to acrylic strips were in the blastospore stage, some with daughter cells and only a very few with pseudohyphae. A stratified random sampling technique (Weibel 1969) was used to enumerate the number of adhering yeast cells. The following criteria were used to standardize counting (i) a budding yeast was considered as a unit cell if the daughter cell was smaller than the mother; (ii) a hypha was counted as a single cell; (iii) clumps and overlapping epithelial cells were not counted. (iv) surface irregularities near the edges of the acrylic strips were avoided as they had artificially high numbers of mechanically lodged yeasts.

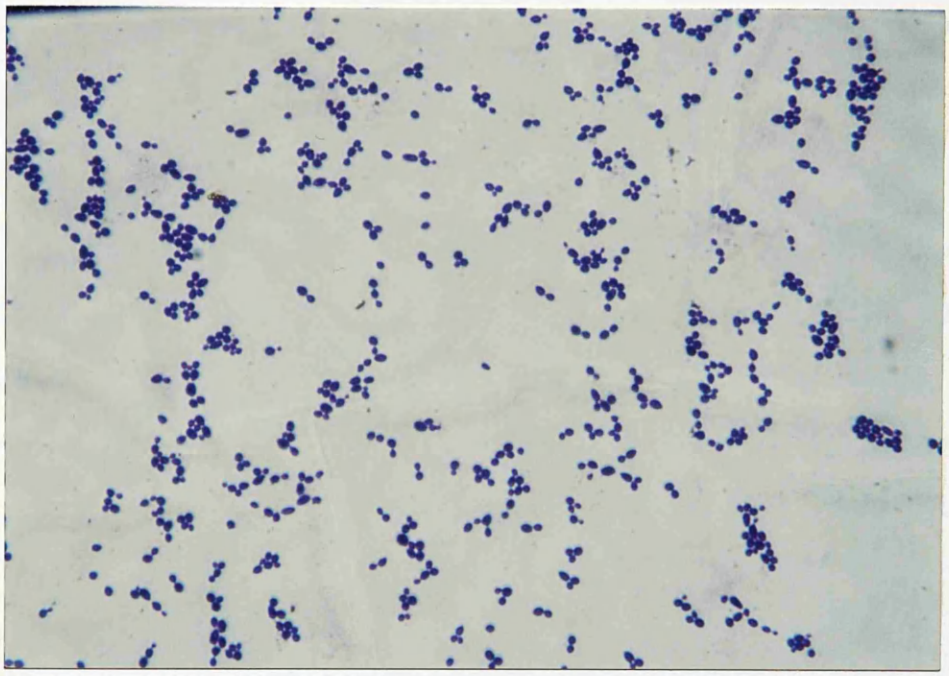
Each polycarbonate filter was examined microscopically and the number of yeast cells which were adherent to 100 BEC's, was determined by light microscopy (Leitz Ortholux microscope Leitz Wetzlar, Germany) at a magnification of 400 times. Counts on the two separate filters were performed for each sample, the mean and standard error were calculated. Each experiment was performed in duplicate on 6 separate occasions to obtain sufficient data for statistical analysis.

The number of adherent yeast cells/mm^2 of the acrylic strips was enumerated (Figure, 3.1). A Leitz Ortholux microscope fitted with a sampling stage which permitted the specimen to be examined at 0.4 mm intervals was used at a magnification of 400 times. A graticule of equal squares which was mounted in the focus of the ocular allowed standardized counts of yeast cells to be made on each strip simulating the haemocytometer principle. Thirty fields were counted for each strip. Since the samples were set up in duplicate for each experiment the mean number of yeasts per 60 fields was finally expressed as yeasts per unit square mm^2 of acrylic. Each experiment was performed in duplicate on 6 separate occasions to obtain sufficient data for statistical analysis.

3.2.6 Statistical analysis:

The student *t* test was used to evaluate differences in yeast adhesion. StatWorks™, a statistics computer package was used (Rafferty *et al.*, 1985). A probability (p) value of <0.05 was considered significant. To evaluate differences between the

groups analysis of variance test (ANOVA) was used. ANOVA is a method of analysing the way in which the mean variable is affected by classifications of the data (Scheffe 1959). This is done by partitioning the total variation in the data into various components. The simplest form of ANOVA is Oneway Analysis of Variance, which is simply an extension of the two sample *t*-test which allows simultaneous comparison of more than two population means. This form of analysis was used in this thesis.



Figure, 3.1. A photomicrograph of *Candida albicans* adhered to acrylic strips. Modified Gram stain, X400 magnification.

3.3 Results.

3.3.1:Adhesion of *Candida albicans* isolates to buccal epithelial cells.

None of the BEC'S examined prior to the addition of the yeast suspension, had naturally attached yeasts after the PBS initial wash.

The adhesion of twenty two *Candida albicans* isolates grown in yeast nitrogen base (YNB) supplemented with (500 mM) sucrose, to BEC's is shown in Table 3.1. All isolates of *C. albicans* used in this study, were able to adhere to BEC's of the same donor. There were substantial variations in the adhesion capacity of the isolates (Figure, 3.2), for example, a significant difference (p <0.001) was found between isolate a15 which produced the highest adhesion value (1569 yeast/100 BEC) compared with isolate a5 which give the lowest value (542 yeast/100 BEC). Table 3.2 shows examples of the statistically significant differences found between the lowest and highest adhesion values of *C. albicans* isolates; values ranged from <0.001 to non-significant (Table 3.2). The variation which occurred in the adhesion of the same isolate to BEC's in different experimental events as represented by the standard errors of the mean lay between 32 and 84 (Table 3.1).

3.3.2:Adhesion of *Candida albicans* to acrylic surfaces

The twenty two *Candida albicans* isolates grown in YNB supplemented with 500 mM sucrose adhered to acrylic surfaces to

varying degrees (Figure 3.3). The results in Table 3.3 show the adhesion values, the means and standard errors, for the above isolates.

When the standard errors of the mean for the adhesion of *C. albicans* isolates to epithelial cells and acrylic surfaces were compared the values obtained for acrylic were somewhat lower (19-49, Table 3.3) than those for BEC's (32-84, Table 3.1). Figure, 3.4, shows that the mean adhesion values for *C. albicans* isolates to BEC's and acrylic surfaces generally followed the same pattern regardless of the nature of the surface.

3.3.3.Adhesion of *Candida* species to buccal epithelial cells.

The adhesion to buccal epithelial cells of seven isolates of *C. tropicalis* (Table 3.4), seven isolates of *C. krusei* (Table 3.5) five isolates of *C. parapsilosis* (Table 3.6), five isolates of *C. guilliermondii*, (Table 3.7), and three isolates of *C. glabrata* (Table 3.8), grown in YNB supplemented with 500 mM sucrose are shown in Tables 3.3-3.8. Figure, 3.5 shows the distribution of the adhesion values of seven *Candida* species, when incubated with epithelial cells. Example of statistically significant differences in the adhesion of the above *Candida* species are recorded in Table 3.9. *Candida tropicalis* shows highly significant variation in adhesion between some of the isolates ($p < 0.001$)..

3.3.4:Adhesion of *Candida* species to acrylic surfaces.

The adhesion results to acrylic strips of the above *Candida* species other than *C. albicans* are shown in Tables 3.10-3.14. When the mean adhesion values to acrylic surfaces of the six different *Candida* species were compared, *C. tropicalis* was the most adherent, while *C. krusei* (Table 3.16) was the least adherent species. Examples of statistical significant counts of the adhesion of the *Candida* species other than *C. albicans* to acrylic strips are recorded in Table 3.15. Figure 3.6 shows the distribution of the adherence values, of *Candida* species, to acrylic strips.

3.3.5.The adhesion of all *Candida* species to both buccal epithelial cells and acrylic surfaces.

The hierarchy of adhesion of *Candida* species to BEC's and acrylic surfaces is shown in Table, 3.16. Statistical analysis of inter-species variation in the adhesion of *Candida* species to BEC's and to acrylic surfaces are shown in Table 3.17 and 3.18 respectively. When the hierarchy of adhesion of *Candida* species to BEC's was studied *C. albicans,* was the most adherent followed by *C. tropicalis C. parapsilosis, C. glabrata, C. guilliermondii* and *C. krusei,* respectively. Whereas in acrylic studies adhesion of *C. tropicalis* was the highest followed by *C. albicans, C. parapsilosis, C. glabrata, C. guilliermondii* and *C. krusei,* respectively.

Table 3.1. Adhesion of *Candida albicans* isolates to 100 buccal epithelial cells after growth in yeast nitrogen base supplemented with 500 mM sucrose, (data from six experiments).

Strain code	Number of yeasts attached to 100 BEC's							
	1	2	3	4	5	6	Mean	±SEM*
a1	795[a]	810	1050	975	836	832	883	43
a2	644	802	464	715	660	570	642	48
a3	987	698	716	642	805	675	754	52
a4	790	1200	857	798	1090	1129	977	75
a5	518	429	740	651	363	549	542	57
a6	735	933	997	1105	914	968	942	50
a7	1299	1142	864	1151	1167	1089	1119	58
a8	1643	1389	1395	1418	1237	1378	1410	54
a9	788	1110	950	690	900	1040	913	64
a10	950	895	980	620	1210	892	924	77
a11	1226	1548	1389	1312	1159	1041	1279	73

[a]=Mean of two results. (Continued on next page.)

± SEM= Standard error of the mean.

Table 3.1 (continued).

Strain code	Number of yeasts attached to 100 BEC's 1	2	3	4	5	6	Mean	±SEM*
a12	1226[a]	1339	1088	1167	992	1324	1189	55
a13	1015	589	765	876	820	680	791	61
a14	1105	1020	1290	999	1043	849	1051	59
a15	1667	1792	1421	1250	1545	1569	1569	84
a16	841	710	690	724	539	642	691	41
a17	792	910	885	774	693	841	816	33
a18	749	905	785	812	670	819	790	32
a19	1298	1183	1241	1656	1335	1272	1331	68
a20	1193	1000	974	780	1031	960	990	54
a21	735	933	997	1105	914	968	942	50
a22	1001	1215	1167	1055	1419	1289	1191	63

[a]=Mean of two results.
± SEM= Standard error of the mean.

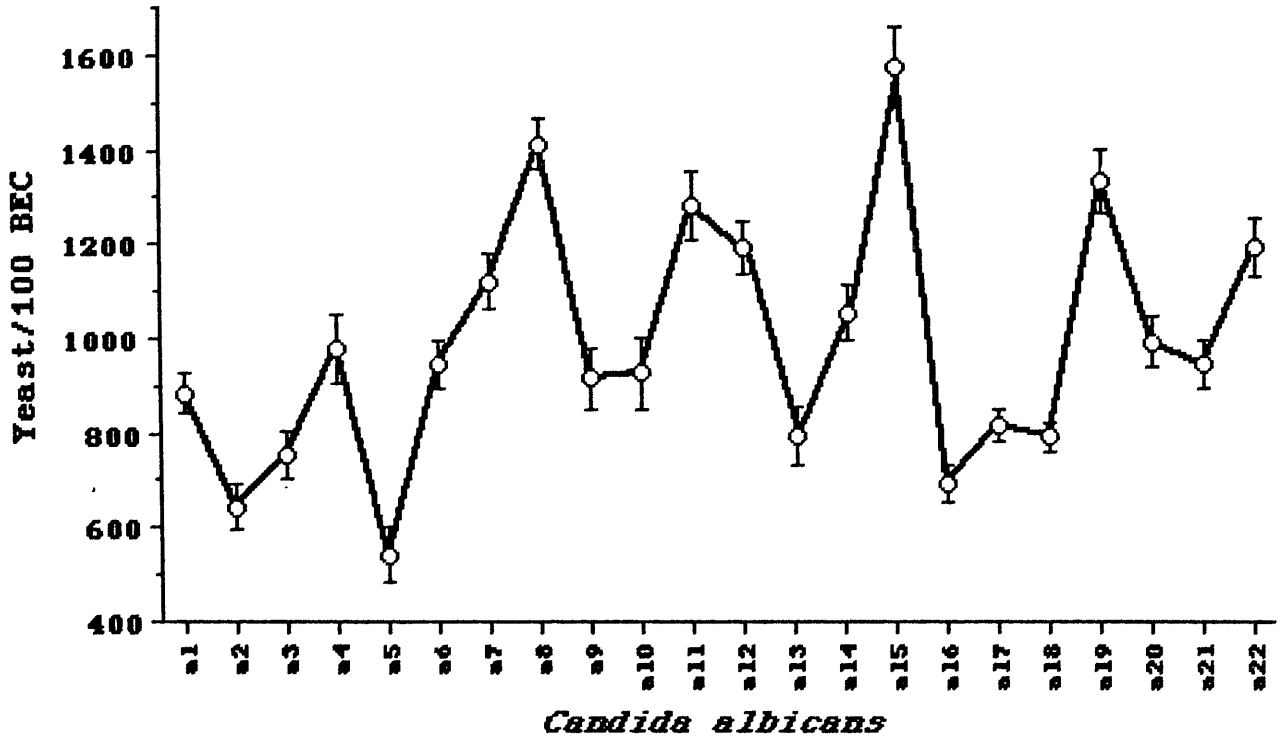


Figure 3.2. Adhesion of 22 *Candida albicans* isolates to buccal epithelial cells. Bars indicate standard error of the mean.

Table 3.2: Statistical significant intra-species differences‡ in the adhesion of 22 strains of *Candida albicans* to buccal epithelial cells and acrylic strips.

Buccal epithelial cells probability		Acrylic strips probability*	
a15 v a16	< 0.001	a15 v a5	< 0.001
a8 v a2	< 0.001	a8 v a16	< 0.001
a20 v a5	< 0.001	a7 v a2	< 0.001
a11 v a1	< 0.005	a20 v a14	< 0.001
a21 v a18	< 0.005	a21 v a18	< 0.001
a12 v a14	< 0.01	a4 v a17	< 0.01
a7 v a17	< 0.05	a11 v a9	< 0.01
a13 v a3	< 0.05	a12 v a3	NS
a23 v a9	NS	a23 v a1	NS
a4 v a10	NS	a10 v a6	NS
a22 v a10	NS	a13 v a22	NS

v=versus.

*Examples of possible significant and non-significant combinations.

‡evaluated by *t* test.

Table 3.3. Adhesion of *Candida albicans* isolates to acrylic strips after growth in yeast nitrogen base containing 500 mM sucrose, (data from six experiments).

Strain code	Number of yeasts attached to 100 BEC's 1	2	3	4	5	6	Mean	±SEM*
a1	356[a]	520	370	280	369	432	388	33
a2	139	237	190	210	189	277	207	19
a3	268	497	349	356	222	360	342	39
a4	331	510	488	399	678	546	492	49
a5	251	124	152	180	162	213	180	19
a6	360	501	381	293	370	439	391	29
a7	618	417	583	569	535	601	554	30
a8	396	681	549	562	535	617	557	39
a9	272	309	270	401	290	302	307	20
a10	369	476	426	408	597	315	432	40
a11	435	601	473	382	459	511	477	30

[a]=Mean of two results.

(Continued on next page.)

± SEM= Standard error of the mean.

Table 3.3 (continued).

Strain code	Number of yeasts attached to 100 BEC's 1	2	3	4	5	6	Mean	±SEM*
a12	339[a]	393	466	578	442	609	471	43
a13	194	246	112	233	301	260	224	27
a14	287	426	510	421	395	453	415	30
a15	431	599	474	682	568	652	568	40
a16	159	281	165	118	171	246	190	25
a17	204	325	290	343	286	341	298	21
a18	259	319	274	189	267	420	288	32
a19	392	564	548	657	610	471	540	39
a20	410	555	531	542	619	579	539	29
a21	356	509	350	401	340	389	391	26
a22	396	465	540	457	435	371	444	24

[a]=Mean of two results.
± SEM= Standard error of the mean.

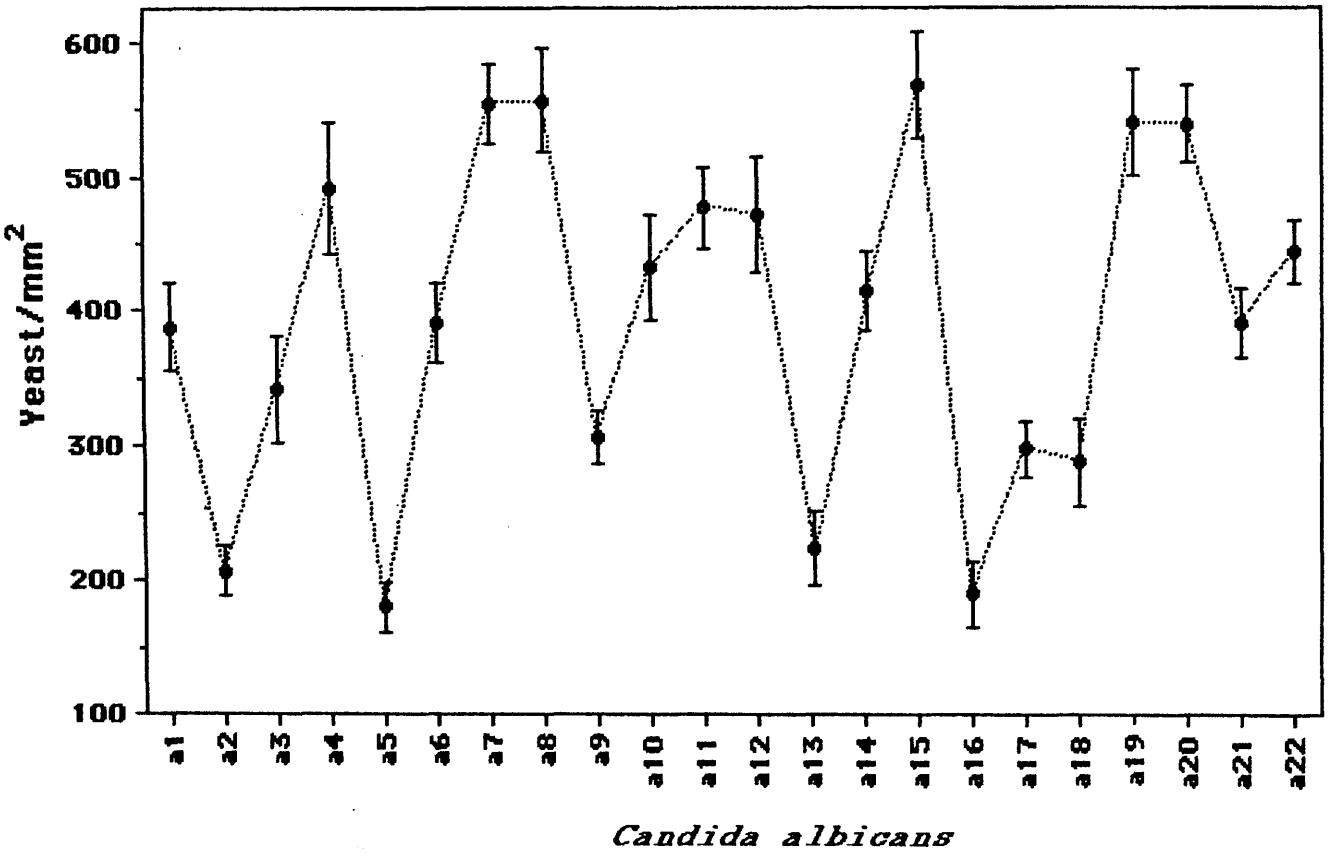


Figure 3.3. Adhesion of 22 *Candida albicans* isolates to acrylic strips. Bars indicate standard error of means

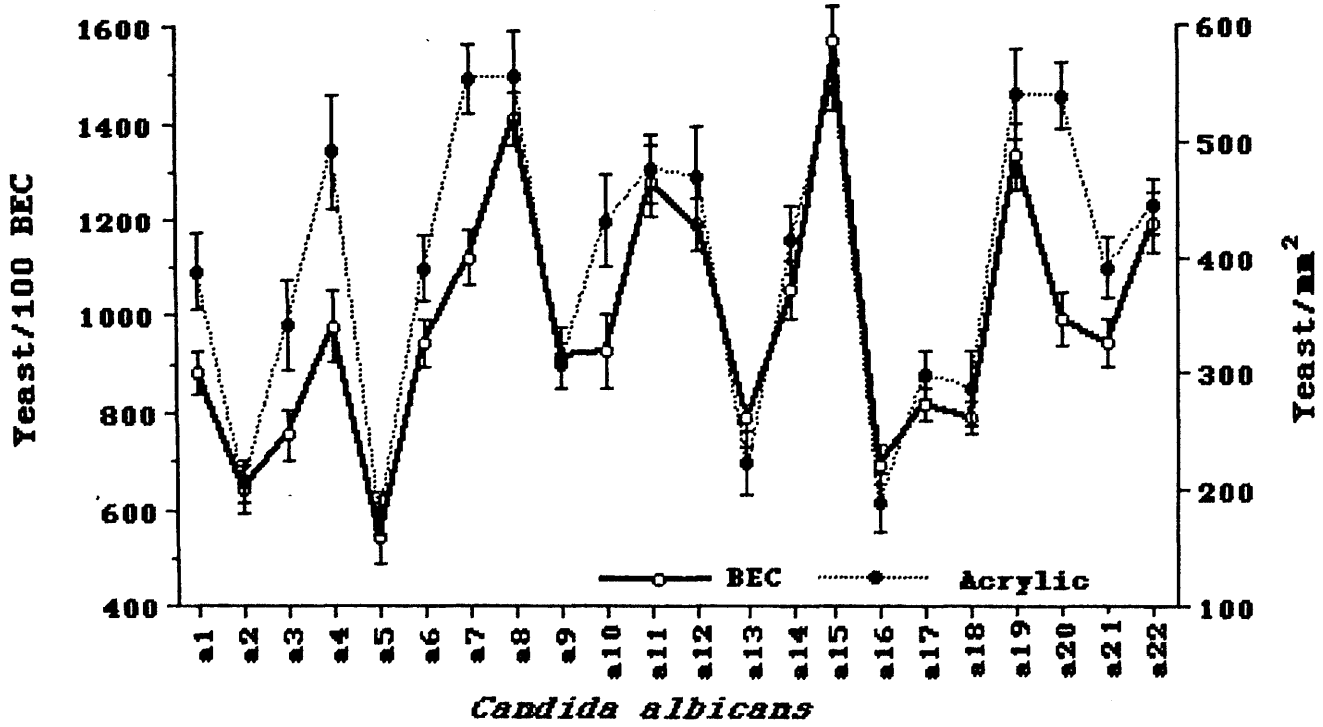


Figure 3.4. A Comparison of adhesion of 22 *Candida albicans* isolates to buccal epithelial cells and acrylic strips. Bars indicate standard error of the mean.

Table 3.4. Adhesion of seven *Candida tropicalis* isolates to 100 buccal epithelial cells after growth in yeast nitrogen base supplemented with 500 mM sucrose, (data from six experiments).

Strain code	Number of yeasts attached to 100 BEC's 1	2	3	4	5	6	Mean	±SEM*
t1	780[a]	660	681	711	539	679	675	32
t2	637	455	522	398	412	515	490	36
t3	310	422	357	441	475	337	390	27
t4	500	368	410	335	316	359	381	27
t5	482	523	412	549	494	690	525	38
t6	645	460	366	431	412	538	475	41
t7	290	347	315	382	269	258	310	20

[a]=Mean of two results.
± SEM= Standard error of the mean.

Table 3.5. Adhesion of seven *Candida krusei* isolates to 100 buccal epithelial cells after growth in yeast nitrogen base supplemented with 500 mM sucrose, (data from six experiments).

Strain code	Number of yeasts attached to 100 BEC's 1	2	3	4	5	6	Mean	±SEM*
k1	105[a]	80	120	96	89	69	93	7
k2	45	75	59	38	29	66	52	7
k3	90	105	82	130	79	92	96	8
k4	130	76	139	102	143	90	110	11
k5	96	85	111	120	83	80	96	24
k6	118	129	86	109	121	70	89	27
k7	115	69	83	92	75	88	87	7

[a]=Mean of two results.
± SEM= Standard error of the mean.

Table 3.6. Adhesion of five *Candida parapsilosis* isolates to 100 buccal epithelial cells after growth in yeast nitrogen base supplemented with 500 mM sucrose, (data from six experiments).

Strain	Number of yeasts attached to 100 BEC's							
code	1	2	3	4	5	6	Mean	±SEM*
p1	180[a]	249	197	393	212	179	235	33
p2	350	278	299	190	240	315	279	23
p3	199	259	387	216	228	230	253	28
p4	390	263	320	250	156	315	282	32
p5	199	305	216	171	412	288	265	36

[a]=Mean of two results.
± SEM= Standard error of the mean.

Table 3.7. Adhesion of five *Candida guilliermondii* isolates to 100 buccal epithelial cells after growth in yeast nitrogen base supplemented with 500 mM sucrose, (data from six experiments).

Strain	Number of yeasts attached to 100 BEC's							
code	1	2	3	4	5	6	Mean	±SEM*
gu1	194[a]	137	213	150	179	148	170	12
gu2	183	179	165	200	198	185	185	5
gu3	196	234	167	211	156	178	190	12
gu4	151	145	182	165	134	123	150	9
gu5	188	152	184	142	129	166	160	10

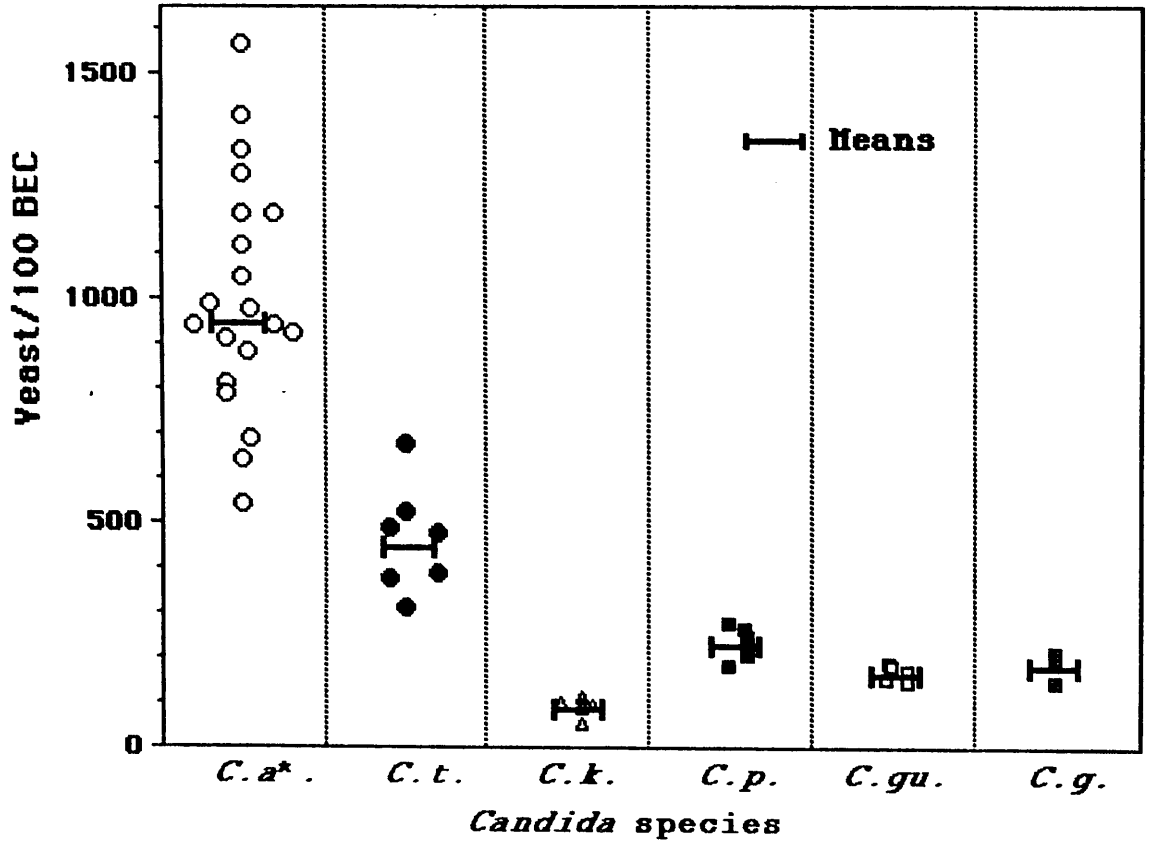
[a]=Mean of two results.
± SEM= Standard error of the mean.

Table 3.8. Adhesion of three *Candida glabrata* isolates to 100 buccal epithelial cells after growth in yeast nitrogen base supplemented with 500 mM sucrose, (data from six experiments).

Strain code	Number of yeasts attached to 100 BEC's 1	2	3	4	5	6	Mean	±SEM*
g1	225[a]	205	184	230	199	219	210	7
g2	122	181	145	158	160	133	150	9
g3	157	173	222	191	168	231	190	12

[a]=Mean of two results.
± SEM= Standard error of the mean.

Figure 3.5. Scatter diagram showing the inter- and intra-species variations in the adhesion of *Candida* species to buccal epithelial cells.



**C.a.=C. albicans, C.t.=C. tropicalis, C.k.=C. krusei, C.p.=C. parapsilosis, C.gu.=C. guilliermondii, C.g.=C. glabrata.*

Table 3.9: Statistical significant intra-species differences‡ in the adhesion of *Candida* species to buccal epithelial cells.

Candida species	probability*
C. tropicalis	
t1 v t7	< 0.001
t4 v t5	< 0.05
t2 v t3	NS
C krusei	
k2 v k4	< 0.05
k5 v k7	NS
k3 v k6	NS
C. parapsilosis	
p1 v p4	NS
p2 v p3	NS
C. guilliermondii	
gu3 v gu4	< 0.05
gu2 v gu5	NS
C. glabrata	
g1 v g2	<0.01

v=versus.

*Examples of possible significant and non-significant combinations.

‡evaluated by *t* test.

Table 3.10. Adhesion of seven *Candida tropicalis* isolates to acrylic strips after growth in yeast nitrogen base supplemented with 500 mM sucrose, (data from six experiments).

Strain code	Number of yeasts attached to mm^2 of acrylic							
	1	2	3	4	5	6	Mean	±SEM*
t1	870[a]	782	850	861	799	810	829	15
t2	676	796	740	756	835	727	755	23
t3	722	567	711	668	681	599	658	25
t4	541	497	532	579	639	510	500	21
t5	748	872	731	826	834	747	793	24
t6	630	724	645	717	683	680	680	15
t7	479	399	515	521	469	437	470	19

[a]=Mean of two results.
± SEM= Standard error of the mean.

Table 3.11. Adhesion of seven *Candida krusei* isolates to acrylic strips after growth in yeast nitrogen base supplemented with 500 mM sucrose, (data from six experiments).

Strain code	Number of yeasts attached to mm^2 of acrylic							
	1	2	3	4	5	6	Mean	±SEM*
k1	142[a]	133	171	110	122	103	130	10
k2	140	114	99	125	68	83	105	11
k3	123	212	135	161	119	150	150	14
k4	135	179	156	217	162	171	170	11
k5	148	125	142	134	199	159	151	11
k6	89	105	149	117	131	90	115	9
k7	105	89	75	110	71	122	95	8

[a]=Mean of two results.
± SEM= Standard error of the mean.

Table 3.12. Adhesion of five *Candida parapsilosis* isolates to acrylic strips after growth in yeast nitrogen base supplemented with 500 mM sucrose, (data from six experiments).

Strain code	Number of yeasts attached to mm^2 of acrylic							
	1	2	3	4	5	6	Mean	±SEM*
p1	422[a]	369	475	286	317	271	357	20
p2	405	410	331	413	372	350	380	14
p3	378	432	399	282	369	465	388	26
p4	424	287	369	330	313	378	350	20
p5	437	346	455	371	398	409	403	17

[a]=Mean of two results.
± SEM= Standard error of the mean.

Table 3.13. Adhesion of five *Candida guilliermondii* isolates to acrylic strips after growth in yeast nitrogen base supplemented with 500 mM sucrose, (data from six experiments).

Strain code	Number of yeasts attached to mm^2 of acrylic							
	1	2	3	4	5	6	Mean	±SEM*
gu1	210[a]	163	191	253	204	230	210	13
gu2	203	189	168	173	234	216	197	10
gu3	210	184	146	197	233	171	190	12
gu4	225	293	217	244	252	270	250	12
gu5	277	301	269	254	235	243	263	10

[a]=Mean of two results.
± SEM= Standard error of the mean.

Table 3.14. Adhesion of three *Candida glabrata* isolates to acrylic strips after growth in yeast nitrogen base supplemented with 500 mM sucrose, (data from six experiments).

Strain code	Number of yeasts attached to mm^2 of acrylic							
	1	2	3	4	5	6	Mean	±SEM*
g1	475[a]	592	436	287	359	448	433	42
g2	286	346	192	281	330	276	285	22
g3	379	266	352	194	378	249	303	32

[a]=Mean of two results.
± SEM= Standard error of the mean.

Table 3.15: Statistical significant intra-species differences‡ in the adhesion of *Candida* species to acrylic strips.

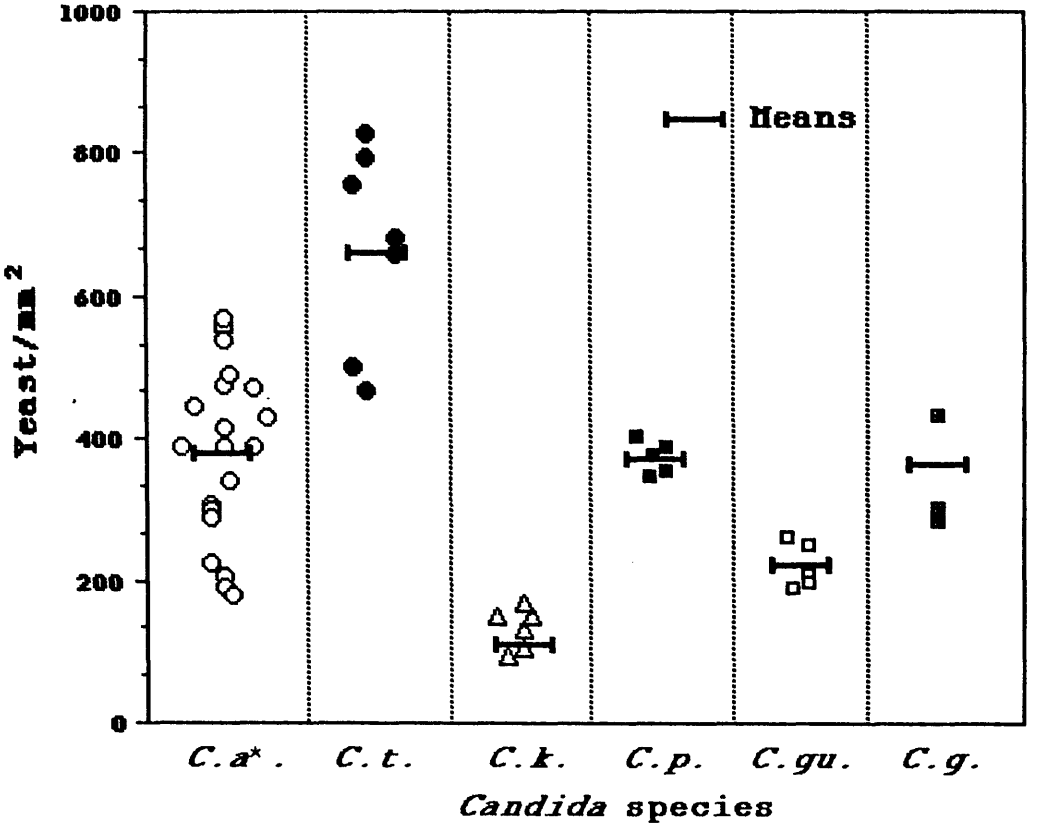
Candida species	probability*	
C. tropicalis		
	t1 v t7	< 0.001
	t4 v t5	< 0.001
	t2 v t3	NS
C krusei		
	k4 v k7	< 0.01
	k2 v k5	NS
	k3 v k6	NS
C. parapsilosis		
	p1 v p3	NS
C. guilliermondii		
	gu2 v gu4	< 0.01
	gu3 v gu5	< 0.01
C. glabrata		
	g1 v g2	< 0.05

v=versus.

*Examples of possible significant and non-significant combinations.

‡evaluated by *t* test.

Figure 3.6. Scatter diagram showing the inter- and intra-species variations in the adhesion of *Candida* species to acrylic strips.



***C.a.=C. albicans, C.t.=C. tropicalis, C.k.=C. krusei, C.p.=C. parapsilosis, C.gu.=C. guilliermondii, C.g.=C. glabrata.**

Table 3.16. Hierarchy of adhesion of *Candida* species to buccal epithelial cells and acrylic surfaces after growth in YNB containing with 500 mM sucrose.

Candida species	Number of isolates	Adhesion to* BEC's+	Acrylic‡
C. albicans	22	988(1)*	395(2)
C. tropicalis	7	464(2)	669(1)
C. parapsilosis	5	262(3)	375(3)
C. glabrata	3	183(4)	340(4)
C. guilliermondii	5	171(5)	222(5)
C. krusei	7	89(6)	131(6)

()*Hierarchy of candidal adhesion.
+Yeast cells/100 BEC's.
‡Yeast cells/mm^2 acrylic strips.

Table 3.17. Statistical analysis of the inter-species variation in the adhesion of *Candida* species to buccal epithelial cells

	*C. a**	*C. t*	*C. k*	*C. gu*	*C. g*	*C. p*
C. a	–	<0.01	<0.001	<0.001	<0.05	<0.001
C. t	<0.01	–	<0.001	<0.005	<0.05	<0.05
C. k	<0.001	<0.001	–	<0.01	<0.005	<0.001
C. gu	<0.001	<0.005	<0.01	–	NS	<0.005
C. g	<0.05	<0.05	<0.005	NS	–	NS
C. p	<0.001	<0.05	<0.001	<0.005	NS	–

**C.a.=C. albicans, C.t.=C. tropicalis, C.k.=C. krusei, C.p.=C. parapsilosis, C.gu.=C. guilliermondii, C.g.=C. glabrata,*
NS=Not Significant.

Table 3.18. Statistical analysis of the inter-species variation in the adhesion of *Candida* species to acrylic strips

	C. a	*C. t*	*C. k*	*C. gu*	*C. g*	*C. p*
C. a	–	<0.05	<0.005	NS	NS	NS
C. t	<0.05	–	<0.001	<0.005	<0.01	<0.005
C. k	<0.005	<0.001	–	<0.005	<0.05	<0.001
C. gu	NS	<0.005	<0.005	–	NS	<0.005
C. g	NS	<0.01	<0.05	NS	–	NS
C. p	NS	<0.005	<0.001	<0.005	NS	–

* *C.a.=C. albicans, C.t.=C. tropicalis, C.k.=C. krusei, C.p.=C. parapsilosis, C.gu.=C. guilliermondii, C.g.=C. glabrata.*
NS=Not Significant.

3.4 Discussion.

The adhesion of *Candida* species to buccal epithelial cells, as well as to acrylic surfaces, has been studied by a number of investigators (Kimura and Pearsall 1978; 1980; Howlett and Squier 1980; King *et al.*, 1980; Samaranayake *et al.*, 1980; Makrides and MacFarlane 1982; Sandin *et al.*, 1982, 1987; Centeno *et al.*, 1983; Cox 1983; Davidson *et al.*, 1984; Calderone *et al.* 1984; Kennedy and Sandin 1988). However, these studies have used only a few strains of *C. albicans* or other *Candida* species compared with the current study, where 22 strains of *C. albicans* and 27 isolates of five other *Candida* species were used.

3.4.1.The influence of culture media and carbohydrates on candidal adhesion.

Many factors have been shown to influence the adhesion of *C. albicans* to both biological and non-biological surfaces *in vitro*. Of those factors, growth conditions (especially, the presence of certain carbohydrates) are probably the most important. Recently Kennedy and Sandin (1988) investigated the adhesion to BEC's of a single strain of *C. albicans* cultivated in 13 media (10 complex, and three synthetic), with varying growth conditions (e.g., temperature). The report indicated that cells grown in undefined media at 37°C were usually less adhesive than those cells grown in defined media at the same temperature. However, for one defined medium (YNB) the production of highly adhesive *Candida* cells was dependent on the type of carbohydrate used in the medium. In general they reported that higher adhesion values

were observed when the yeast cells were grown in defined media (depending on the carbohydrate used) and/or at 25°C. For instance, for one defined medium (YNB) the production of highly adhesive *Candida* cells was dependent upon the type of carbohydrate used. For example, galactose produced a higher number of adhering cells than glucose. Moreover, significant differences in adhesion to BEC's were noted when *C. albicans* was grown in the same complex medium from different manufacturers and in different batches of medium from the same manufacturer. A similar finding has been reported by Odds, Hall and Abbott (1978).

In the current study, the defined medium, yeast nitrogen base supplemented with 500 mM sucrose was used to cultivate *Candida* isolates for adhesion assays. Sucrose was used instead of galactose in this study to compare the adhesion of yeast strains to the test surfaces, because it is the most commonly consumed dietary sugar, and most of strains tested were isolated from the human mouth.

Differences in the ability of the same *Candida* isolates to attach to both BEC's and acrylic surfaces have been noted when cells of *C. albicans* were cultivated in different carbohydrates such as galactose, sucrose, and maltose (Douglas, Houston and McCourtie 1981; Samaranayake and MacFarlane 1982b). It has also been suggested that the adhesion of pathogenic species but not isolates from healthy carriers was enhanced by high sugar concentrations (Critchley and Douglas 1985, for further details see. 3.4.11).

3.4.2 Adhesion of *Candida albicans* to Buccal epithelial cells.

Although BEC's have been commonly used in studying the adhesion of *Candida*, it is recognized that care must be taken in standardizing the epithelial cells in such assays. Substantial day-to-day variation in the adhesion of a single strain of *C. albicans* to BEC's collected from the same donor have been reported (Kearns, Davies and Smith 1983; Sandin *et al.*, 1987; Tobgi, Samaranayake and MacFarlane 1987; and Kennedy and Sandin 1988). It seems likely that much of this is due to variations in the surface structure of the epithelial cells. However, BEC's are usually chosen for *in vitro* assays because they are derived from a natural mucosal surface, and are easily obtained and maintained for adhesion investigations. Therefore, possible variations in BEC's have been minimized as far as possible, in this study by collecting the cells from one donor at the same time each day (i.e., before breakfast). However, in spite of these precautions, small differences were found between the adhesion of the same isolate of *C. albicans* to the BEC's from the same donor on different occasions as shown in Table 3.1. Nevertheless, such variations in adhesion were less in acrylic studies (Table 3.3). Due to these variations in adhesion of *Candida* to BEC's and differences in growth conditions and media compositions, used by different workers meaningful comparisons between adhesion studies are difficult. In addition there are very few comparable studies in the literature which have

investigated a similar number of strains.

While accepting the problems with the BEC's adherence assay discussed above, it is still worth comparing the results of this study with that of others. McCourtie and Douglas (1984) studied the adhesion of nine *C. albicans* strains grown in YNB supplemented with 500 mM sucrose, to BEC's. Seven strains of the above nine were also used in the current study and were grown under the same conditions, although the source of the BEC's was different. A comparison of the results of two studies are shown in Table 3.19, and Figure 3.7, and a number of differences are evident. In general, the values obtained by this study were higher than those of McCourtie and Douglas (1984). Variations occurred not only in the extent of adhesion but were also seen in the rank order of *Candida* isolates. For instance, GDH 2036 (a2), was ranked sixth in the current study, but was first in their study. However, the rank of the other strains were somewhat similar. These variations may be related to the fact that different epithelial cell donors were used and the cells had varying susceptibility to yeast adhesion. The yeast cells used in the last two assays, were grown in YNB supplemented with 500 mM sucrose. Barrett-Bee *et al.* (1985) investigated the adhesion of four *C. albicans* isolates grown in Sabouraud's dextrose broth to BEC's. Their adhesion results ranged from 117-1174 yeast cells/100 BEC's. Similarly, in the current study variations in the adhesion were also observed and ranged between 542-1569 yeast cells/100 BEC's. Interestingly, the same strain (NCPE, 3153) used by McCourtie and Douglas (1984), was also used by both

Barrett-Bee *et al.*, (1985) and in this study. The results obtained are; 210 (McCourtie and Douglas 1984), 1174 (Barrett-Bee *et al.*, 1985) and 913 yeast cells/100 BEC's by the current study. Ghannoum *et al.*, (1986) investigated the adhesion of three strains of *C. albicans* to BEC's, grown in YNB supplemented with 2.5 percent glucose, and in contrast to the results of the present study and those of other workers, the range of adhesion was very similar, 523, 570 and 565 yeast cells/100 BEC's.

3.4.3. Adhesion of *Candida albicans* to acrylic surfaces.

Chronic atrophic candidosis is a common complication in elderly denture wearers. This suggests that colonization of the acrylic denture surfaces serves as a reservoir for infection. Adhesion of *C. albicans* to non-biological materials has been studied *in vivo* and *in vitro* and attachment of *C. albicans* to acrylic strips, has been examined to help elucidate the early events in the pathogenesis of denture related candidosis. Therefore, Samaranayake and MacFarlane (1980) devised a method to measure adhesion of *C. albicans* to denture acrylic surfaces. However, comparatively few studies (McCourtie and Douglas 1981; 1984; Miyake *et al.*, 1986; Samaranayake and MacFarlane 1980; Samaranayake, McCourtie and MacFarlane 1980) have investigated the adhesion of a large number of *C. albicans* isolates to acrylic surfaces, despite the potential relevance of such adhesion for denture stomatitis.

Although the adhesion assay using acrylic strips is relatively simple, care must be taken to ensure that strips are maintained vertically in the wells of the flat-bottomed plastic serology plates used in the assay. These is a tendency for the strips to either float on the surface of the yeast suspension or lie horizontally in the bottom of the plastic well. In the first case, few yeasts will attach to the strip whereas, in the second case heavy attachment may well occur.

McCourtie and Douglas (1981) have studied the adhesion to acrylic strips, of three *C. albicans* strains (the same strains used also in the current investigation) and grown in yeast nitrogen base supplemented with 500 mM sucrose. Table 3.20 compares the results obtained by both McCourtie and Douglas (1981), with those described in this study. Although, the results of the current investigation display a higher level of adhesion, both studies show the same rank order of adhesion by strains to acrylic strips. The difference between the two studies could be due to variation in media (YNB media were supplied by Difco, in both studies), although this is unlikely. Another possibility is the difference in the concentration of yeast suspensions used which were 0.5 to 1.0×10^6 in the McCourtie and Douglas (1981) study and 4.0 to 4.5×10^7 cells/ml in the current study. In a subsequent study McCourtie and Douglas (1984) investigated the adhesion to acrylic strips of nine *C. albicans* strains grown in YNB supplemented with 500 mM sucrose, the yeast cell count in both studies being about 1.2×10^7 cells/ml. Seven strains of the above

nine were also used in the current study and grown under the same conditions. The carbohydrate used in both studies was 500 mM sucrose. The results of the two studies are compared in Table 3.21. Most of the strains show a similar level of adhesion to the acrylic in both investigations, and the differences in the ranked order of the strains were slight.

3.4.4. Adhesion of *Candida albicans* to epithelial cells and acrylic surfaces.

The current study shows that most *C. albicans* strains behave in a similar manner in attaching to both the acrylic and BEC's surfaces (Figure, 3.4). For instance, *C. albicans* strain a15 gave the highest mean adhesion values to BEC's as well as acrylic, whereas isolate a5 gave the lowest mean adhesion values, compared with other isolates used. However, a few strains (e.g., a4 and a20, Table, 3.22) reacted differently and scored a higher value in the ranked order for adhesion to acrylic compared with BEC's. In general, however, the standard error of the mean in the acrylic studies was less than that for BEC's when overall adhesion results were compared ($p < 0.001$)

The only study that can be compared with the current investigation is that of McCourtie and Douglas (1984). They compared the adhesion of nine strains of *C. albicans* grown in YNB supplemented with 500 mM sucrose to both BEC's and acrylic surfaces. A comparison of the results are shown in Table 3.23 and Figure, 3.7. The mean ratio of *C. albicans* adhesion to BEC's

compared with acrylic strips of the present study (BEC's : Acrylic = 2.85) was higher than that reported in the McCourtie and Douglas study (BEC's : Acrylic = 1.16). No significant difference was found between the two studies as far as adhesion to acrylic was concerned. A highly significant difference ($p<0.001$) was obtained for the adhesion to BEC's when the two studies were compared. The adhesion values obtained in the current study were higher than those reported by McCourtie and Douglas (1984). These results show a significant difference in the adhesion of these nine strains of *C. albicans* to BEC's but not to acrylic strips. From these results one may conclude that acrylic strips are more stable for adhesion assays than BEC's. This is not unexpected as acrylic is an inert, non-biological substrate compared with BEC's whose surface composition may vary diurnally as well as among donors.

3.4.5. Mechanisms of candidal adhesion to buccal epithelial cells and acrylic surfaces.

The mechanisms responsible for attachment of *Candida* to buccal epithelial cells and acrylic strips are not fully understood. Jones (1984) has described specific adhesion as a two-component system that depends as much on the number and distribution of host surface receptors as it does on the extent of adhesin production by the microorganism. There is now considerable evidence that surface mannoprotein is responsible for the attachment of *C. albicans* to epithelial cells (Douglas 1985, 1987; Kennedy 1987). However, ultrastructural studies do

suggest that at last two morphological surface structures of *C. albicans* normally exist, floccular and fibrillar, and it is very likely, that these structures represent distinct adhesins (Kennedy 1987). It has been found that the floccular outer layer mediates the adhesion of *C. albicans* to oral mucosal cells (Howlett and Squier 1980; Douglas, Houston and McCourtie 1981; McCourtie and Douglas 1981). More recently, Critchley and Douglas (1987a), reported the partial purification of an adhesin from extracellular polymeric material isolated from yeast culture supernatants. These studies provide evidence that the protein portion of the mannoprotein adhesin is more important than the carbohydrate moiety in mediating attachment to buccal cells. Moreover, the same workers, (1987b) studied the effect of various lectins and sugars on the adhesion of *C. albicans* to buccal epithelial cells and indicated that there are at least two types of adhesion mechanisms and that glycosides containing **L**-fucose or **N**-acetyl-**D**-glucosamine can function as epithelial cell receptors for *C. albicans*.

Miyake *et al.*, 1986 found a strong correlation existed between the adhesion capacities of *Candida* species to acrylic surfaces and hydrophobicity. However, ion-bridging bonds have also been suggested to play a role in the adhesion of *C. albicans* to acrylic (McCourtie and Douglas 1981). McCourtie and Douglas (1981), reported that the addition of divalent cations e.g., calcium (Ca^{+2}), to assay mixtures caused an increase in *Candida* adhesion to acrylic. Thus, ion-bridging mechanisms, in addition to cell surface hydrophobicity and other unknown factors (Rotrosen

et al., 1986), may play a role in the adhesion of *C. albicans* to acrylic surfaces.

3.4.6. Adhesion of *Candida* species (other than *Candida albicans*) to buccal epithelial cells.

There is evidence from a number of experimental animal studies for a hierarchy of relative virulence among the pathogenic *Candida*; species, *C. tropicalis* isolates are usually pathogenic, *C. parapsilosis* emerges as low-grade pathogen, *C. pseudotropicalis* (*C. kefyr*) occasionally causes morbidity, while *C. glabrata, C. guilliermondii* and *C. krusei* seldom reveal pathogenic attributes (Odds 1988). Differences in virulence may be reflected in the ability of an organism to adhere to human epithelial cells *in vivo*. The results of this study revealed that the order of mean adhesion values for *Candida* species, (other than *C. albicans*) to 100 BEC's was as follows: *C. tropicalis* 464, *C. parapsilosis* 263, *C. glabrata* 183, *C. guilliermondii* 171, and *C. krusei* 89, (Table 3.18). This is in agreement with the order of hierarchy of relative virulence among pathogenic *Candida* species as described above. King Lee and Morris (1980), investigated the *in vitro* adhesion capabilities of five *Candida* species cultivated in 1 per cent phytone peptone broth supplemented with a final concentration of 1 mg of glucose. They concluded that *C. tropicalis* and *C. stellatoidea* isolates adhered in moderate numbers, *C. parapsilosis* adhered only to a slight degree to BEC's and the other species (*C. guilliermondii, C. krusei* and *C. pseudotropicalis*) failed to adhere to isolated

BEC's. Ray *et al*, (1984) investigated five *Candida* species (*C. stellatoidea*, *C. tropicalis*, *C. parapsilosis*, and *C. krusei*), grown in Sabouraud's broth at 25°C for adhesion to BEC's and reported that *C. stellatoidea* exhibited marked adhesion and was pathogenic in a rodent experimental model. The remaining species showed little or no adhesion, and were nonpathogenic in animal experiments. In another study (Critchley and Douglas 1985), a single strain of *C. tropicalis*, showed substantially increased adhesion to buccal epithelial cells after growth in YNB supplemented with 500 mM galactose, compared with the control where the same isolates grown in YNB was supplemented with only 50 mM glucose. A second strain of *C. tropicalis* as well as single strains of *C. stellatoidea*, *C. parapsilosis*, *C. pseudotropicalis*, and *C. guilliermondii* grown in the same medium showed little or no increased adhesion to BEC's compared with the control (50 mM glucose).

3.4.7 Adhesion of all *Candida* species to buccal epithelial cell's.

In the present study, the adhesion of *C. albicans*, to BEC's (the species most often implicated in human infection), was significantly greater than was the adhesion of the other *Candida* species (including *C. tropicalis*, a pathogen of increasing clinical importance) to buccal epithelial cells (Figure 3.5). King, *et al.*, (1980) reported that *C. albicans* was the most adherent species to BEC's, followed by *C. tropicalis*, *C. stellatoidea*, while *C. parapsilosis* adhered only to a slight degree. However,

in other study (Ray *et al.*, 1984), it was reported that only *C. albicans* and *C. stellatoidea* when grown in Sabouraud's broth at 25°C, exhibited marked adhesion to BEC's, whereas *C. tropicalis*, *C. parapsilosis*, *C. guilliermondii* and *C. krusei* showed little or no adhesion. Barrett-Bee *et al.*, (1985) investigated two *Candida* species (4 strains of *C. albicans*, and one strain of *C. parapsilosis*, grown in Sabouraud's dextrose agar) and found that the adhesion of the *C. albicans* isolates to BEC's to be far greater than that of *C. parapsilosis* Critchley and Douglas (1985) studied the adhesion of six *Candida* species, and found that when *Candida* was grown in YNB supplemented with 50 mM glucose, the subsequent adhesion was in the following order: *C. tropicalis*, *C. albicans*, *C stellatoidea*, *C. parapsilosis*, *C. guilliermondii* and *C. pseudotropicalis*. Interestingly, when these strains were grown in YNB containing 500 mM galactose their order was changed and *C. albicans* adhered in greater numbers than the other species. Table 3.24, shows the ranked order of adhesion of *Candida* species to BEC's obtained by the current study and that produced by Critchley and Douglas, (1985). Both studies used YNB supplemented with 500 mM sucrose. There is similarity in both studies in the descending rank order of adhesion to BEC's, of the four *Candida* species. In general, the figures obtained in this study revealed adhesion values which were a little higher than those reported by Critchley and Douglas (1985).

3.4.8.Adhesion of *Candida* species (other than *Candida albicans*) to acrylic.

Very few studies have reported the adhesion of *Candida* species to non-biological surfaces. In the present study, the adhesion to acrylic strips of *C. tropicalis*, the pathogen of increasing clinical importance, was significantly greater than the adhesion of other *Candida* species (including *C. albicans*, the species most often implicated in human infection, Figure, 3.6). The hierarchy of adhesion of *Candida* species to acrylic strips produced by this study was in the following order: *C. tropicalis*, *C. parapsilosis*, *C. glabrata*, *C. guilliermondii*, and *C. krusei*. (Table, 3.16). Critchley and Douglas (1985) studied the adhesion of six *Candida* species to acrylic strips, and reported that the adhesion order was *C. tropicalis*, *C stellatoidea*, *C. parapsilosis*, *C. guilliermondii* and *C. pseudotropicalis*. In an other study, Miyake *et al* (1986) investigated the adhesion of seven laboratory strains and 18 clinical isolates of *Candida* species, grown on Sabouraud's glucose agar. The rank order produced by Miyake *et al.* (1986) was as follows: *C. tropicalis*, *C. glabrata*, *C. krusei*, *C. guilliermondii*, *C. pseudotropicalis*, and *C. parapsilosis*. The conflicting results (Table, 3.25) obtained by these three studies may be explained by some of the technical problems discussed earlier.

3.4.9 Adhesion of all *Candida* species to acrylic.

Minagi *et al.*,(1985) correlated the adhesion of *Candida* species to denture resin materials with varying degrees of hydrophobicity, and reported that *C. tropicalis* was more adherent than *C. albicans* to a variety of denture base materials. They concluded that *C. tropicalis* bound preferentially to surfaces of increasing hydrophobicity compared with other *Candida* species. On the other hand, McCourtie and Douglas (1981 and 1984) found that the adhesion of *C. albicans, C. pseudotropicalis, C. stellatoidea* or *C. guilliermondii* strains cultured in 50 mM glucose, were considerably less adherent than strains of *C. tropicalis* or *C. parapsilosis* to acrylic strips (Critchley and Douglas, 1985). Interestingly, Miyake *et al* (1986) reported that the adhesion of *C. albicans* cells to acrylic surfaces was less than that of the other six *Candida* species grown on Sabouraud's glucose slopes. Table 3.25, shows the hierarchy of adhesion obtained from three different studies. The observed diversity in the adhesion of these species to acrylic is not only in number of attached cells but also in the rank order; for instance, *C. albicans* was ranked first by Critchley and Douglas (1985), second by the current study, and last in the work reported by Miyake *et al* (1986). The reasons for these differences between the above studies are not clear, although the medium used by Miyake, *et al.*, (1986) (ie., Sabouraud's glucose agar) is different from those used by others.

3.4.10. Adhesion of all *Candida* species to both epithelial cells and acrylic surfaces.

Critchley and Douglas (1985) studied the adhesion of six *Candida* species (two strains of *C. tropicalis* and a single strain of *C. albicans, C. stellatoidea, C. parapsilosis, C. pseudotropicalis* and *C. guilliermondii*) to both epithelial cells and acrylic surfaces. They found that the adhesion of *C. albicans,* grown in YNB containing 500 mM galactose, to BEC's or to acrylic strips *in vitro* was promoted. However, only a single strain of *C. tropicalis* showed substantially increased adhesion to buccal cells (but not to acrylic surfaces) after growth in YNB supplemented with 500 mM galactose. However, after growth in YNB containing 50 mM glucose, *C. tropicalis and C. parapsilosis* were significantly more adherent to acrylic than the glucose-grown *Candida* species, including *C. albicans.* The order of adhesion of *Candida* species to both surfaces was compared in the current study, and revealed that the mean adhesion values of *C. albicans* to BEC's was much greater than other species whereas that of *C. tropicalis* was greater to acrylic surfaces (Table 3.16). Statistical analysis of the results within the *Candida* species to BEC's and to acrylic strips are shown in Tables, 3.17 and 3.18 respectively. It is difficult to compare the adhesion of each isolate to BEC's and acrylic as the measurement units used in the studies were different, (ie., adhesion to 50 BEC's and yeast/mm^2 of acrylic).

3.4.11. A comparison of the adhesion results of strains from active infections with those from asymptomatic carriers.

McCourtie and Douglas, (1984).studied the adhesion and virulence of nine strains of *C. albicans*, seven strains of which were isolated originally from active infectious and two strains were isolated from asymptomatic carriers. The adhesion of *C. albicans* strains isolated from active infections, grown in either 500 mM sucrose or galactose was enhanced up to 5 and 11 fold, respectively, whereas, strains isolated from asymptomatic carriers grown in the same sugars, showed only small increases in adhesion. They also demonstrated that the adhesion of yeasts grown in 500 mM galactose showed marked variations between the infectious and asymptomatic strains whereas sucrose grown strains displayed less considerable variations in contrast with galactose. Furthermore, they stated that isolates from both sources grown in 50 mM glucose, did not show significant variations in the adhesion to the BEC's. McCourtie and Douglas (1981 and 1984) also reported that the adhesion of 'infectious' strains of *C. albicans* to acrylic surfaces was greatly promoted by growth in YNB medium supplemented with 500 mM galactose. Although, it is beyond the scope of this study to investigate the variations in strains isolated from different sources, the findings of the current study show that the results for *Candida* strains from candidosis patients tend to give higher values compared with the few isolates from asymptomatic carriers

(Figure, 3.8). Some strains, (e.g., a13), cultured from a patient with chronic atrophic candidosis, produced a mean count of 791 yeasts/100 BEC's, whereas, strain a2, isolated from an asymptomatic individual, produced 642 yeasts/100 BEC's (Table 3.17). Kearns Davies and Smith (1983) investigated the adhesion of ten *C. albicans* isolates and detected small differences in adhesion between four laboratory strains of *C. albicans* that differed in virulence for mice, also between three of subcultured isolates from cases of oral thrush and between three isolates from the mouths of healthy carriers; all isolates being cultured directly on Lee's agar containing glucose (the concentration of the glucose was not recorded). However, Kearns *et al.*, stated that the differences were small and did not consistently occurred. Barrett-Bee *et al.*, (1985) investigated the adhesion to BEC's of four strains of *C. albicans*; two reference laboratory strains were isolated from symptomatic patients and the other two were isolated from the mouth of an asymptomatic carrier. The yeasts were grown in Sabouraud's dextrose agar and subsequent adhesion studies failed to discriminate between the asymptomatic and infective strains.

Variations between the results of these and other investigations quoted above reveals that a standard test assay is greatly needed to overcome the problems due to the use of different methods by different workers.

3.4.12. Conclusion.

The results of this study reveals extreme variations in the adhesion of different *C. albicans* isolates either to BEC's or acrylic surfaces. However, the former surface shows higher variations.

Candida albicans demonstrated higher affinity to BEC's than other *Candida* species, whereas, *C. tropicalis* showed elevated adhesion to acrylic strips. Furthermore, *C. albicans* *C. tropicalis* and *C. parapsilosis* demonstrated a higher significant differences ($p<0.001$) in their adhesion to BEC's and acrylic surfaces, while other species show less significant differences ($p<0.05$).

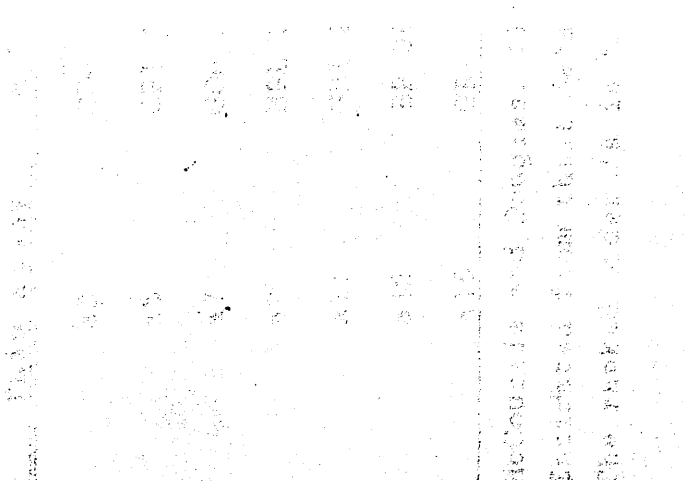


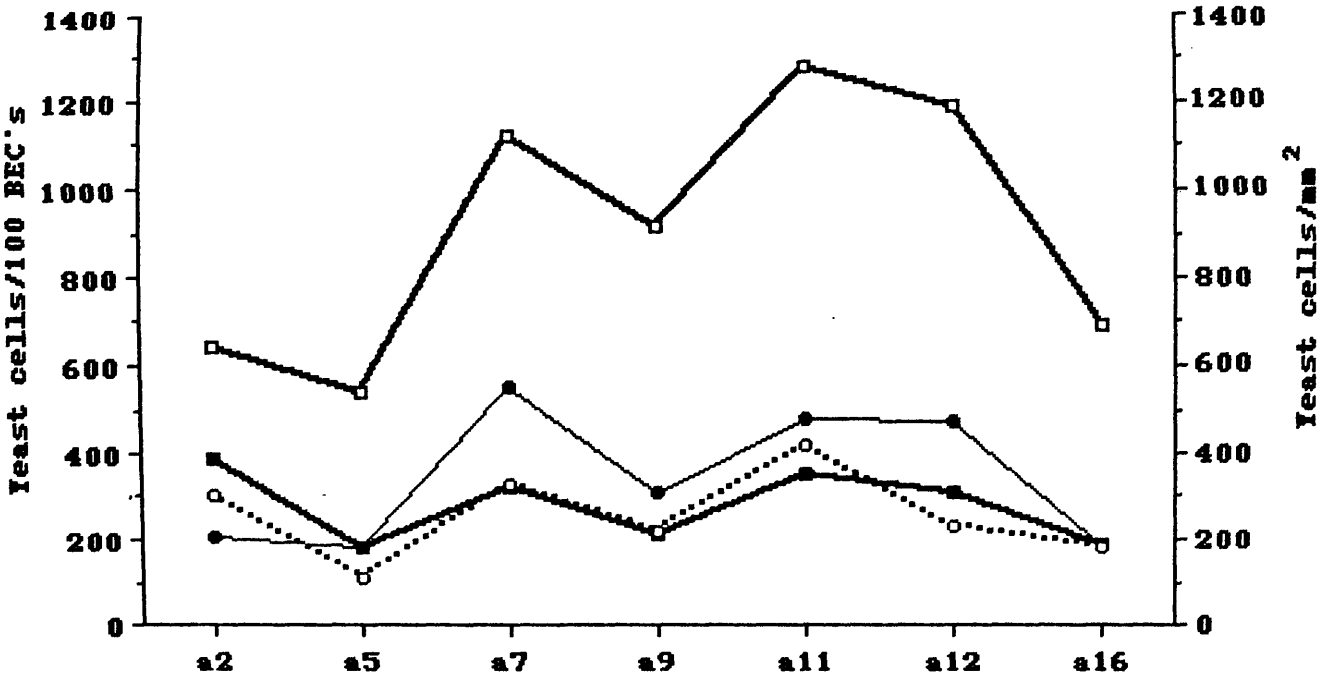
Table 3.19: A comparison of the adhesion of *Candida albicans* strains (grown in 500 mM sucrose) to different buccal epithelial cells, used in this study, and the study by McCourtie and Douglas, (1984) (M&D).

Code used		Results	
This study	M & D*	This study	M & D*
a2	GDH 2036	642(6)+	390‡(1)
a5	GRI 681	542(7)	180(7)
a7	GDH 2346	1119(3)	320(3)
a9	MRL 3153	913(4)	210(5)
a11	GRI 2773	1279(1)	350(2)
a12	BP 3968	1189(2)	310(4)
a16	GRI 682	691(5)	190(6)

*McCourtie and Douglas, (1984).

‡Estimated from their data.

+The ranked order is in brackets.



Candida albicans

Figure, 3.7: The adhesion of seven strains of *C. albicans* grown in 500 mM sucrose containing YNB, to BEC's —□— and acrylic strips —●— this study and to BEC's —■— and acrylic strips ·····o····· McCourtie and Douglas study (1984).

Table 3.20: A comparison of the adhesion of *Candida albicans* strains (grown in 500 mM sucrose) to acrylic strips, used in this study and the study by McCourtie and Douglas, (1981).

Code used		Results	
This study	M & D*	This study	M & D*
a7	GDH 2346	554	348
a9	MRL 3153	307	240
a12	BP 3968	471	249

*McCourtie and Douglas, (1981).

Table 3.21: A comparison of the adhesion of *Candida albicans* strains (grown in 500 mM sucrose) to acrylic strips, used in this study and the study by McCourtie and Douglas, (1984).

Code used		Results	
This study	M & D*	This study	M & D*
a2	GDH 2036	207(5)+	300‡(3)
a5	GRI 681	180(7)	110(7)
a7	GDH 2346	554(1)	330(2)
a9	MRL 3153	307(4)	220(5)
a11	GRI 2773	477(2)	420(1)
a12	BP 3968	471(3)	230(4)
a16	GRI 682	190(6)	180(6)

*McCourtie and Douglas, (1984).

‡Estimated from their data.

+The ranked order is in brackets.

Table 3.22. The ranked order of *Candida albicans* adhesion to buccal epithelial cells and acrylic strips.

	Adhesion of *Candida albicans* to				
	BEC's‡	Acrylic*		BEC's‡	Acrylic*
a1	883(15)	388(14)	a12	1189(6)	471(8)
a2	642(21)	207(20)	a13	791(17)	224(19)
a3	754(19)	342(15)	a14	1051(8)	415(11)
a4	977(10)	492(6)	a15	1569(1)	568(1)
a5	542(22)	180(22)	a16	691(20)	190(21)
a6	942(11)	391(12)	a17	816(16)	298(17)
a7	1119(7)	554(3)	a18	790(18)	288(18)
a8	1410(2)	557(2)	a19	1331(3)	540(4)
a9	913(14)	307(16)	a20	990(9)	539(5)
a10	924(13)	432(10)	a21	942(12)	391(13)
a11	1279(4)	477(7)	a22	1191(5)	444(9)

‡Yeast/100 BEC's *Yeast/mm^2

Table 3.23: The adhesion of *Candida albicans* strains (grown in 500 mM sucrose) to acrylic strips, used in this study and the study by McCourtie and Douglas, (1984).

Code used by		Adhesion of *C. albicans* to BEC's		Acrylic	
This study	M & D*	This study	M & D*	This study	M & D*
a2	GDH 2036	642(6)	390‡(1)	207(5)	300‡(3)
a5	GRI 681	542(7)	180(6)	180(7)	110(7)
a7	GDH 2346	1119(3)	320(3)	554(1)	330(2)
a9	MRL 3153	913(4)	210(5)	307(4)	220(5)
a11	GRI 2773	1279(1)	350(2)	477(2)	420(1)
a12	BP 3968	1189(2)	310(4)	471(3)	230(4)
a16	GRI 682	691(5)	190(7)	190(6)	180(6)

*McCourtie and Douglas, (1984).

‡Estimated from their data.

The ranked order is in brackets.

Table 3.24: The ranked order of adhesion to BEC's of *Candida* species produced by two studies using 500 mM of sucrose (this study and the study by C.D.‡ studies), are shown between brackets.

***Candida* species**	**This Study**	**R.O.**	**C.D. Study**	**R.O.**
C. albicans	988◊	1	792◊	1
C. tropicalis	464	2	358*	2
C. parapsilosis	262	3	264	3
C. guilliermondii	171	5	35	4
C. glabrata	183	4	-	
C. krusei	89	6	-	

‡ Critohley and Douglas, 1985.

*Mean results of two strains.

◊Yeast cells/100 BEC's.

R.O=The ranked order.

Table 3.25: The ranked order of adhesion to acrylic surfaces of *Candida* species produced by three studies using 500 mM of sucrose (this study and C.D.‡ study) and Sabouraud's glucose agar (Miyake *et al.*, 1986).

Candida species	This study		C.D study		Miyake *et al.*, study	
	Mean	R.O.	Mean	R.O.	Mean¤	R.O.
C. albicans	395◊	2	1088◊	1	114.9*	6
C. tropicalis	669	1	399*	2	1465.2	1
C. parapsilosis	375	3	184	3	501.2	5
C. guilliermondii	222	5	103	4	707.8	4
C. glabrata	340	4	-		1096.8	2
C. krusei	131	6	-		956.6	3

‡ Critchley and Douglas, 1985.

*Mean results of two strains.

¤Mean number of adherent cells/ten high power fields.

◊Yeast cells/mm^2.

R.O=The ranked order.

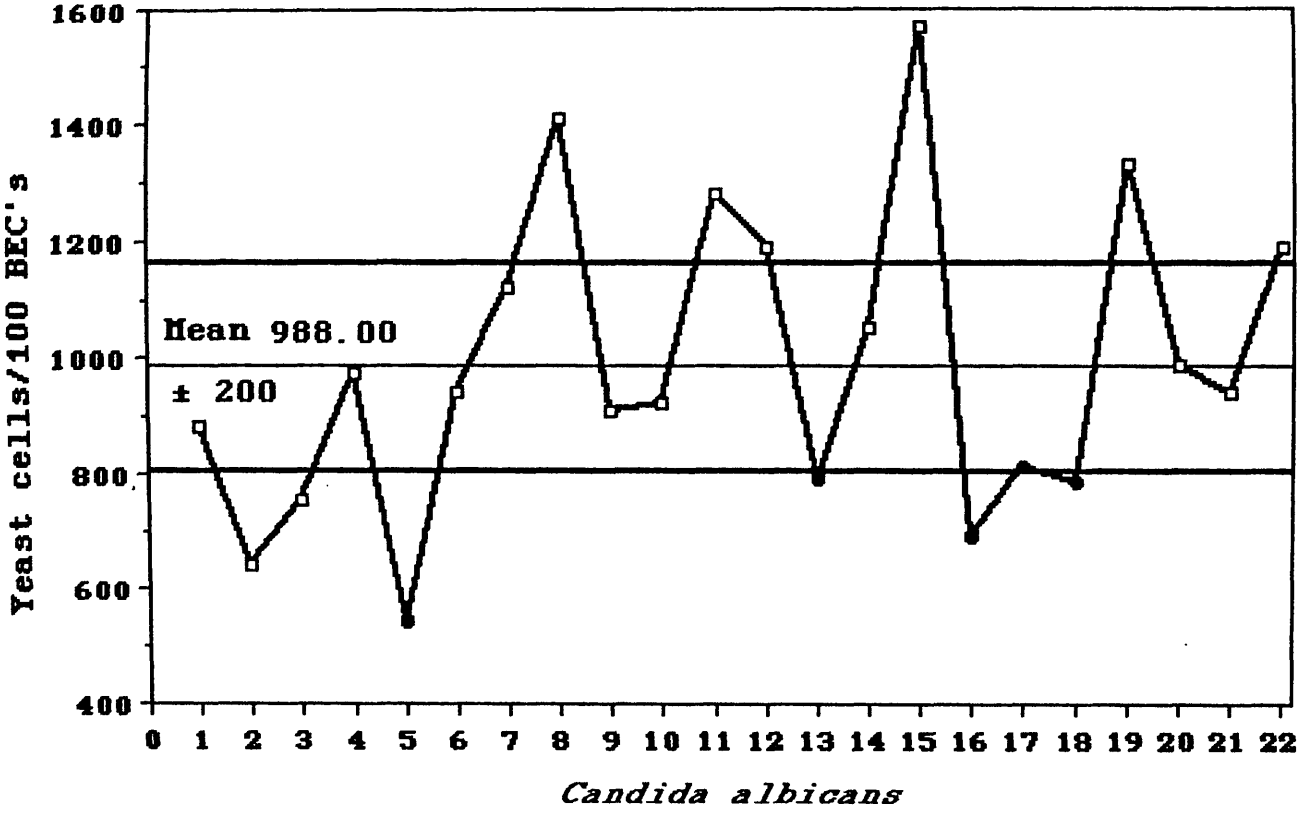


Figure 3.8. The BEC adhesion of *Candida albicans* strains isolated from asymptomatic carriers • and from candidosis patients ▫. Note that the adhesion of most of asymptomatic carrier (•) isolates, is below 200 of the mean.

CHAPTER FOUR

THE POTENTIAL OF *Candida* SPECIES TO PRODUCE PHOSPHOLIPASES AND PROTEASES.

4.1. Introduction.

There are a number of studies on the pathogenesis of *Candida* infections, which focus on the problem of the product(s) responsible for the initiation and progression of infection (Chattaway, Odds and Barlow 1971; Iwata 1977; Cutler *et al.* 1972). For instance, Odds (1988), has named more than 40 enzymes, which have an obvious role in yeast metabolism as catabolic enzymes. Of these, phospholipases and proteinases, in particular, have been implicated in the mechanisms of *C. albicans* pathogenicity (Staib 1967; Samaranayake, Raeside and MacFarlane 1984; Rüchel, Böning and Borg 1986).

Phospholipase is known to degrade phospholipids, the essential structural components of cell walls and intracellular membranes. It is generally accepted that these membranes are not static and that there is a constant metabolic turnover of phospholipids with subsequent production of lysophospholipid. Phospholipase is known to damage cell membranes. Phospholipases are an active component of snake venoms (Volwerk and Haas 1982), and are frequently found associated with cell membranes and the membrane-bound vesicles of yeasts (Odds 1988).

The activity of phospholipase in *C. albicans* was first demonstrated by Costa *et al.*, (1967) and subsequently, the same

authors found phospholipase activity in many pathogenic strains of *C. albicans*. This group of investigators also identified two enzyme activities, phospholipase A and phospholipase C in *C. albicans*, whereas no evidence was found for the presence of phospholipase B and D. A few years later, Pugh and Cawson (1975) found that *C. albicans* also possessed another enzyme, lysophospholipase, which has the ability to act on lysolecithin. However, the phospholipase activity of the different candidal isolates could not be easily compared due to the lack of a simple quantitative assay technique. In 1982 Price Wilkinson and Gentry, demonstrated a simple plate method, for the detection of the phospholipase activity of *C. albicans*, using Sabouraud's dextrose agar, supplemented with egg yolk. This method has been used to study the effect of the pH and carbohydrates on the phospholipase production by *C. albicans* (Samaranayake, Raeside and MacFarlane 1984).

Candida albicans is also known to produce other enzymes such as proteases. Staib (1965), for instance, detected proteolytic enzymes in *C. albicans* using both an auxanographic and a serum-protein-agar method. The *C. albicans* proteinases are of two types, an extracellular proteinase with a pH optimum of 3.5 to 4 and an intracellular neutral protease with a pH optimum 6.6, (Remold *et al.* 1968, Chattaway Odds and Barlow 1971 and Samaranayake, Hughes and MacFarlane 1984), the former consists of up to three separate enzymes, depending on the strain investigated (Rüchel 1981). Serum antibodies to one of these proteases have also been demonstrated in patients with

systemic candidosis (MacDonald and Odds 1980a) and the enzyme has been localized by indirect immuno-fluorescence in tissue lesions (MacDonald and Odds 1980b). Therefore, it has been suggested that proteases elaborated by *C. albicans* isolates contribute to their virulence (Remold Fasold and Staib.1968). Indeed, MacDonald and Odds (1983) found that a non-proteinase-producing mutant of a proteinase-producing *C. albicans* strain, was considerably less lethal than the parent strain. It has also been suggested that other environmental factors may regulate the activity of this group of enzymes. For instance, Staib (1967) found that buffering of the medium suppressed proteolysis, while, Chattaway Odds and Barlow (1971) found that purified yeast-proteinases have an optimum pH of 3.2 and do not exhibit any enzymatic activity at pH 5.0 or above. More recently, Samaranayake, Hughes and MacFarlane (1984) have investigated the production of proteases by *C. albicans* in batch cultures of human saliva supplemented with glucose and demonstrated a positive correlation between acid production and salivary proteolysis. Rüchel *et al.*, (1986) supported this view and found that the proteolytic activity of *C. parapsilosis* (a clinical isolate) was dependent on the supply of glucose (≥ 1 percent) as opposed to a proteolytic reference strain of *C. albicans*, which did not require glucose.

The above data indicate clearly that the phospholipase and proteolytic activity of *Candida* species vary widely. The main aims of the present study therefore, were to demonstrate the phospholipase and proteinase activity of a large number of

Candida isolates obtained from patients with active infection and healthy individuals. A subsidiary aim was to compare the phospholipase and protease activity with the other pathogenic attributes of the organism.

4.2 Material and methods.

4.2.1. *Candida* Isolates

Forty-nine clinical isolates of *Candida* species, *C. albicans*, 22, *C. glabrata*, 3 , *C. tropicalis*, 7, *C. parapsilosis*, 5, *C. krusei*, 7 and *C. guilliermondii*, 5 (See Maintenance of *Candida* species Chapter Two) were used in this study. Yeast cells were grown on Sabouraud's dextrose agar (SDA, Oxoid). Plates were prepared, as recommended by the manufacturer, inoculated and incubated aerobically at 37°C for 24 hours. A loopful of the confluent growth was transferred to 10 ml of 1 percent (w/v) Mycological Peptone Medium (Oxoid) supplemented with 50 mM glucose (British Drug House) and incubated at 37°C on an orbital incubator (Gallenkamp) at 100 rpm.for 18 hours. The growth was harvested by centrifugation at 1500 g for 10 minutes and washed twice in sterile phosphate buffered saline (PBS, pH 7.2). Next a yeast suspension of 1.0×10^7 yeast per ml was prepared in PBS. The number of yeast cells per ml was regulated, in all the experiments, by microscopic counting in a haemocytometer counting chamber (Hawksley, England), (See, 2.5.1).

4.2.2. Preparation, inoculation and incubation of plates for phospholipase activity.

The method of Price, Wilkinson and Gentry (1982) with the modification of Samaranayake, Raeside and MacFarlane (1984), was used. The test medium consisted of SDA, (Oxoid Ltd, Basingstoke, England) supplemented with 1 M sodium chloride, 0.005 M calcium chloride (BDH, Poole, UK) and 8 percent sterile egg yolk (Oxoid, UK). The egg yolk was first centrifuged at 500 g for 15 minutes. The sterile supernatant was made up to its original volume in sterile distilled water and incorporated into the sterile SDA. Prior to inoculation, plates were kept at 37°C for two hours to remove the excess surface moisture which may affect the diameter of the colonies. A Ridgeway-Watt A 400 multi-point inoculator (Denley Instruments Ltd, Billinghurst, Sussex, England) (Figure 4.1) was then used to inoculate the yeast onto the test medium (4 samples per plate, Figure 4.2). One positive and one negative reference isolate was included in each experiment to act as controls and to ensure that different batches of SDA (Oxoid) did not affect enzyme activity. All plates were incubated aerobically in a humid chamber (an aerobic-jar containing 10 ml of water with the lid kept open throughout the experiment) at 37°C for approximately, 7 days. Each isolate was tested in duplicate on at least three different occasions, in all the experiments.

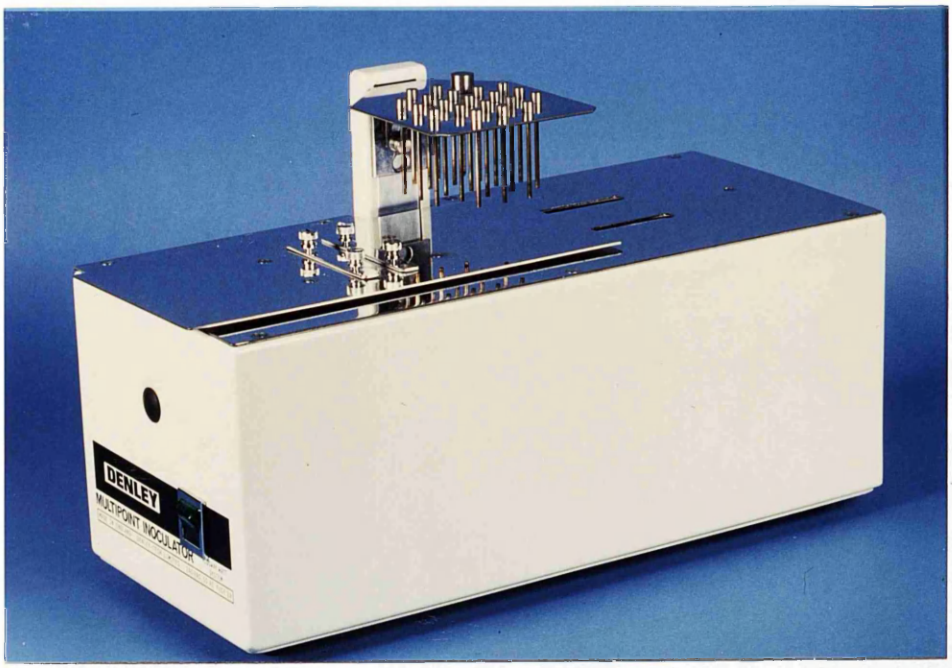


Figure 4.1. Multi-inoculator, used to inoculate *Candida* species on phospholipase and proteinase media

4.2.3. Measurement of phospholipase activity.

The dense white precipitation around the colonies of phospholipase-positive isolates was distinctive and well defined on the initially translucent medium. The plates were read with a zone reader (Luckham Ltd, West Sussex, England) which allowed the diameter of the colonies and precipitation zones to be measured on a magnified scale. Phospholipase activity (P_a value) was measured as follows:

$$\text{Phospholipase activity } (P_a \text{ value}) = 100 - \left(\frac{\text{The mean diameter of the colony (C)}}{\text{The mean diameter of colony and precipitation zone (CZ)}} \times 100 \right)$$

Thus, for example, when P_a=0.00 the isolate under test was phospholipase-negative and when P_a=40, the isolate was producing a considerable amount of phospholipase. The mean diameter of the colony and the precipitation zone (CZ) is estimated by the addition of the vertical and the horizontal diameter of each colony / precipitation zone and dividing this value by two. The latter procedure compensates for the irregularity of the colony / precipitation zone (see Figure 4.3).

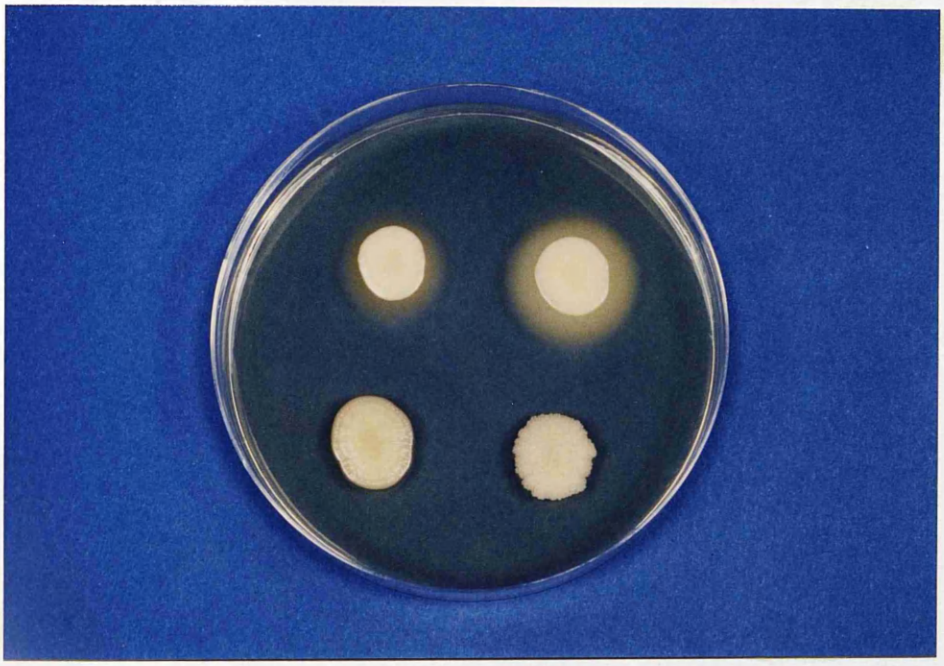
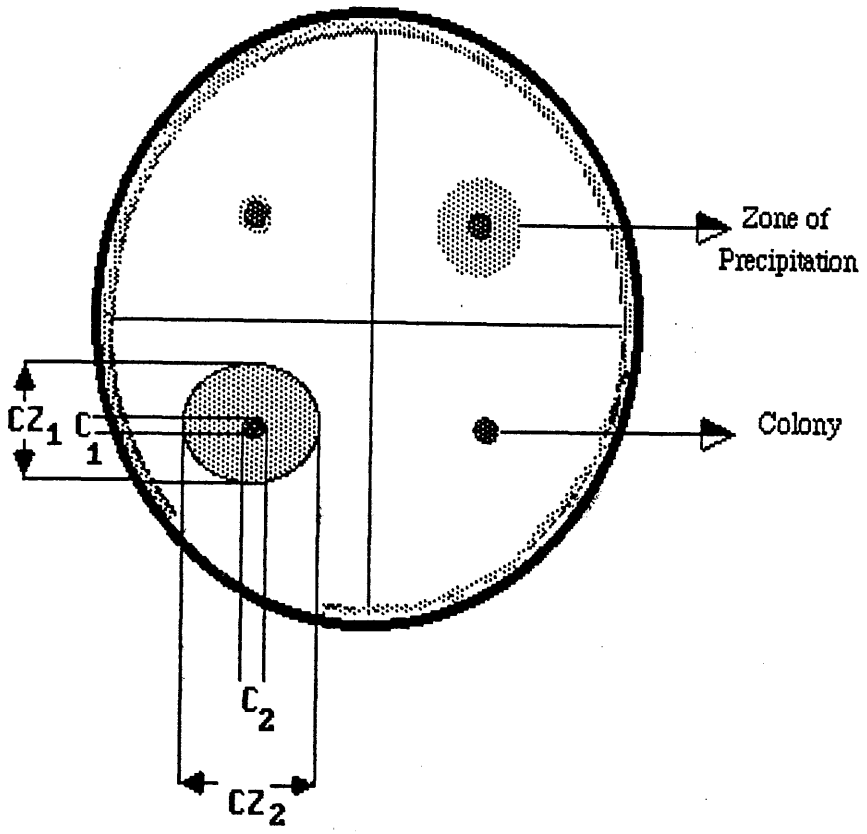


Figure 4.2. Phopholipase produced by *Candida albicans* positive isolates



$$C^{*} = (C_1 + C_2)/2$$

$$CZ = (CZ_1 + CZ_2)/2$$

$$P_a \text{ Value} = 100 - [(C/CZ) \times 100]$$

Figure 4.3. Measurement of Phospholipase activity,

*Where C is the diameter of the colony and CZ is the mean readings of colony diameter and the diameter of precipitation zone

4.2.4. Preparation, inoculation and incubation of plates for proteinase.

The basic medium consists of KH_2PO_4, $MgSO_4$, sucrose (BDH, Poole, Dorset) and agar (Oxoid Ltd, Basingstoke, England), (for preparation and autoclaving the basal medium). The medium was distributed in 100 ml quantities, cooled and stored at 4°C. When required a bottle of medium was remelted, cooled to 50°C in a water bath, and 20 ml of a 1 percent solution of either bovine albumin or IgA (both from Sigma, London, Poole, Dorset) in sterile distilled water (pH 6.8) were added to each bottle. The medium was immediately mixed thoroughly, with the avoidance of bubble formation, by rolling the bottles between the hands. Twelve milliliter aliquots of the protein agar medium were poured into sterile plastic Petri dishes, and were kept at room temperature until the agar solidified. Then the plates were placed in an incubator at 37°C for two hours to remove the excess surface moisture. A Ridgeway-Watt A 400 multi-point inoculator (see above) was then used to inoculate the yeast onto the test medium (see Figure 4.3). One positive and one negative reference isolate were included in each experiment to act as controls and to ensure that different batches of SDA did not affect enzyme activity. All plates were incubated aerobically in a humid chamber (an aerobic-jar containing 10 ml of water with the lid kept open throughout the experiment) at 37°C for approximately, 120 hours. All experiments were repeated on three separate occasions with duplicate determinations on each occasion.

Protein stain.

For the preparation of the protein staining solution, 12.5 grams of Naphthalene Black 10 B (BDH, Poole, UK.) were mixed in 900 ml of methanol and 100 ml of glacial acetic acid (BDH, Poole, UK.). The decolorizing solution was prepared by mixing 750 ml glacial acetic acid and 250 ml phenol crystals (liquid) in 5000 ml distilled water (Staib, 1965).

Staining procedure

The plates were flooded with the naphthalene black solution for 20 minutes. Next the naphthalene black was decanted and the plates decolorized, by pouring 10 ml of the decolorizing solution on to the plate-surface and leaving this for 15 hours at room temperature. The decolorizing solution was replaced thrice, at approximately, 2, 13 and 15 hours. The protein in the medium was stained with naphthalene black, while the areas affected by proteolysis due to *Candida* proteinase, were translucent. The proteinase-positive translucent colonies were distinctive and well defined (Figure, 4.4).

4.2.5. Measurement of proteinase activity.

The clear translucent zones around the colonies of proteinase-positive isolates was distinctive and well defined on a back drop of naphthalene black stained agar.

The plates were read with a zone reader (Luckham Ltd, West Sussex, England) which allowed the diameter of the colonies and translucent zones to be measured on a magnified scale.

Proteinase activity (Pr_a value) was assessed by calculating the mean of two readings (measured vertically and horizontally) of the translucent zone diameter CZ, (including the colony zone, C) Figure 4.5. The zones were coded, as follows: ≥ 3 mm =3, >2-3 mm =2, 1-2 mm =1, 0-<1 mm =0

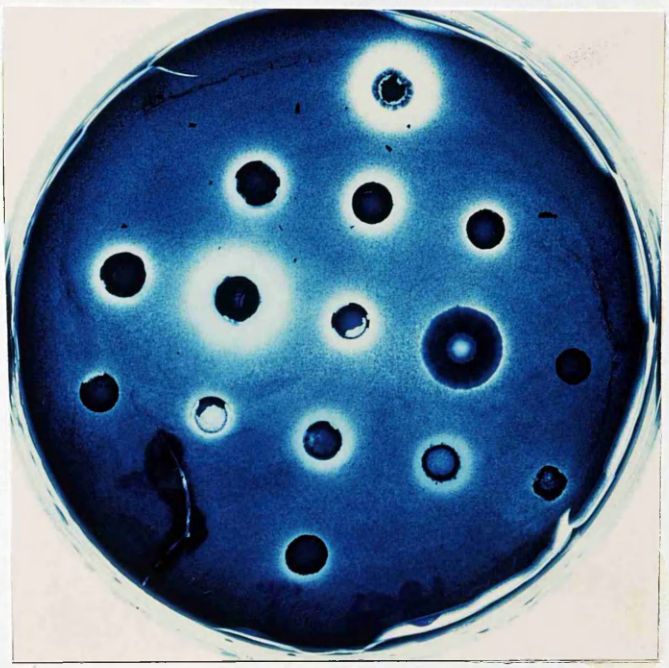
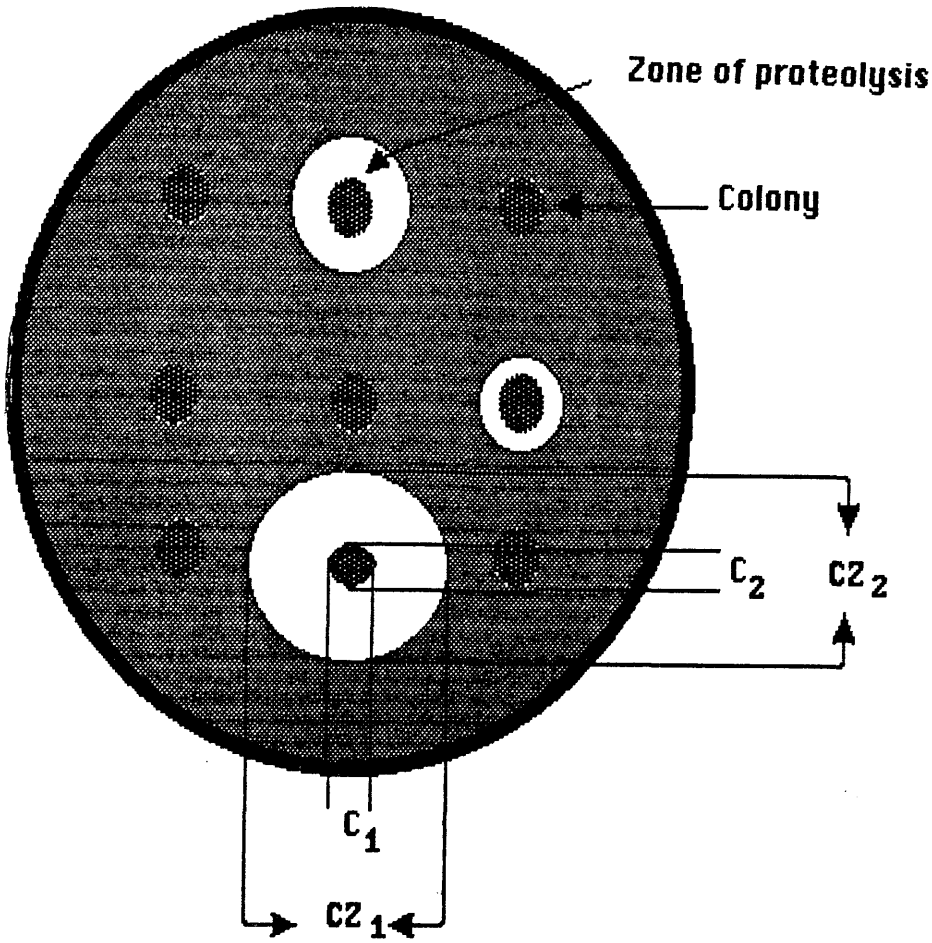


Figure 4.4. Proteinase positive and negative strains of *Candida albicans*



$$C^{*} = (C_1 + C_2)/2$$

$$CZ = (CZ_1 + CZ_2)/2$$

$$Pr_a \text{ Value} = (CZ - C)/2$$

Figure 4.5. Measurement of Proteinase activity
The zones were coded, as follows: ≥ 3 mm =3, >2-3 mm =2, 1-2 mm =1, 0-<1 mm =0

*Where C is the diameter of the colony and CZ is the mean readings of colony diameter and the diameter of the precipitation zone

4.3 Results.

4.3.1. Phospholipase activity of *Candida albicans* isolates.

Phospholipase activity (P_a values) of 22 *Candida albicans* isolates, is shown in Table 4.1. All *C. albicans* isolates (100 percent) were positive for phospholipase activity. The positive isolates produced a distinct zone of precipitation around the colony which could be easily quantified (Figure 4.2 and 4.3). The distribution of the phospholipase activity of *C. albicans* isolates demonstrated that zones of precipitation varied depending upon the isolates under test (Table 4.1); for example, strain a8 shows the highest phospholipase production, while strain a5 shows the lowest (Figure, 4.6). The distribution of phospholipase activity of 22 *C. albicans* isolates is further demonstrated in the histogram shown in Figure 4.7, in which most of the isolates (72.7 %) were able to produce a phospholipase activity value of >36 (See measurement of phospholipase section, 4.2.3).

4.3.2. Phospholipase activity of *Candida* species.

All isolates of the other *Candida* species (3 *C. glabrata*, 7 *C. tropicalis*, 5 *C. parapsilosis*, 7 *C. krusei* and 5 *C. guillier-mondii*,) failed to show phospholipase activity under the test conditions used in these experiments.

4.3.3. Proteinase activity of *Candida albicans* isolates.

The proteolytic activity of the 22 *C. albicans* isolates were studied with two protein substrates, bovine serum albumin (BSA), (Table 4.2) and IgA, (Table 4.3). In these experiments each substrate was used as the sole nitrogen source. The results indicate that 68.2 percent of *C. albicans* isolates utilized BSA, whereas 45.5 percent utilized IgA as the sole source of protein *in vitro* (Table 4.4).

4.3.4. Proteinase activity of *Candida* species.

Only *C. parapsilosis*, and *C. tropicalis* isolates demonstrated detectable proteinase activity. The results for *C. parapsilosis* are shown in Tables 4.5 and 4.6 and that of *C. tropicalis* in Tables 4.7 and 4.8. The other *Candida* species, i.e., *C. glabrata*, *C. krusei* and *C. guilliermondii*, were unable to produce detectable proteinase activity under these conditions. Tables 4.9 and 4.10 give a summary of the distribution and percentage of proteinase activity of *Candida* species with BSA and IgA, respectively. The relative proteinase activity of *C. albicans*, *C. tropicalis*, and *C. parapsilosis*, using either BSA or IgA are presented in Figure 4.8.

Table 4.1.The Phospholipase Activity (P_a) produced by *Candida albicans* isolates.

C.albicans Isolates	Phospholipase activity (P_a) (1)*		(2)		(3)		Mean	±SEM
a 1	46.9	43.6	48.1	49.1	46.9	48.9	47.3	0.8
a 2	36.1	35.3	41.9	42.3	41.0	42.8	39.9	1.4
a 3	35.8	36.4	41.2	44.0	39.6	40.8	39.6	1.3
a 4	46.0	46.8	49.5	50.5	47.5	50.1	48.4	0.7
a 5	19.9	19.5	22.5	21.9	22.2	21.8	21.3	0.5
a 6	35.0	35.6	39.8	40.4	36.4	39.0	37.7	0.9
a 7	39.8	42.4	49.9	50.7	46.7	47.7	46.2	1.8
a 8	44.2	45.0	55.1	53.5	51.2	49.8	49.8	1.8
a 9	40.5	40.9	47.5	48.7	45.9	46.5	45.0	1.4
a 10	37.7	38.5	42.8	43.2	38.0	40.4	40.1	1.0
a 11	41.8	42.4	50.0	48.2	43.3	44.5	45.0	1.4

*Experiment number; each isolate was tested in duplicate on each experiment.

Table 4.1. (Continued).

C. albicans Isolates	Phospholipase activity (P_s) (1)*		(2)		(3)		Mean	±SEM
a 12	37.0	38.8	45.3	46.9	41.4	40.8	41.7	3.8
a 13	24.8	25.2	31.2	30.4	30.1	31.5	28.9	1.2
a 14	30.1	29.3	33.9	34.5	31.5	33.9	32.2	0.9
a 15	37.1	37.9	42.0	42.6	39.8	41.2	40.1	0.9
a 16	25.0	24.4	27.0	27.6	21.8	24.2	25.0	0.9
a 17	33.8	34.4	38.9	37.5	36.2	37.0	36.3	0.8
a 18	28.1	28.5	32.7	32.1	29.5	29.1	30.0	0.8
a 19	36.1	36.9	38.8	39.2	36.6	37.4	37.5	0.5
a 20	26.3	27.5	30.0	28.8	27.8	28.8	28.2	0.5
a 21	41.1	39.9	44.9	45.9	43.2	44.2	43.2	0.9
a 22	40.8	42.2	46.7	47.5	45.2	46.4	44.8	1.1

*Experiment number; each isolate was tested in a duplicate on each experiment.

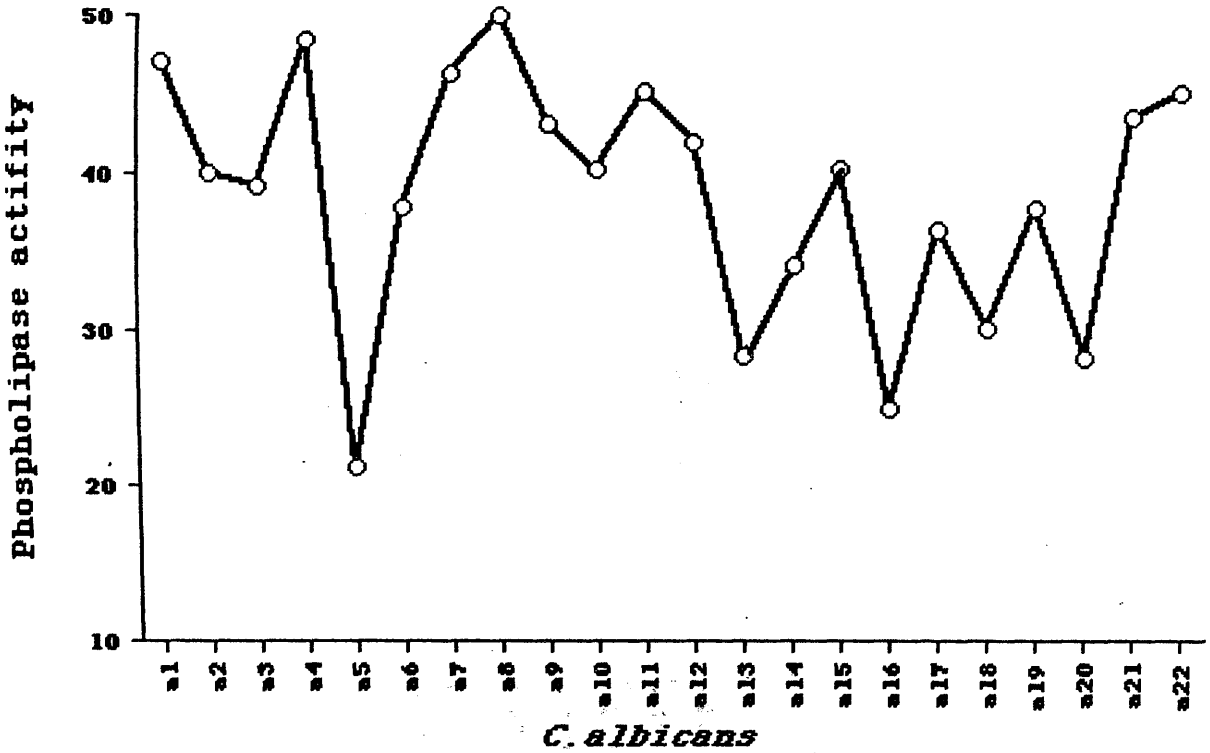


Figure 4.6. Phospholipase activity (P_a) of 22 *Candida albicans* isolates

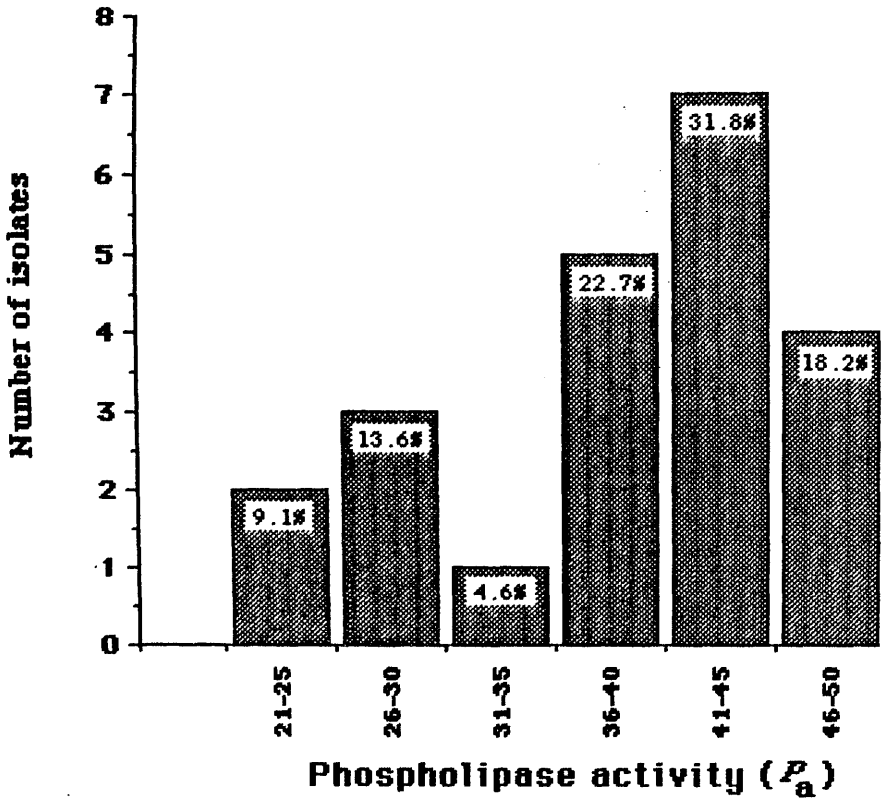


Figure 4.7. The distribution of phospholipase activity of 22 isolates of *Candida albicans*.

Table 4.2. The Proteinase activity (Pr_a) of 22 *Candida albicans* isolates on bovine serum albumin as the sole nitrogen source.

C. albicans Isolates	Proteinase activity (Pr_a) (1)*		(2)		(3)		Mean
a1	1	1	0	1	1	1	0.8
a2	0	0	0	0	0	0	0.0
a3	0	0	1	1	1	1	0.7
a4	1	1	1	1	1	1	1.0
a5	2	2	3	3	3	3	2.7
a6	0	0	0	0	0	0	0.0
a7	0	0	1	1	0	0	0.3
a8	3	3	3	3	2	2	2.7
a9	1	1	1	1	1	1	1.0
a10	0	0	1	1	0	0	0.0
a11	0	0	0	0	0	0	0.0

*Experiment number; each isolate was tested in duplicate on each occasion.

Table 4.2. (Continued).

C. albicans Isolates	Proteinase activity (Pr_a) (1)*		(2)		(3)		Mean
a12	1	1	1	1	1	1	1.0
a13	1	1	2	2	2	1	1.5
a14	1	1	1	1	1	1	1.0
a15	3	3	3	3	3	3	3.0
a16	0	0	1	1	0	1	0.5
a17	1	1	1	1	1	1	1.0
a18	0	0	0	0	0	0	0.0
a19	3	3	3	3	2	2	2.7
a20	1	1	1	1	1	1	1.0
a21	1	1	0	0	1	1	0.7
a22	0	0	1	1	0	0	0.3

*Experiment number; each isolate was tested in duplicate on each occasion.

Table 4.3. The Proteinase Activity (Pr_a) of 22 *Candida albicans* isolates using IgA as the sole nitrogen source.

C. albicans Isolates	Proteinase activity (Pr_a) (1)*		(2)		(3)		Mean
a1	0	0	0	0	1	1	0.3
a2	0	0	0	0	0	0	0.0
a3	1	1	1	1	1	1	1.0
a4	1	1	1	1	1	1	1.0
a5	2	1	2	2	2	1	1.7
a6	0	0	0	0	0	0	0.0
a7	1	1	0	0	0	0	0.3
a8	2	2	2	2	2	2	2.0
a9	0	0	0	0	0	0	1.0
a10	1	1	0	0	1	1	0.3
a11	0	0	0	0	0	0	0.0

*Experiment number; each isolate was tested in duplicate on each occasion.

Table 4.3. (Continued).

C. albicans Isolates	Proteinase activity (Pr_a) (1)*		(2)		(3)		Mean
a12	0	0	0	0	0	0	0.0
a13	1	1	1	1	1	1	1.0
a14	0	0	0	0	0	0	0.0
a15	3	3	2	3	3	3	2.8
a16	0	0	0	0	1	1	0.3
a17	0	0	0	0	0	0	0.0
a18	0	0	0	0	0	0	0.0
a19	3	2	2	3	3	3	2.7
a20	2	2	2	2	2	2	2.0
a21	0	0	0	0	0	0	0.0
a22	2	2	2	2	1	1	1.7

*Experiment number; each isolate was tested in duplicate on each occasion.

Table 4.4. A comparison of the proteinase activity of 22 *Candid albicans* isolates on bovine serum albumin and IgA, used as the sole nitrogen source.

Protein substrate	Degree of proteolysis*				Positive
	0	1	2	3	%
Bovine serum albumin	7	9	2	4	68.2
IgA	12	4	4	2	45.5

Table 4.5. The Proteinase activity (Pr_a) of five *Candida parapsilosis* isolates with bovine albumin as the sole nitrogen source.

Candida parapsilosis	Isolates	Proteinase activity (Pr_a) (1)*		(2)		(3)		Mean
C. parapsilosis	p1	1	1	1	1	1	1	1.0
	p2	3	3	2	2	2	2	2.3
	p3	1	1	2	2	1	1	1.3
	p4	2	2	2	2	2	2	2.0
	p5	2	2	1	1	1	1	1.3

*Experiment number; each isolate was tested in duplicate on each occasion.

Table 4.6. The Proteinase activity (Pr_a) of five *Candida parapsilosis* species with IgA as the sole nitrogen source.

Candida parapsilosis	Isolates Code	Proteinase activity (Pr_a) (1)*		(2)		(3)		Mean
C. parapsilosis	p1	2	2	1	1	1	1	1.3
	p2	2	2	3	3	2	2	2.3
	p3	2	2	2	2	2	2	2.0
	p4	3	3	3	3	3	3	3.0
	p5	2	2	1	1	2	2	1.7

*Experiment number; each isolate was tested in duplicate on each occasion.

Table 4.7. The Proteinase activity (Pr_a) of seven *Candida tropicalis* isolates with bovine albumin as the sole nitrogen source.

Candida tropicalis	Isolates	Proteinase activity (Pr_a) (1)*		(2)		(3)		Mean
C. tropicalis	t1	1	1	2	2	1	1	1.3
	t2	0	0	1	1	1	1	0.7
	t3	0	0	0	0	0	0	0.0
	t4	2	2	1	1	1	1	1.3
	t5	0	0	1	1	1	1	0.7
	t6	1	1	1	2	1	1	1.2
	t7	0	0	0	0	0	0	0.0

*Experiment number; each isolate was tested in duplicate on each occasion.

Table 4.8. The Proteinase activity (Pr_a) of seven *Candida tropicalis* isolates with IgA as the sole nitrogen source.

Candida tropicalis	Isolates Code	Proteinase activity (Pr_a) (1)*		(2)		(3)		Mean
C. tropicalis	t 1	3	3	2	2	2	2	2.3
	t 2	2	2	1	1	2	2	1.7
	t 3	0	0	0	0	0	0	0.0
	t 4	3	3	2	2	2	2	2.3
	t 5	1	1	1	1	1	1	1.0
	t 6	2	2	1	1	2	2	1.7
	t.7	0	0	0	0	0	0	0.0

*Experiment number; each isolate was tested in duplicate on each occasion.

Table 4.9. The proteinase activity of 49 isolates of *Candida* species with bovine serum albumin as the sole nitrogen source.

Candida species	Number of isolates	Degree of bovine serum albumin proteolysis*				Positive percentage
		0	1	2	3	
C. albicans	22	7	9	2	4	68.2
C. tropicalis	7	2	5	0	0	71.4
C. parapsilosis	5	0	3	2	0	100 0
C. glabrata	3	3	0	0	0	0 0
C. krusei	7	4	0	0	0	0 0
C. quilliermondii	5	3	0	0	0	0 0

*Semi-quantitative assessment of proteolysis (See text for details).

TABLE 4.10. Proteinase activity, of 49 isolates of *Candida* species with IgA as the sole nitrogen source.

Candida species	Number of isolates	Degree of IgA albumin proteolysis* 0	1	2	3	Positive percentage
C. albicans	22	12	4	4	2	45.5
C. tropicalis	7	2	1	4	0	85.7
C. parapsilosis	5	0	1	3	1	100.0
C. glabrata	3	3	0	0	0	0.0
C. krusei	7	4	0	0	0	0.0
C. guilliermondii	5	3	0	0	0	0 0

*Semi-quantitative assessment of proteolysis (See text for details).

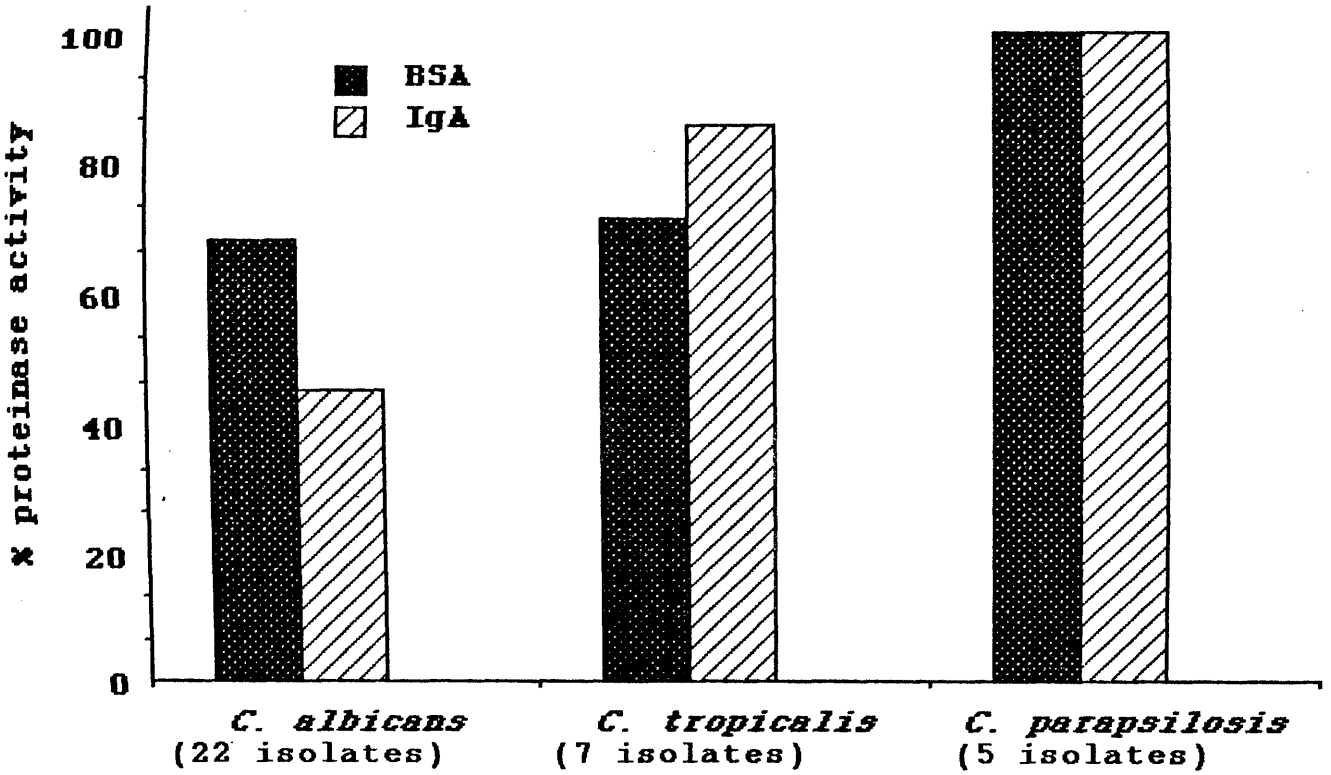


Figure 4.8. The percentage of proteinase positive isolates of *Candida albicans, C. tropicalis and C. parapsilosis* with either bovine albumin or IgA used as the sole nitrogen source

4.4.Discussion

The part played by phospholipase and proteinase activity in the pathogenesis of *Candida albicans* infections is unknown. In order to obtain information about this topic, the phospholipase and proteinase activity of *C. albicans* (22 strains), *C. tropicalis* (7), *C. parapsilosis* (5), *C. glabrata* (3), *C. krusei* (7) and *C. guilliermondii* (5) were studied. The results of the phospholipase activity of the latter two species have not been reported before.

4.4.1.Phospholipase activity.

It was revealed that only *C. albicans* isolates are able to produce a detectable levels of phospholipase activity *in vitro* whereas, the other species tested produced negative results. These results are in agreement with Samaranayake, Raeside and MacFarlane (1984) who reported that only *C. albicans* isolates were able to produce this enzyme, but not isolates of *C. tropicalis, C. glabrata* or *C. parapsilosis*.

The results of this study have demonstrated that 100 per cent of *C. albicans* isolates used, were phospholipase positive. This figure is greater than that of Price, Wilkinson and Gentry (1982) who reported that 55 per cent of blood culture isolates, 50 per cent of wound isolates and 30 per cent of urine isolates were phospholipase positive. The variations between the current results and those of Price *et al.*, (1982) may be due to differences in the incubation period which was two days in the latter study and seven days in this investigation. The longer incubation

period may have enhanced phospholipase production and subsequent detection. Indeed, in a recent study, Samaranayake, Raeside and MacFarlane (1984) detected the enzyme in 79 per cent of 22 oral isolates of *C albicans*, by incubating them for 4 days. It would therefore, seem that the longer the incubation period -as in this study- the greater the expression of phospholipase activity detected using the method of Price, Wilkinson and Gentry (1982). More recently, Williamson, Samaranayake and MacFarlane (1986) used phospholipase activity as a criterion for biotyping *C. albicans*, and tested 100 oral isolates for phospholipase activity. The results revealed that 97 per cent of the *C. albicans* were phospholipase producers with varying degrees of activity.

The cytochemical localization of phospholipase in *C. albicans* has been demonstrated by Pugh and Cawson (1975), using the substrate lecithin. They reported that the distribution of enzyme activity varies during the cell cycle of *C. albicans*. In an early synchronous culture and before the start of budding, enzyme activity is localized in numerous granules in the centre of the cell. These granules then move towards the periphery of the cell at the region where bud will develop. In older cultures the enzyme localization is different. In yeast cells from a day old culture the deposit formed by enzyme activity was found at the periphery of the cells and in small vesicles attached or lying close to the cell membrane and then secreted into the medium. Furthermore, Banno, Yamada and Nozawa (1985) have reported that phospholipase activity was found to accumulate in the culture filtrate with age of growth of yeast cells. These ultrastructural

finding tend to agree with the observation that the age of the culture is important in the expression of phospholipase activity of the yeasts.

Recently, Barrett-Bee *et al.*, (1985) detected the enzyme in four *C. albicans* isolates (two from an asymptomatic carrier while, the clinical sources of the other two isolates were not recorded) with variable phospholipase concentrations. They reported that these variations were correlated with the pathogenicity in mice. In addition, the study revealed that 40 per cent of the enzyme was detected in cell free supernatant of *C. albicans* cultures grown in Sabouraud's dextrose broth for 10 hours at 37 ^{o}C. This indicates that a significant proportion of the enzyme is extracellular and could be detected by the plate method described in this study.

The present study indicates no variations in phospholipase activity between isolates collected from oral or vaginal cavities, which agrees with the results of Price and Cawson (1977), and Samaranayake, Raeside and MacFarlane (1984). However, isolates of *C. albicans* collected from apparently active candidosis cases were able to produce higher quantities of the enzyme than those isolated form asymptomatic individuals. Thus the isolates coded a5, a16, and a18 which were collected, from oral and vaginal cavities of individuals who were apparently asymptomatic produced a relatively low concentrations of the enzyme whereas other strains isolated from active candidosis, e.g., a1 and a8, produced higher concentrations of phospholipase (Table, 4.1).

The ability to secrete phospholipase by *C. albicans*, but not by other *Candida* species can be considered as a potential determinant of virulence as *C. albicans* is known to be the most pathogenic *Candida* species (Odds 1988). Further, this enzyme may play an important role in facilitating germ-tube formation and host tissue penetration by candidal hyphae. Therefore, the results of the present investigation support the earlier suggestion by Pugh and Cawson 1975, and Samaranayake, Raeside and MacFarlane 1984, that phospholipases may be implicated in the pathogenicity of *C. albicans*.

4.4.2.Proteinase activity.

As well as phospholipases, proteinases, too have been implicated in the *Candida* pathogenicity. The most suggestive evidence for the involvement of extracellular proteinases in the virulence of *C. albicans* has come from extensive studies by two main groups, MacDonald and associates in UK, and Rüchel and colleagues in West Germany.

By means of auxanographic method, Staib in 1965, could detect the proteolytic activity of *C. albicans*. In this method, the organism was mixed with 10 ml, of a basic agar medium (the organism was a constituent of the medium), and when solidified, a protein substrate was inoculated on to the surface of the agar plates as a nitrogen source. The plates after being incubated for 48 hours, were stained with a protein stain which stains the protein intensely blue (Staib 1965).

The other technique used by the same author was the serum agar protein method. A protein substrate was added as a nitrogen source to an agar medium (the same basic medium of the auxanographic method) and the test organisms were incubated on the agar surface of the solidified medium and incubated for five days. The agar surface was later stained with a protein stain (Staib 1965)

MacDonald and Odds (1980b) used a proteinase detection method in which a growth medium containing bovine serum albumin is inoculated with the organism. The cultures after being incubated at 37□C for 5 days, were centrifuged and proteolysis determined by measuring the absorbence of the soluble peptides.

In the present study, using serum agar protein method (Staib1965), 49 *Candida* isolates (*C. albicans* 22 isolates, *C. tropicalis* 7, *C. krusei*, 7, *C. parapsilosis*, 5, *C. guilliermondii* 5, and *C. glabrata* 3) were investigated for the production of proteinase. Results indicated that when BSA was used as the sole protein substrate, 68.2 per cent of the strains tested demonstrated a positivity. However, only 45.5 per cent of *C. albicans* possessed proteinase activity when IgA was used as the sole nitrogen source. The former figure, is similar to that of Staib (1965), who used an agar medium (pH 4.6-5.5) supplemented with human serum albumin as the only protein source, for *C. albicans* proteinase detection. They found that 75 per cent of 100 isolates of *C. albicans* were able to produce the enzyme.

A number of other workers have studied the protease activity of *C. albicans* isolates from oral and non-oral sources. For instance, Budtz-Jörgensen.(1971), using the auxanographic method, reported that proteinase activity was present in 59 per cent (26/44) of *C. albicans* isolates from the palatal mucosa of 20 denture wearers with clinically normal palates, and patients with candida-induced denture stomatitis, grown in a medium supplemented with 1 per cent human serum protein for 72 hours. He also investigated 62 *C. albicans* isolates by the serum protein agar method (pH 5.0). When incubated for 72 hours, proteinase was produced by 78 percent (49/62) of the isolates. Recently, MacDonald (1984) evaluated the secretion of proteinase by three *C. albicans* isolates the sources of which were not recorded, grown for 72 hours in liquid cultures supplemented with bovine serum albumin (pH 3.2) using the method described by MacDonald and Odds (1980b). All three isolates produced proteinase.

Schreiber *et al.*, (1985) studied 75 *C. albicans* isolates (clinical isolates were grouped into I. isolates from patients with invasive disease. II.patients who were suspected of having severe candidal disease, III. isolates from patients who were judged clinically to have superficial infections, and group IV. isolates from colonized patients with no apparent infection) grown for two and seven days in medium containing BSA (pH 3.2) using MacDonald and Odds (1980b) method. They found that 63 percent (37/59) and 97 percent (73/75) of isolates produced the enzyme after two and seven days growth respectively. More recently, Ghannoum and Abu Elteen (1986) have demonstrated proteinase in 100

percent (53/53) of clinical isolates of *C. albicans* incubated for seven days using the method of MacDonald and Odds (1980b). From the above data, it would appear that two in three *C. albicans* isolates have the ability to produce proteinases which breakdown serum protein agar.

The use of immunoglobulin A (IgA) as a sole nitrogen source and as a target for *Candida* proteinase, has not been reported previously. Immunoglobulin A appears selectively in the sero-mucous secretions such as saliva, and secretions of the lung, genito-urinary and gastro-intestinal tracts. organs in which *Candida* usually exerts its effects. Immunoglobulin A contributes in the defense mechanisms of the exposed external surfaces of the body against attack by microorganisms. It is a dimer stabilized against proteolytic activity by combination with another protein - the secretory component which is synthesized by local epithelial cells (Roitt, 1980). This may in part, explain the difficulty in IgA breakage by *C. albicans* proteinase, as only 45.5 per cent of the IgA was destroyed by the proteinase compared with 68.2 per cent of the BSA.

One function of IgA is to inhibit the adhesion of microorganism to the surfaces of mucosal cells. The present results indicate *C. albicans* has the ability to breakdown this host defense barrier to a significant extent and possibly initiate an infective process.

Seventy five per cent of *C. tropicalis*, 100 percent of *C. parapsilosis* and none of the other species investigated (*C.*

glabrata, *C. krusei* and *C. guilliermondii*) produced proteinase. This supports the general pattern presented by other workers that *C. albicans*, *C. tropicalis* and *C. parapsilosis* are able to produce the enzyme. For instance, MacDonald (1984) has reported that proteinase was produced by 100 per cent of *C. tropicalis* (3/3) and 33.3 per cent of *C. parapsilosis* (1/3) but not by *C. pseudotropicalis*, (0/3) *C. krusei* (0/3) *C. guilliermondii* (0/3) or *C. glabrata* (0/3). On the other hand, Schreiber *et al* , (1985) reported the production of the enzyme by 17 per cent (1/6) of *C. tropicalis* 67 per cent (4/6) of *C. parapsilosis* and 13 per cent (1/8) of *C. glabrata*. The variations between the results of different studies may be due to changes, in the pH, incubation time, the composition of the media (e.g., Vitamins) and the type and purity of the protein used (Staib, 1965).

The results from the current study do not indicate a correlation between the source of the isolates and the production of proteinase. These observations support the results of previous workers. For instance, Staib (1965) collected two isolates from the same patient, which gave different results on serum protein agar, and an isolate from an asymptomatic carrier, caused an intensive lysis of human albumin *in vitro*. Budtz-Jörgensen (1971), stated that no relationships was found between the severity of the inflammatory condition of mucosa and the proteolytic activity of the *C. albicans* strains used.

Other too have observed that the amount of proteinase produced *in vitro*, by proteolytic strains of *C. albicans* isolates, does not correlate with the degree of *Candida* invasion (Staib 1965; and Schreiber *et al.*, 1985). These results agree with the present study where no correlation could be determined with proteinase activity and the clinical state of the patient from whom the organism was isolated.

Among the *Candida* species, *C. albicans* clearly is of foremost medical importance, followed by *C. tropicalis* (Hurley *et al.*, 1986 and Odds 1988). The dominance of these two species reflect their prevalence as commensals (Odds 1988). However, the factors favouring the persistence of these yeasts on mucous membranes are not yet clearly identified. It is possible that the proteinase secreted by most isolates of both species favour candidal persistence and virulence (Odds 1985b). Proteases may be also produced by *C. parapsilosis*, in which a correlation between proteolytic activity and virulence has not been established, as yet. In spite of its proteolytic potential, the virulence of *C. parapsilosis* is low *in vitro* (Borg *et al.*, 1984) as well as in humans and animals (Bistoni *et al.*, 1984; Dyess Garrison and Fry 1985). Isolates of *C. parapsilosis* were less cytotoxic in cell-culture than any of the tested isolates of *C. albicans* and *C. tropicalis* (Borg *et al.*, 1984). The lack of virulence may be related to the fact that *C. parapsilosis* did not produce proteinase under *in vitro* conditions (Rüchel Böning and Borg 1986). In mice, no specific titre, could be detected after infection with *C. parapsilosis* (Borg and Rüchel 1988). However,

anti-proteinase antibody was readily detectable after infection with *C. albicans* and *C. tropicalis*. Thus, Rüchel, Böning and Borg (1986), suggested that a lack of induction of the enzyme under conditions of infection might account for the low virulence of most isolates of *C. parapsilosis*.

In conclusion, it seems, that there is no visible correlation between the *in vitro* and *in vivo* production of *Candida* proteinase. This may suggest that there is more than one type of proteinase included in *Candida* pathogenicity mechanisms. Namely, an extracellular proteinase which can be detected *in vitro*, as well as an intracellular proteinase, bounded to the yeast cells and released *in vivo* possibly during germ-tube formation and penetration of the host tissues.

CHAPTER FIVE

THE CANDIDACIDAL ACTIVITY OF LYSOZYME

5.1. Introduction.

Lysozyme (muramidase) is presumed to contribute to the antimicrobial defence mechanisms of several body fluids (Mandel 1979) and is also present in phagocytic cells (Elsbach 1980). Its bacteriolytic activities were discovered by Fleming as early as 1922. Since then, several functions have been recognized for this enzyme, Namely, (i) modulating the inflammatory reaction (Gordon *et al*, 1979), (ii) enhancement of phagocytosis (Klockars and Roberts 1976), (iii) lysozyme hydrolyses peptidoglycan of Gram-negative bacteria after being rendered susceptible by antibodies (IgA) plus complement (Brown *et al.*, 1976; Roitt 1980), and (iv) inactivation of viruses (Arimure 1973).

Lysozyme is delivered to the oral cavity by salivary secretions and the concentration of lysozyme in saliva ranges from 1.5 to 57 μg of human lysozyme equivalents ml^{-1} (Petit and Jolles 1963; Raeste and Touompo 1976; Stuchell and Mandel 1983). Indeed, plaque fluid has been reported to contain up to 15 times the level of lysozyme that is present in whole saliva (Cole *et al.* 1981). The antimicrobial properties of lysozyme may be important in the regulation of the oral microflora where growth conditions are not ideal and are highly variable (Shannon, Suddick and Dowd 1974). In fact, it has been shown that lysozyme exerts a selective bacteriostatic or bactericidal action on oral commensals such as

Streptococcus mutans, Actinomyces viscosus, Haemophilus actinomycetemcomitans and *Capnocytophaga gingivalis,* (Iacono *et al.,* 1980; 1983; 1985).

Although several workers have studied the effect of lysozyme on bacteria only three groups have studied its effect on *Candida albicans* and other *Candida* species (Collins and Pappagianis 1974; Kamaya 1970; Marquis *et al.,* (1982). Thus Kamaya (1970) used a relatively crude qualitative assay to estimate the candidacidal activity of lysozyme against six *Candida* species including *C. albicans.* Collins and Pappagianis (1974) used a turbidimetric assay to investigate the fungicidal activity of lysozyme in the presence of the polyene antibiotic Amphotericin B. More recently, Marquis *et al.,* (1982) have demonstrated ultrastructural damage to *C. albicans* exposed to lysozyme which is concentration dependent. Therefore, due to the lack of knowledge about the interaction of lysozyme and *Candida* species the aim of this study was to examine the antifungal activity of lysozyme against 49 isolates of: *C. albicans, C. krusei, C. parapsilosis, C. tropicalis, C. guilliermondii* and *C. glabrata* using a sensitive assay procedure.

5.2. Materials and methods.

5.2.1. Candidal isolates and growth conditions.

Sixty six clinical isolates of *Candida* species, 22 *C. albicans*, and 20 *C. glabrata*. (This is the only occasion in this thesis when 20 strains of *C. glabrata* were used), 7 *C. tropicalis*, 5 *C. parapsilosis*, 7 *C. krusei*, and 5 *C. guilliermondii*, was investigated (See Maintenance of *Candida*, Chapter Two). Yeast cells were grown on Sabouraud's dextrose agar (SDA; Oxoid) plates, incubated aerobically at 37°C for 24 hours. A loopful of the confluent growth was transferred to 10 ml of Sabouraud's broth medium (Difco) supplemented with 4 per cent glucose (British Drug House) and incubated at 37°C on an orbital incubator at 100 rpm for 18 hours. The growth was harvested by centrifuging the culture at 1500 g for 10 minutes. The cells were washed twice in 10 ml of sterile phosphate buffered saline (pH 7.2) adjusted with PBS to give a final suspension of 2-2.5 X 10^7 yeast cells per ml. The number of yeast cells per ml was regulated, in all the experiments, by means of a haemocytometer counting chamber (Hawksley, England), (See section, 2.5.1, Chapter Two).

5.2.2. Lysozyme

Hen egg white lysozyme (Enzyme Commission 3.2.1.17) crystallized three times by the manufacturer, was obtained from Sigma Chemical Company (Poole, UK). A stock solution of lysozyme was prepared (4000 $\mu g\ ml^{-1}$) by dissolving 40 mg of lysozyme in 10

ml of sterile distilled water. The stock solution was aliquoted in 1-ml quantities, refrigerated and used within 7 days.

5.2.3 Fungicidal assay:

The influence of sterile distilled water, and phosphate buffered saline on the anti-candidal activity of lysozyme.

In order to study the influence of sterile distilled water (pH 6.8 SDW), and phosphate buffered saline (pH 7 PBS) on the anti-candidal effect of 2μg/ml lysozyme on *Candida* species, the yeast suspensions prepared as above (Section 5.2.1) were washed and resuspended either in SDW or PBS and the assays performed as follows.. Experiments were carried out in bijou bottles containing 0.25 ml of 4μg/ml lysozyme and either SDW or PBS. To each bottle 0.25 ml of *C. albicans* suspension was added to yield a total volume of 0.5 ml and a lysozyme concentration of 2μg/ml. The bottles were incubated at 37°C in an orbital incubator (Gallenkamp) at 80 rpm for 5 hours. The control contained, 0.25 ml of the above candidal suspension (Section, 5.2.1), and 0.25 ml SDW or PBS, but no lysozyme; the latter too was incubated similar to the test sample. Subsequently, a sample of each suspension was inoculated on to Sabouraud's agar using the spiral plater system as described previously (Section 2.5.2), at 0, 1, 2, 3, 4, and 5 hours.

Dose response study and the inoculation procedure.

A dose response study was carried to investigate the effect of lysozyme on the viability of *Candida* species by using the method of Iacono *et al* (1980). To assess the relationship between the concentration of lysozyme and its fungicidal effect on *Candida,* the experiment was carried out with a single *C. albicans* isolate (a1). Serial dilutions of the enzyme from 4000 $\mu g\ ml^{-1}$ to 0.5 $\mu g\ ml^{-1}$ were made in bijou bottles containing 0.25 ml sterile distilled water. To each bottle 0.25 ml of *C. albicans* suspension prepared as above (Section 5.2.1) were added to yield a total volume of 0.5 ml. The bottles were incubated at 37°C in an orbital incubator (Gallenkamp) at 80 rpm. for 5 hours.

Using a spiral plater system (Spiral System Marketing: Baltimore, USA) 50 µl of each sample was inoculated on to SDA plate and incubated at 37°C for 18 hours. The resultant number of colony forming units (CFU's) (see section 2.5.1) of *C. albicans* (Figure 5.1) were then counted using a Gallenkamp colony counter (Gallenkamp). This experiment was repeated on three different occasions.

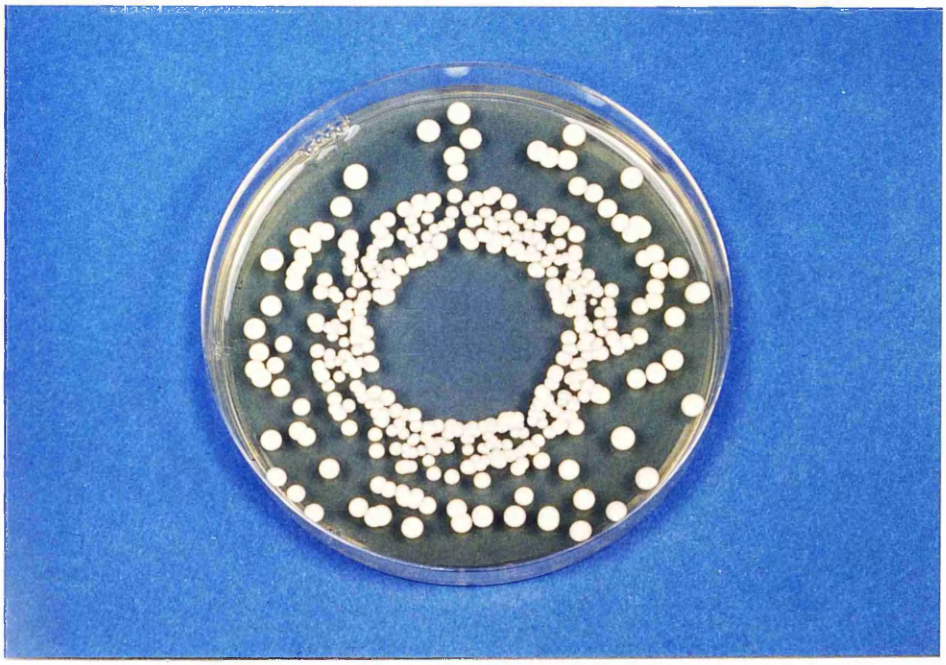


Figure 5.1. The use of spiral plater in counting lysozyme treated *Candida* species

The susceptibility of *Candida* species to lysozyme.

Data from the above dose-response study was used to compare the effect of a standard concentration of lysozyme on the relative growth inhibition of the examined *Candida* species. This part of the study was preformed by incubating 0.25 ml of the appropriate yeast suspension containing approximately $5\text{-}6 \times 10^7$ yeast/ml with 0.25 ml of lysozyme solution (final lysozyme concentration, 2 μg/ml) for 5 hours. Subsequently the number of CFU's was estimated as described previously. A suspension of 0.25 ml of the investigated *Candida* species in an equal volume of sterile distilled water was included as the control on each occasion the experiment was performed and the number of CFU's estimated.

5.2.4 Measurement of Fungicidal Activity of Lysozyme (F_{ly})

The fungicidal activity of lysozyme denoted F_{ly} was determined by computing the logarithmic ratio of the number of CFU obtained from the test suspension and number of the CFU from the control suspension, as follows.

Fungicidal activity of lysozyme

$$(F_{ly}) = -\text{Log} \frac{\text{CFU Test Suspension}}{\text{CFU Control Suspension}}$$

Thus the higher the F_{ly} value for a particular strain the more sensitive is the strain to lysozyme. For instance, F_{ly} value of zero means that the isolate in question is not susceptible to the activity of lysozyme and any value above zero indicates the degree of susceptibility to the enzyme.

All experiments with each organism were repeated in triplicate on three separate occasions.

5.2.5. Statistical analysis

Differences between the F_{ly} values of various *Candida* species were compared using the Kolmogorov-Smirnov test as it is a sensitive non-parametric test for demonstrating the differences in location skewness and dispersion (Massey 1951).

The regression was used to test the validity of the relationship between lysozyme concentration and candidal CFU's (Rafferty *et al.*, 1985).

5.3. Results.

5.3.1. Relationship between the period of exposure to phosphate buffered saline, and sterile distilled water on the fungicidal activity of lysozyme.

Results shown in Table 5.1 and Figure 5.2 indicate a marked inhibition of the lytic action of lysozyme when suspended in PBS, while that suspended in SDW was highly active against the yeast compared with the lysozyme-free control under similar conditions.

Incubation of *C. albicans* in sterile distilled water supplemented with lysozyme resulted in a significant loss of yeast viability after 5 hours (Figure 5.2).

5.3.2. Relationship between lysozyme concentration and fungicidal activity.

The sensitivity of *Candida albicans* isolates to a variety of lysozyme concentrations as indicated by the reduction in the CFU's after exposure to the enzyme is shown in Figure 5.3. The effect of lysozyme on yeast viability was apparent at an enzyme concentration greater than 0.5 µg/ml. Subsequently the number of CFU's decreased exponentially in a dose dependent manner until an enzyme level of approximately 30 µg/ml of lysozyme was reached. Very few yeasts survived at enzyme concentration between 30 to 1000 µg/ml and to eliminate all yeasts in the suspension a concentration of greater than 1000 µg/ml of lysozyme was required (Figure 5.3).

Linear regression analysis showed a significant positive correlation between the lysozyme concentration and CFU's. ($r = 0.96$, $p < 0.001$).

5.3.3. The fungicidal effect of lysozyme on *Candida* species.

The results of the relative potency of lysozyme in killing the six selected *Candida* species are shown in Tables 5.2 - 5.8 and Figure 5.4. Table 5.9 indicates percentage of F_{ly} values of 68 isolates of *Candida* species tested.

The results demonstrate that some *Candida* species such as *C. glabrata* are least susceptible to lysozyme while other such as *C. krusei* are very susceptible. However, there is also evidence of a difference in susceptibility to the enzyme among the isolates within a given species as exemplified by the wide range of Fly values of *C. tropicalis C. parapsilosis* (Table, 5.9). The examined *Candida* species were susceptible to lysozyme in the following order: *C. krusei* > *C. parapsilosis* > *C. tropicalis* > *C. guilliermondii* > *C. albicans* > *C. glabrata*

There was a significant difference between F_{ly} values of *C. glabrata* and the following *Candida* species *C. albicans* ($p < 0.001$), *C. guilliermondii* ($p < 0.15$) and *C. krusei* ($p < 0.01$). Similarly, F_{ly} values of *C. albicans* and *C. krusei* were significantly different ($p < 0.01$). However, there were no significant differences between *C. albicans* and *C. tropicalis*, *C. parapsilosis*, or *C. guilliermondii* (Kolmogorov-smirnov test was used).

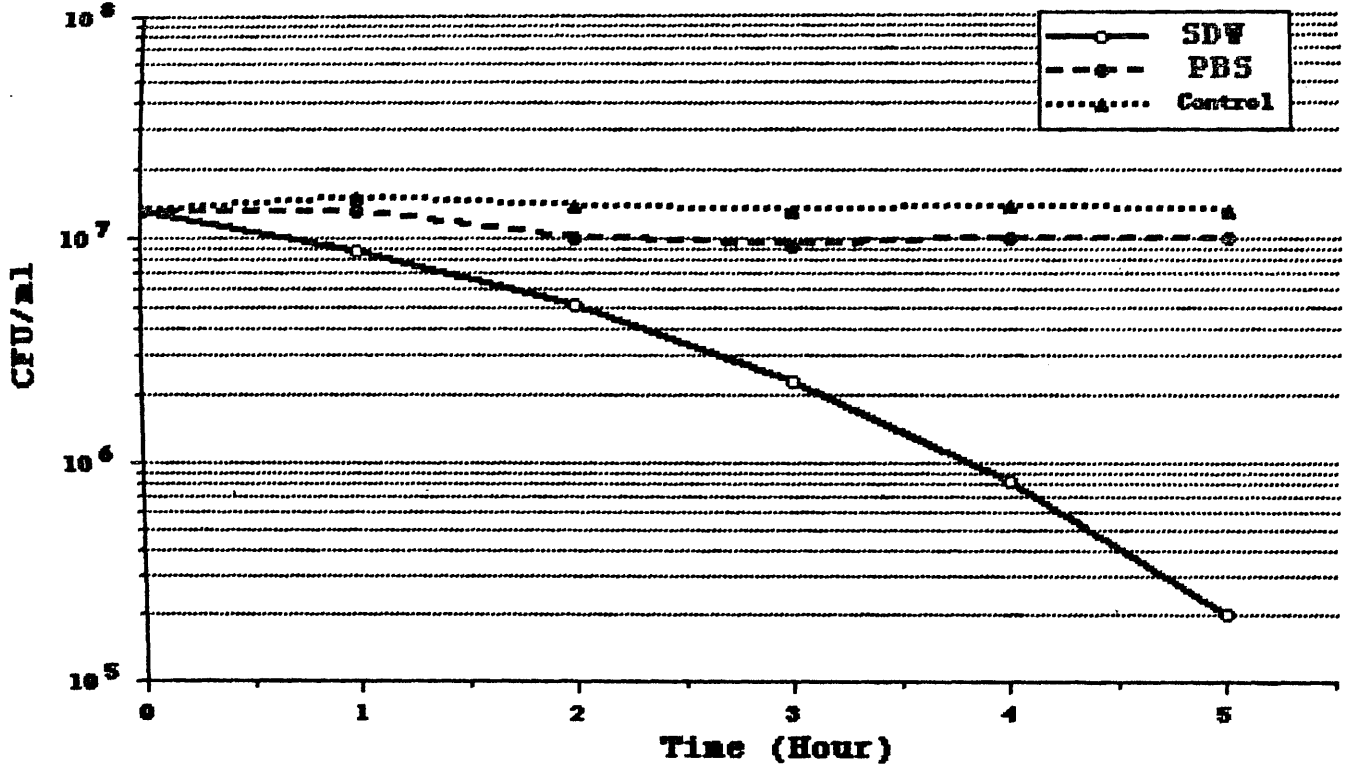


Figure 5.2. The relative susceptibility of *Candida albicans* strain a1 to either sterile distilled water (SDW —o—) or phosphate buffered saline (PBS --•--) containing 2 mg/ml lysozyme; control (SDW ···▲···) containing no lysozyme.

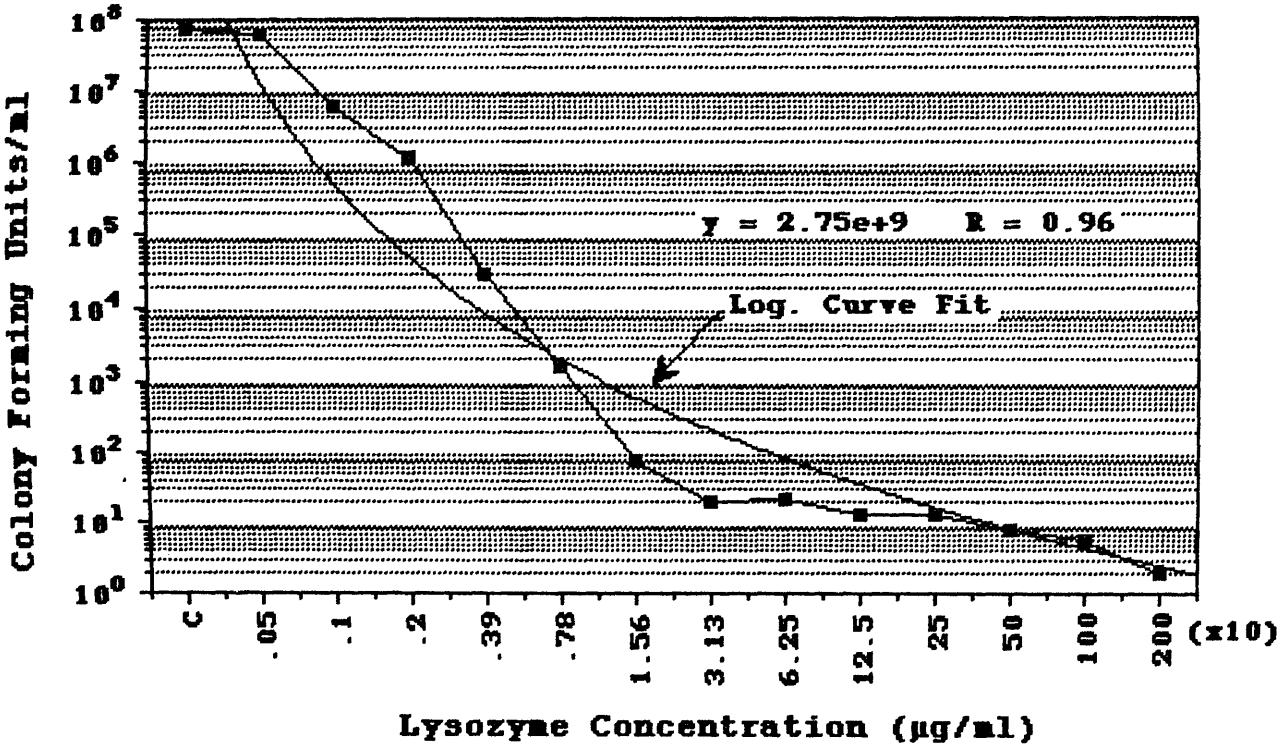


Figure 5.3. The susceptibility of *Candida albicans* strain a1 to a variety of lysozyme concentrations after 5 hours exposure to the the enzyme. Each circle represents the mean of three separate experiments. C=Control.

Table 5.1. The effect of sterile distilled water (SDW) and phosphate buffered saline (PBS) containing 2 μg/ml lysozyme on *Candida albicans* (a1).

Time (hours)	SDW containing lysozyme	PBS containing lysozyme	SDW Control
0	1.3×0^7	1.3×10^7	1.3×10^7
1	8.7×0^6	1.3×10^7	1.5×10^7
2	5.1×0^6	1.0×10^7	1.4×10^7
3	2.3×0^6	9.3×10^6	1.3×10^7
4	8.3×0^5	9.9×10^6	1.4×10^7
5	2.0×0^5	1.0×10^7	1.3×10^7

Table 5.2. The effect of 2 μg/ml lysozyme in distilled water on 22 *Candida albicans* strains.

	Experiment number			
Strain number	1	2	3	Mean
a1	4.9*	5.2	4.9	5.0
a2	3.1	2.7	3.0	2.9
a3	3.2	3.3	3.1	3.2
a4	2.3	2.3	2.4	2.3
a5	3.1	3.2	3.5	3.3
a6	2.9	3.1	3.1	3.0
a7	2.0	2.2	2.2	2.1
a8	1.7	2.0	2.0	1.9
a9	1.2	1.4	1.4	1.3
a10	2.2	1.9	2.0	2.0
a11	1.8	1.6	1.7	1.7

*(F_{1y}), please see the text.

Table 5.2. (Continued).

Strain number	Experiment number 1	2	3	Mean
a12	2.0*	2.4	2.5	2.3
a13	2.6	2.4	2.5	2.5
a14	2.2	1.9	2.1	2.1
a15	2.5	2.4	2.4	2.4
a16	2.5	2.4	2.3	2.4
a17	1.1	1.2	1.2	1.2
a18	2.0	2.3	2.3	2.2
a19	2.5	2.6	2.6	2.6
a20	2.6	2.4	2.7	2.6
a21	3.0	3.4	3.4	3.3
a22	1.3	1.3	1.8	1.5

*(F_{1y}), please see the text.

Table 5.3. The effect of 2 μg/ml lysozyme in distilled water on 20 *Candida glabrata* strains

	Experiment number			
Strain number	**1**	**2**	**3**	**Mean**
g1	1.5*	1.4	1.3	1.4
g2	0.3	0.3	0.3	0.3
g3	0.0	0.0	0.0	0.0
g4	0.0	0.0	0.0	0.0
g5	0.1	0.1	0.1	0.0
g6	1.0	1.2	1.4	1.2
g7	1.6	2.0	1.8	1.8
g8	0.3	0.4	0.4	0.4
g9	0.0	0.0	0.0	0.0
g10	0.0	0.0	0.0	0.0

*(F_{1y}), please see the text.

Table 5.3. (Continued)

Strain number	Experiment number 1	2	3	Mean
g11	0.0*	0.0	0.0	0.0
g12	0.0	0.0	0.0	0.0
g13	0.1	0.1	0.1	0.1
g14	0.4	0.4	0.4	0.4
g15	0.0	0.0	0.0	0.0
g16	0.1	0.1	0.1	0.1
g17	0.1	0.1	0.1	0.1
g18	0.4	0.5	0.5	0.5
g19	0.1	0.1	0.0	0.0
g20	0.1	0.1	0.1	0.1

*(F_{1y}), please see the text.

Table 5.4. The effect of 2 μg/ml of lysozyme in distilled water on 7 *Candida tropicalis* strains.

	Experiment number			
Strain number	**1**	**2**	**3**	**Mean**
t1	4.3*	4.5	4.2	4.3
t2	3.3	3.2	3.1	3.2
t3	3.7	3.6	3.8	3.8
t4	1.5	1.0	2.0	1.5
t5	5.2	4.7	4.9	4.9
t6	1.0	2.0	2.0	1.7
t7	3.5	3.3	3.8	3.5

*(F_{ly}), please see the text.

Table 5.5. The effect of 2 μg/ml of lysozyme in distilled water on 7 *Candida krusei* strains.

Strain number	Experiment number 1	2	3	Mean
k1	4.6*	4.3	4.2	4.4
k2	4.8	4.6	4.7	4.7
k3	4.2	4.9	4.3	4.5
k4	4.4	3.9	4.3	4.6
k5	4.7	4.8	4.7	4.7
k6	4.1	4.2	4.5	4.3
k7	4.5	4.7	4.7	4.7

*(F_{ly}), please see the text.

Table 5.6. The effect of 2 μg/ml of lysozyme in distilled water on 5 *Candida parapsilosis* strains.

	Experiment number			
Strain number	**1**	**2**	**3**	**Mean**
p1	3.9*	3.8	3.8	3.8
p2	2.7	2.9	2.6	2.8
p3	1.7	1.4	1.1	1.4
p4	4.5	4.1	4.3	4.3
p5	4.4	4.5	4.4	4.4

*(F_{ly}), please see the text.

Table 5.7. The effect of 2 μg/ml of lysozyme in distilled water on 5 *Candida guilliermondii* strains.

Strain number	Experiment number 1	2	3	Mean
gu1	4.2*	4.6	4.7	4.4
gu2	0.2	0.2	0.2	0.2
gu3	5.0	4.8	5.0	4.9
gu4	0.3	0.3	0.5	0.4
gu5	4.3	4.9	4.5	4.6

*(F_{ly}), please see the text.

Table 5.8. Mean of F_{ly} values of six different *Candida* species.

Candida isolates	number of isolates	F_{ly} values Mean (± SD)
C. albicans	24	2.5* (± 0.80)
C. glabrata	20	0.3 (± 0.50)
C. tropicalis	07	3.3 (± 1.29)
C. krusei	07	4.5 (± 0.18)
C. parapsilosis	05	3.3 (± 1.28)
C. guilliermondii	05	2.9 (± 2.38)

*(F_{ly}) please see the text. SD=Standard deviation.

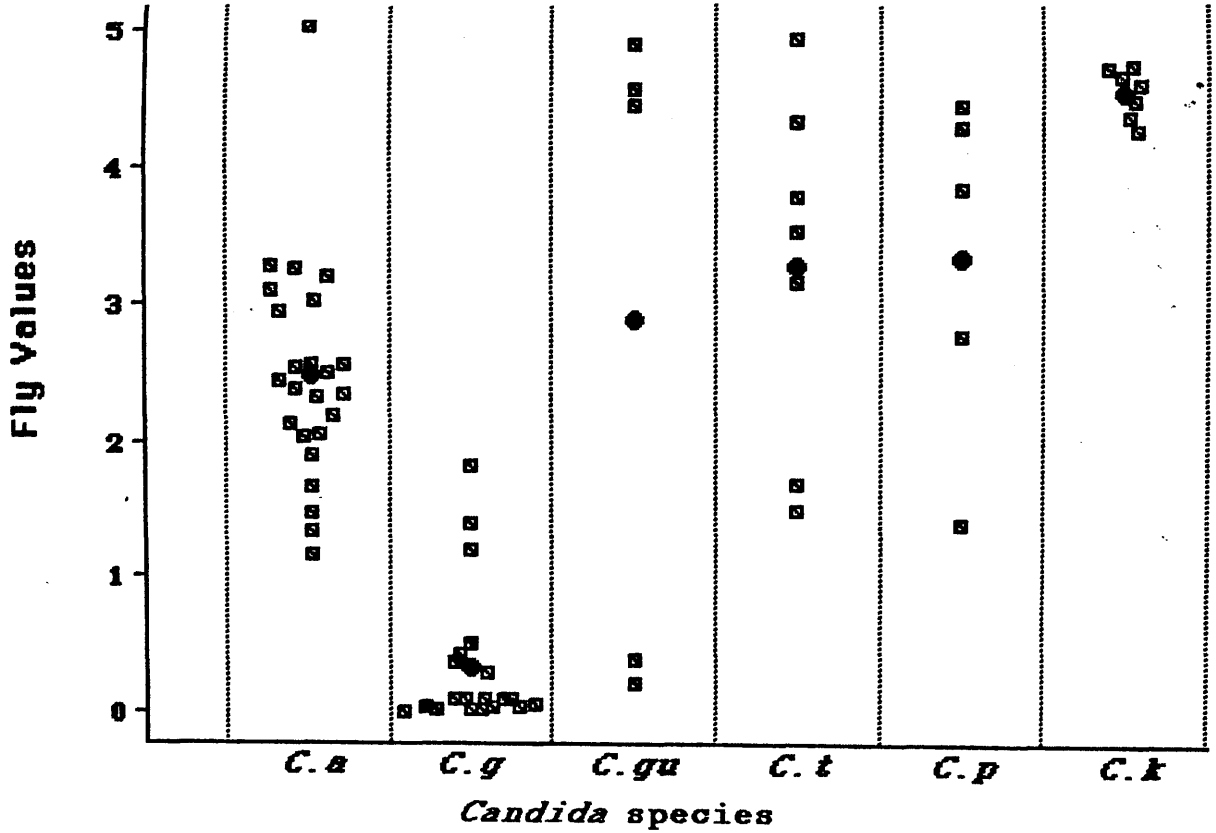


Figure 5.4. A summary of the lytic activity of lysozyme for different *Candida* species. Each square represents the mean of three separate experiments.
● =Mean.
C.g =C. glabrata, C.a =C. albicans, C.gu =C. guilliermondii, C.t =C. tropicalis, C.p =C. parapsilosis, C.k =C. krusei

Table 5.9. The percentage distribution of F_{ly} values among different *Candida* species.

Candida isolates	number of isolates	% with F_{ly} values >5	>4-5	>3-4	>2-3	>1-2	>0-1
C. albicans	**24**	4.2	0.0	20.8	**54.2**#	20.8	0.0
C. glabrata	**20**	0.0	0.0	0.0	15	**75**	10
C. tropicalis	**7**	0.0	28.6	**42.9**	0.0	28.6	0.0
C. krusei	**7**	0.0	**100**	0.0	0.0	0.0	0.0
C. parapsilosis	**5**	0.0	**40**	20	20	20	0.0
C. quilliermondii	**5**	0.0	**60**	0.0	0.0	0.0	**40**

*(F_{ly}) please see the text. SD=Standard deviation. #Highest values in bold

5.4.Discussion.

The results of this study indicate that *Candida* species are either inhibited or destroyed *in vitro* by the action of lysozyme. The results contradict those of some previous workers who were unable to demonstrate the fungicidal activity of lysozyme in the absence of either glucose or amphotericin B in the suspending medium (Collins and Pappagianis 1974; and Kamaya 1970). Thus, Kamaya (1970) found that addition of 0.1 percent (5.5 mM) glucose to the medium was required to potentiate the candidacidal activity of lysozyme on five clinical isolates of *C. albicans* and Collins and Pappagianis (1974) could not inhibit the growth of three isolates of *C. albicans* by lysozyme without the addition of amphotericin B. Both Kamaya (1970), and Collins and Pappagianis (1974) used distilled water suspensions of yeasts and hen egg white lysozyme at a concentration > 2000 µg/ml. The results of this study however, agree with those of Marquis *et al* (1982) who demonstrated fungicidal activity of hen egg white lysozyme when *C. albicans* was suspended in a medium containing malt and beef extract and glycerol.

Interaction of lysozyme with cell membrane components has been described. Membranes composed of beef heart structural protein and soybean phospholipids readily bound lysozyme (Romoeo and de Benard 1966). This association of lysozyme with the artificial membrane was electrostatic, as washing the complex with 0.01 M tris acetate buffer in 0.25 M sucrose failed to elute bound lysozyme while washing with 0.15 M potassium chloride eluted 85 per cent of the bound enzyme.(Joos and Carr 1969). Lysozyme is a reactive protein

that has been shown to complex with a variety of acidic polymers including RNA and DNA (Skarnes and Watson 1955); hence, the presence of lysozyme within cells might lead to disruption of normal function.

A significant dose response relationship between lysozyme concentration and fungicidal activity ($r = 0.96$, $p < 0.001$) was found in this study which similar to that described by Marquis *et al* (1982). Nevertheless, Kamaya (1970) could not demonstrate such a relationship even in the presence of added glucose which enhanced the fungicidal potency. However, as is the present study, the latter investigator did find variations in the degree of susceptibility of different *C. albicans* isolates to lysozyme. Interestingly, strain variations in the susceptibility of bacteria such as *Streptococcus mutans* to lysozyme have been previously demonstrated, and it is thought that extracellular polysaccharide synthesized by the bacteria may play a role in this phenomenon (Iacono *et al.*, 1980). The strain variation in susceptibility of *C. albicans* isolated could be explicable in similar terms as *C. albicans* is known to elaborate an outer surface layer depending on the growth media (Samaranayake and MacFarlane 1980; Douglas Houston McCourtie 1981; Kennedy and Sandin 1988), age of the culture (Pugh and Cawson 1978) and the source of the isolate (Masler *et al.*, 1966). Further studies are required to ascertain the role of the extracellular cell wall components in the susceptibility of *C. albicans* to lysozyme.

The dramatic reduction in the fungicidal activity of lysozyme when suspended in PBS in this study is worthy of note. This may be

due to the presence of inorganic salts in the medium which are known to adsorb the highly cationic lysozyme (Collins and Pappagianis 1974). It implies that *in vivo* biological fluids such as saliva may modulate the enzyme potency by virtue of their inorganic ionic content.

When the effect of lysozyme on a variety of *Candida* species was examined *C. glabrata* and *C. albicans* were found to be most resistant to the enzyme and *C. krusei* the most sensitive. As mentioned above, this may reflect the subtle variations in the cell wall composition between various *Candida* species. Indeed some workers have suggested that *C. krusei* should be re-classified into a different genus based on the ultrastructure and chemical composition of the cell wall and co-enzyme Q numbers (Hagler and Ahearn 1981; Yamada and Kondo 1972).

In clinical terms, the relatively high resistance of *C. albicans* to lysozyme may at least partly explain their high oral and vaginal carriage rate in healthy individuals, as opposed to the rare presence of more lysozyme sensitive *Candida* species such as *C. krusei* (Odds 1988). On the other hand, the increased oral carriage of *C. albicans* as well as the more lysozyme sensitive *C. glabrata*, a species observed in patients with reduced salivary flow due to irradiation (Martin Al-Tikriti and Bramley 1981) cytotoxic therapy (Main *et al* 1984; Samaranayake *et al.*, 1984) or disease such as Sjögren's syndrome (MacFarlane and Mason 1973) could be due, at least partly to the concomitant reduction in salivary lysozyme.

There are no studies in the literature investigating the biochemical mechanisms by which lysozyme may interact with fungal cell walls. However, lysozyme (ß-1→4-glycan hydrolase) basically interacts with bacteria by hydrolysing ß-1→4-glycosidic linkages of the cell wall peptidoglycan although, many bacteria contain lysozyme-sensitive mucopeptide and yet are resistant to lysis by this enzyme (Gould and Hitchins 1963). Several investigators have reported that certain gram-positive microorganisms which are normally resistant to the lytic action of hen egg white lysozyme, undergo gross cellular lysis in the presence of this enzyme together with either sodium chloride, or sodium dodecyl sulfate (Iacono *et al.*, 1980; Goodman *et al.*, 1981). Also it has been reported (Pollock *et al* , 1976) that a significant yeast lysis was observed at salt molarities within normal salivary concentrations of lysozyme.

It is interesting to speculate on the mechanism by which lysozyme exerts its fungistatic or fungicidal properties. Due to the extremely high cationic charge of lysozyme it is positively charged at all physiological pH's (Petit and Jolles 1963) and therefore, the enzyme may avidly bind to the yeast cell wall mannans and thereby, influence yeast growth in some, as yet unknown, manner. Alternatively lysozyme may exert its effect by activation or deregulation of autolytic enzymes as lysozyme receptors on yeast cell walls may be important in the regulation of autolysin formation. However, as yeast cell walls do not possess ß-1→4-linkages (Odds 1988) it is possible that lysozyme may act on yeasts by a non-enzymatic manner. Nevertheless, Marquis *et*

al.(1982) argue that the damage may be the result of enzymatic hydrolysis of the n-glycosidic bonds that link polysaccharides and structural proteins of the cell wall.

It has been mentioned that lysozyme acts on the glycosidic linkages between n-acetylmuramic acid and n-acetylglucosamine residues of the bacterial cell wall peptidoglycan. Goodman *et al.*, (1981), demonstrated a peptidoglycan loss of *Staphylococcus. mutans*, during hen egg white lysozyme-inorganic salt lysis. Thus, both the enzyme and inorganic salts may effect the glycosidic linkages in the bacterial cell wall. In which case the absence of this linkage from candidal cell wall may explain, in part, why PBS did not enhance candidal inhibition by lysozyme. Whatever this mechanism may be it is tempting to speculate that lysozyme may be actively involved in modulating the yeast population in the oral cavity. However, much remains to be discovered about the molecular mechanisms by which this enzyme operates in both bacterial and yeast cell walls and the factors that are involved in lysozyme mediated inhibition of candidal growth.

CHAPTER SIX

CANDIDACIDAL ACTIVITY OF RAT BRONCHOALVEOLAR LAVAGE FLUID

6.1. Introduction.

The role of many non-specific inhibitors of *Candida albicans* present in host body fluids especially those bathing the human mucosal surfaces in the pathogenesis of candidosis, have attracted the interest of many investigators (Samaranayake Hughes, Weetman and MacFarlane 1986; Nugent and Fick, 1987; Tobgi, Samaranayake and MacFarlane 1988). Thus, in chapter five the effect of lysozyme on several *Candida* species was examined and the results demonstrated that lysozyme may act as an inhibitor of candidal growth in the oral cavity. Also, it has been shown recently, that bronchoalveolar lavage fluid (BLF) may exert an anti-candidal effect.(Nugent and Fick 1987). This property of BLF may partly explain the fact that pneumonia due to *C. albicans* is very unusual, despite the general increase in all types of fungal infections in recent years (Odds 1988).

The major process by which large particles such as *Candida albicans* and other fungi are cleared from the lungs is probably via the mucocilliary transport system (Green 1968), although additional clearance mechanisms must exist for those large particles which evade the mucocilliary action, (Peterson and Calderone 1977). These observations therefore, suggest the

presence of potent lung defences against *Candida* because the isolation of yeasts from sputum samples are relatively uncommon (Rosenbaum, Barber and Stevens 1974; and Hurley *et al.*, 1986).

Recently, Nugent and Pesanti (1982) monitored intra-alveolar killing of staphylococci by use of BLF and obtained evidence that inhaled staphylococci are killed mainly outside alveolar macrophages. Therefore, this *in vitro* investigation was performed to obtain further information about the effect of macrophages-free bronchoalveolar lavage fluid collected from rat lungs on the viability of different *Candida* species.

6.2. Materials and methods.

6.2.1. Candidal isolates and growth conditions.

Forty nine clinical isolates of *Candida* species, 22 *C. albicans*, 3 *C. glabrata*, 7 *C. tropicalis*, 5 *C. parapsilosis*, 7 *C. krusei*, and 5 *C. guilliermondii*, were investigated (See Maintenance of *Candida* species Chapter Two). Yeast cells were grown on Sabouraud's dextrose agar plates (SDA; Oxoid) incubated aerobically at 37°C for 24 hours. A loopful of the confluent growth was transferred to 10 ml of Sabouraud's broth medium (Difco) supplemented with 4 percent glucose (British Drug House) and incubated at 37°C on an orbital incubator at 100 rpm for 18 hours. The growth was harvested by centrifuging the culture at 1500 g for 10 minute. The cells were washed twice in 10 ml of

sterile phosphate buffer saline (PBS pH 7.2) and adjusted with PBS to give a final concentration of $2\text{-}2.5 \times 10^7$ yeast cells per ml. The number of yeast cells per ml was regulated, in all the experiments, by means of a haemocytometer counting chamber (Hawksley, England), (see Chapter Two, section, 2.5.1)

6.2.2. Animals

Twelve young adult male Sprague Dawley rats, 200-300 grams in weight (Bantin and Kingman, Glasgow) were used. These animals were involved in preliminary experiments to study oral candidosis and therefore, were split into two groups; a control group comprising five animals and a test group, comprising seven animals. The latter group, received tetracycline hydrochloride, a carbohydrate rich diet and challenged with *Candida albicans* (a15) as described below

6.2.3. Carbohydrate rich diet

The carbohydrate rich diet comprised 62 percent carbohydrate (Sucrose, 39%, Glucose, 16% and Fructose 7%), 33 percent proteins (Casein, 26%, Gelatin, 6% and Methionine, 0.78%) vitamins and minerals.

6.2.4. Tetracycline hydrochloride

Two concentrations of tetracycline hydrochloride were used in distilled water, 0.1 and 0.01 percent. The former was given to the rats during week 0, and the latter used for the remainder of the experiment (Russell and Jones 1973), (See Table 6.1)

Table 6.1. The animal experimental schedule.

Week Number	Treatment
WEEK 0	0.1% tetracycline hydrochloride* Carbohydrate-rich diet
WEEK 1	Three oral inoculations of *C. albicans* (mon., Wed., & Fri.) 0.01% tetracycline hydrochloride Carbohydrate-rich diet
WEEK 2	Two inoculations of *C. albicans* (mon., & Fri.) 0.01% tetracycline hydrochloride Carbohydrate-rich diet
WEEK 3	Two inoculations of *C. albicans* (mon., & Fri.) 0.01% tetracycline hydrochloride Carbohydrate-rich diet
WEEK 4	Two inoculations of *C. albicans* (mon., & Fri.) 0.01% tetracycline hydrochloride Carbohydrate-rich diet
WEEK 5	0.01% tetracycline hydrochloride Carbohydrate-rich diet Animals killed at the end of this week.

*40 ml per day per animal.

6.2.5. The experimental schedule

The experimental schedule used for the test group of rats is shown in Table 6.1. Before the test group rats were challenged with the yeast, they were given 40 ml/day of a 0.1 percent aqueous solution of tetracycline hydrochloride and 30 gm of the carbohydrate rich diet per animal per day, for seven days (week 0). During week 1 the rats continued to receiving the same carbohydrate diet, but the concentration of tetracycline hydrochloride in the drinking water was reduced to 0.01 percent, and this was used (40 ml/day) throughout the experimental period. The mouths of the rats were inoculated with 0.2 ml of the candidal suspension (See section 6.2.1) by means of a tuberculin syringe. Anesthetics were not necessary during this procedure. Three doses of candida were given in the first week and this was reduced, subsequently, to two doses until the end of the week 4.

The control group of animals consisted of five rats who received no carbohydrate-rich diet, no tetracycline hydrochloride and were not inoculated with the yeast.

6.2.6 Bronchoalveolar Lavage fluid

Rats in both the test and control groups were killed by overdose of intraperitoneal barbiturate (Nembutal) at the end of week 5 (in the case of the test rats, one week after the last inoculation with *Candida*). The lungs, heart, and trachea were

carefully, dissected out as a single block into a numbered, sterile Petri-dish. A tracheotomy was made with a blunt 22-gauge needle, and then a plastic catheter was inserted into the trachea and secured with a tie. Each lung was infused twice with 5 ml of sterile PBS (pH 7.2) and the resultant lavage was aspirated. The lavage fluid was collected into a conical tube and centrifuged immediately at 300 g for 10 minutes to remove macrophages and large particles. The supernatant was aliquoted (1 ml) into small sterile bottles which were, marked and stored at -20 ^{o}C. The clear, cell-free (centrifuged) lavage fluid was used for the assay as described below.

6.2.7 Candidacidal assay

The effect of bronchoalveolar lavage fluid (BLF) on the viability of *Candida* species was tested by adding 0.25 ml of BLF and 0.25 ml of a yeast suspension containing 2-2.5 x 10^6 yeast per ml, (prepared as above 6.2.1) to a bijou bottle, giving a total volume of 0.5 ml. The bottles were incubated at 37 ^{o}C in an orbital incubator at 80 rpm. (Gallenkamp) for 0, 30, 60 and 120 minutes.

Each sample was examined microscopically for presence of clumps of yeast cells. Using a spiral plater system (Spiral system Marketing; Baltimore, USA), 50 µl of each sample were inoculated on to a SDA plate and incubated at 37^{o}C for 18 hours. In each experiment the resultant number of viable yeasts were then counted using a Gallenkamp colony counter (Gallenkamp). Each experiment was repeated on three different occasions.

6.2.8. Comparison of serum, and bronchoalveolar lavage fluid on *Candida albicans*

It is known that serum contains heat-stable agglutinating factors that cause a reduction in the viable count of *Candida*, which appear to be due to clumping of the yeast cells rather than due to a true microbicidal action (Louria *et al.*, 1972). Therefore, serum was included as a control using the above candidal suspension (2-2.5 x 10^6 yeast cells/ml) as follows: 0.25 ml of the yeast suspension was mixed with an equal volume of undiluted sterile horse serum (replacing BLF) (Gibco, Glasgow, UK) in a bijou bottle. Phosphate buffered saline was included as a serum and BLF-free control. The three bottles-the test suspension in BLF and serum, and control suspension in PBS were then incubated and treated in exactly the same manner as the test mentioned above (6.2.7). Samples and the number of viable yeasts estimated.

6.2.9. The effect of heat-inactivation on the anti-candida effect of bronchoalveolar lavage fluid

In order to eliminate heat-labile proteins (e.g., complement), a heat inactivation study was preformed by incubating the BLF in a water bath at 56°C for 30 minutes prior to the assay procedure. Inactivated BLF (0.25 ml) from test rat R2 was incubated with 0.25 ml of *Candida albicans* suspensions (strains a2, a11 and a15) containing approximately 2-2.5 x 10^6 yeast per ml, for 120 minutes. Subsequently the number of CFU's was

estimated as described above. A suspension of 0.25 ml of the investigated *Candida* species in an equal volume of unheated BLF was included as the control on each occasion the experiment was preformed. This experiment was repeated in duplicate on three occasions with each *Candida* isolate.

6.2.10 The effect of bronchoalveolar lavage fluid concentration, on candidacidal activity

In order to assess the relationship between the concentration of BLF and its candidacidal effect, the following experiment was carried out with a single *C. albicans* strain.(a15). Two-fold dilutions of BLF (R2) from neat to 1:100, were made in bijou bottles containing 0.25 ml sterile distiled water. To each bottle 0.25 ml of the *C. albicans* suspension containing approximately $2\text{-}2.5 \times 10^6$ yeast per ml (prepared as above 6.2.1) was added to yield a total volume of 0.5 ml. The bottles were incubated at 37 °C in an orbital incubator at 80 rpm. for 120 minutes. A control with the BLF, replaced by 0.25 ml of PBS was used

Each experiment was repeated on three different occasions. Data from this dose-response study was used to compare the effect of a standard concentration of BLF on the relative growth inhibition of the *Candida* species examined.

6.2.11. Assessment of variation in the anti-candida activity of bronchoalveolar lavage fluid from different rats

Candida albicans strain (a15) was used to assess possible variations in the anti-candida activity of BLF from different animals. Bronchoalveolar lavage fluids from 7-test and 5-control rats (see section 6.2.2) were tested using the candidacidal assay method described above (see section 6.2.7) in which a 0.25 ml of the particular BLF and 0.25 ml of the candidal suspension containing 2-2.5 x 10^6 yeast/ml were mixed in a bijou bottle. The bottles were incubated at 37°C in an orbital incubator at 80 rpm. for 0, 30, 60 and 120 minutes. The results for the anti-candida effect of BLF from the 12 rats were then compared.

6.2.12. The anti-candidal effect of bronchoalveolar lavage fluid on the viability of *Candida* species

The effect of BLF from two rats (R3 and R4) on the following yeast species was investigated: 22 *C. albicans,* 7 *C. krusei,* 7 *C. tropicalis,* 5 *C. parapsilosis,* and 5 *C. guillier-mondii.* Aliquots (0.25 ml) of the different yeast suspensions (2-2.5 x 10^6 yeast/ml), were added to 0.25 ml of the BLF from the rats R 3 or R 4 in a bijou bottle, then incubated at 37 °C in an orbital incubator at 80 rpm. for 0 and 120 minutes. The candidal assay was carried out as described above (6.2.7).

6.2.13 Statistical analysis

The *t* -test was used in case of two group analysis. Whereas ANOVA test was used in comparison of more than two population means. The significant confidence intervals of the results were tested at 95 percent, and the means were recorded.

6.3. Results

6.3.1. A comparison between serum and bronchoalveolar lavage fluid on the viability of three *Candida albicans* isolates

The results shown in Figures 6.1, 6.2, 6.3 illustrate the effect of BLF and serum compared with the control PBS, on the viability of *C. albicans* isolates (a2, a11, and a15). Note the drop in the survival rate of *C. albicans* when exposed to BLF compared with the serum and PBS (Figures, 6.1, 6.2, 6.3). Candida agglutination did not occur during the incubation of the yeast cells ($2\text{-}2.5 \times 10^6$ yeast/ml) with either the BLF or PBS (control) as assessed microscopically (Olympus microscope, X400 magnification). However, when higher concentrations of yeast suspension ($2\text{-}2.5 \times 10^7$ yeast cells per ml) was used, a higher number of clumped yeast cells were observed with serum. Also BLF in higher yeast concentration demonstrated very little agglutination compared with the serum. Therefore, a candidal suspension of $2\text{-}2.5 \times 10^6$ yeast/ml was used, and significant differences ($p < 0.001$) (Tables 6.2, 6.3 and 6.4).were observed when either PBS control or the serum were compared with the active BLF. However, when the serum was compared with the PBS control, no significant difference was found with strain a11, while strains a2 and a15 did show a significant differences ($p = <0.05$) Tables 6.2, 6.3 and 6.4.

6.3.2. A comparison of the effect of bronchoalveolar lavage fluid from the 5 control and 7 test rats on *Candida albicans* isolate (a15)

The data in Tables, 6.5 and 6.6 show the results of BLF from 5-control (untreated) rats on *Candida albicans* strain a15, and Figure 6.4 demonstrates the survival rate of a15 when incubated with BLF from control rats, over 30, 60, and 120 minutes, compared with 0 time. Increase in the period of incubation resulted in a proportional reduction in the percentage viable cells exposed to BLF of all rats. Tables, 6.7 and 6.8 show the effect of BLF from 7-test rats (who received a high carbohydrate rich diet, tetracycline and were challenged with *C. albicans*) (see section 6.2.2), and Figure 6.5 illustrates the anti-candidal effect of BLF from the seven test-rats on *C. albicans* a15 at 30, 60, and 120 minutes compared with the 0 time. As in the previous experiment, increased period of exposure to BLF resulted in decrease in viability of the *Candida*.

Table, 6.9, shows multiple comparisons of pair analysis of the results for the BLF activity from 5-control rats. Results revealed no individual variations between BLF from control-rats in terms of their anti-*Candida* activity on *C. albicans* strain (a15). However, the statistical multiple comparisons of pair analysis of the 7-test rats (Table, 6.10), indicated slight variations in these anti-*Candida* activity (on *C. albicans* strain a15), especially with BLF from rat 4 of this group. For instance, the BLF from latter (R4) was less candidacidal when

compared with BLF from either rat R1, R2, R3 or R6. In comparing the mean percentage survival rates of the yeasts effected by BLF from the test and control rats, (Table, 6.11) significant differences between the potency of BLF from individual rats were seen. Thus, 12 out of 35 pair-comparisons demonstrated significant differences (Table, 6.11). However, when the overall mean candidacidal activity of BLF from control and test rats were compared no significant difference was found. Table, 6.12, and Figure, 6.6, show no statistical difference between the mean of candidacidal effect of the two groups on isolate a15. ANOVA and *t*-test were implicated in these analysis.

6.3.3. The effect of heat-inactivated bronchoalveolar lavage fluid on the viability of three *Candida albicans* isolates

The next series of experiments were performed to assess the relative potency of normal and heat-inactivated BLF in suppressing the growth of three selected *Candida albicans* strains (a2, a11 and a15). The results are shown in Tables 6.13, 6.14, 6.15, and Figure 6.7. Generally, heat-inactivated BLF from rat 2 (test rat) was less effective in killing or suppressing the viability of *C. albicans* isolates a2, a11 and a15 compared with normal BLF from the same rat. Figure, 6.7 shows decreased susceptibility of *C. albicans* isolates a2, a11 and a15 on exposure to the heat inactivated BLF when compared

with normal BLF Table, 6.16. There was a significant difference between the PBS control and candidacidal effect of heat treated BLF. There were also significant differences between PBS control and untreated BLF ($p<0.05$). However, there was no significance between untreated and heat-inactivated BLF although heat-inactivated BLF demonstrated a lowered killing potency.

6.3.4 The effect of bronchoalveolar lavage fluid concentration on the viability of *Candida albicans* isolate (a15)

The sensitivity of a *Candida albicans* isolate (a15) to a variety of BLF concentrations measured by the reduction in the CFU's after exposure to the fluid was tested. Table, 6.17 shows the relationship between the concentration of BLF and its inhibitory effect on the viability of the *C. albicans* a15. Results indicated that the BLF at a dilution of less than 1/32 could significantly reduce the viability of the yeast. Figure 6.8 shows the dose response curve for BLF from test rat R2, and yeast viability.

6.3.5 The effect of bronchoalveolar lavage fluid on 22 isolates of *Candida albicans*

The effect of BLF from two test rats (R3 and R4) on the survival of 22 *C. albicans* isolates, is shown in Tables 6.18 and 6.19.respectively. Highly statistically significant difference in the susceptibility of the isolates to the BLF from rat 3 was noted. Table, 6.21 shows examples of the significantly

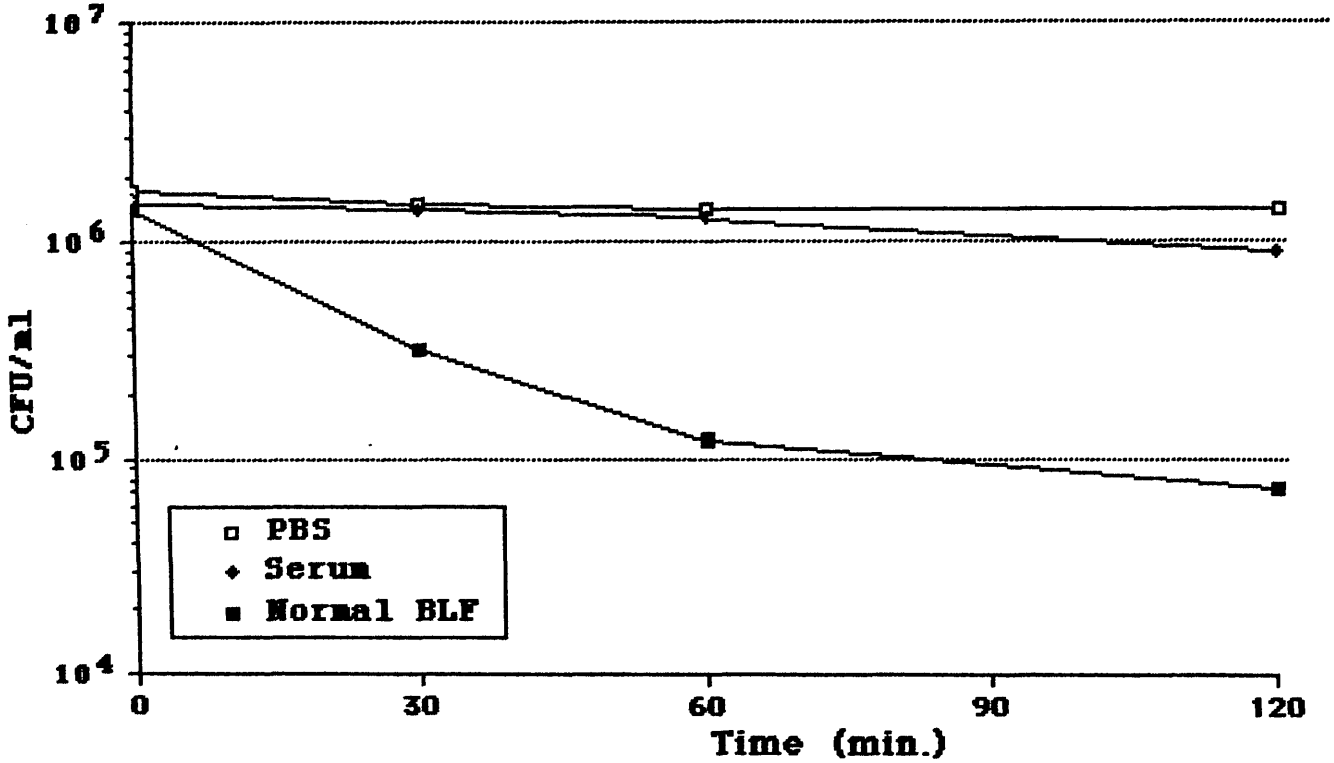


Figure 6.1. The survival of *Candida albicans* strain a2, when exposed to normal bronchoalveolar lavage fluid (BLF) from rat R2 together with phosphate buffered saline (PBS) and serum controls.

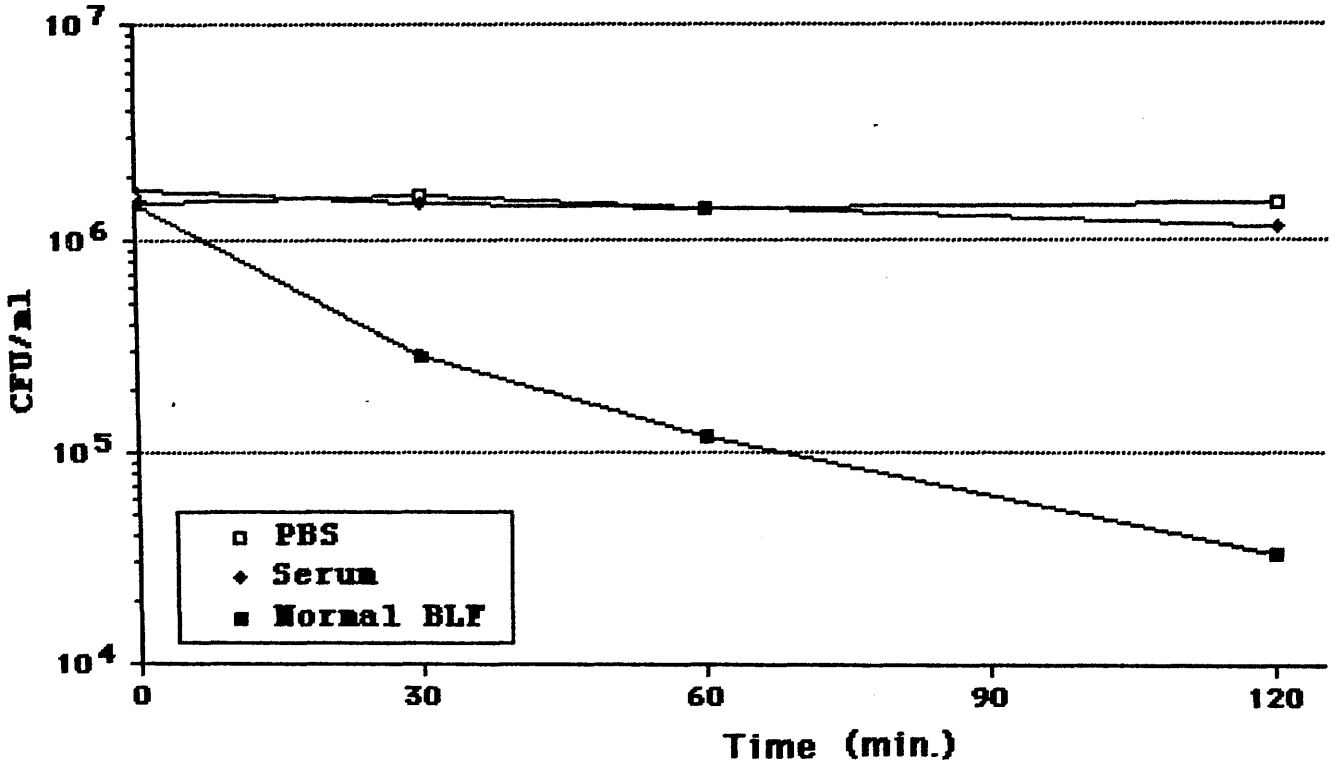


Figure 6.2. The survival of *Candida albicans* strain a11, when exposed to normal bronchoalveolar lavage fluid (BLF) from rat R2 together with phosphate buffered saline (PBS) and serum controls.

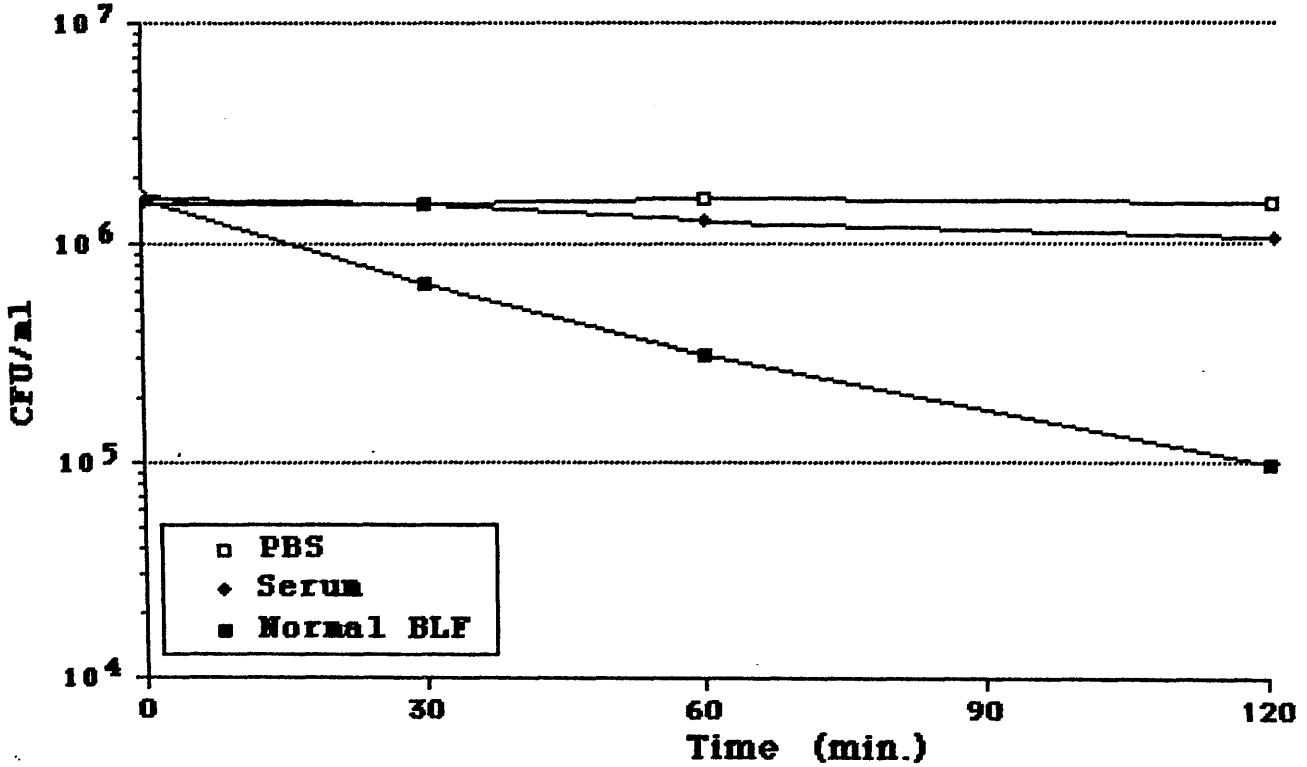


Figure 6.3. The survival of *Candida albicans* strain a15, when exposed to normal bronchoalveolar lavage fluid (BLF) from rat R2 together with phosphate buffered saline (PBS) and serum controls.

Table 6.2. A Statistical comparison of the effect of serum, and normal bronchoalveolar lavage fluid (BLF) from rat 2 for 120 minutes. on *Candida albicans* isolate a2.

Comparison	Significance	*t*-test
Control (PBS)* V Serum	S	$p < 0.05$
Control (PBS)* V Normal BLF	S	$p < 0.001$
Serum V Normal BLF	S	$p < 0.001$

***PBS control contains no BLF.**

Table 6.3. A Statistical comparison of the effect of serum, normal bronchoalveolar lavage fluid (BLF) from rat 2 for 120 minutes. *Candida albicans* isolate all.

Comparison	Significance	*t*-test
Control* V† Serum	NS	$p > 0.05$
Control* V Normal BLF	S	$P < 0.001$
Serum V Normal BLF	S	$P < 0.001$

***PBS control contains no BLF.**

†v = versus

Table 6.4. A Statistical comparison of the effect of serum, and normal bronchoalveolar lavage fluid (BLF) from rat 2 for 120 minutes. *Candida albicans* isolate a15.

Comparison	Significance	*t*-test
Control (PBS)* v Serum	S	$P < 0.05$
Control (PBS)* v Normal BLF	S	$P < 0.001$
Serum v Normal BLF	S	$P < 0.001$

***PBS control contains no BLF.**

v = versus

Table 6.5: The candidacidal effect of bronchoalveolar lavage fluid (BLF) of 5-control rats (see section 6.2.2) on *Candida albicans* strain a15.

Rat number	Time 0 minutes			Time 30 minutes		
	1‡	2	3	1	2	3
RC1	$9.7x10^5$*	$9.0x10^5$	$8.7x10^5$	$6.8x10^5$	$7.2x10^5$	$6.4x10^5$
RC2	$7.0x10^5$	$6.7x10^5$	$6.4x10^5$	$4.2x10^5$	$4.4x10^5$	$4.6x10^5$
RC3	$7.2x10^5$	$7.5x10^5$	$7.1x10^5$	$5.9x10^5$	$6.6x10^5$	$6.3x10^5$
RC4	$7.4x10^5$	$6.8x10^5$	$6.5x10^5$	$4.2x10^5$	$3.6x10^5$	$3.9x10^5$
RC5	$8.5x10^5$	$7.9x10^5$	$8.2x10^5$	$5.8x10^5$	$5.4x10^5$	$5.9x10^5$

‡Experiment number *Colony forming units/ml.

Table 6.5: (Continued).

Rat number	Time 60 minutes			Time 120 minutes		
	1‡	2	3	1	2	3
RC1	$4.1x10^5$	$4.6x10^5$	$3.9x10^5$	$1.0x10^5$	$9.8x10^4$	$9.6x10^4$
RC2	$3.0x10^5$	$2.7x10^5$	$2.5x10^5$	$4.0x10^4$	$3.6x10^4$	$3.3x10^4$
RC3	$3.9x10^5$	$4.1x10^5$	$3.7x10^5$	$8.2x10^5$	$9.0x10^5$	$8.6x10^5$
RC4	$1.7x10^5$	$2.1x10^5$	$1.9x10^5$	$5.1x10^4$	$5.5x10^4$	$5.9x10^4$
RC5	$4.2x10^5$	$3.6x10^5$	$3.3x10^5$	$1.7x10^5$	$1.5x10^5$	$1.4x10^5$

‡Experiment number *Colony forming units/ml.

Table 6.6: The mean candidacidal affect of bronchoalveolar lavage fluid of 5-control rats (see section 6.2.2) on *Candida albicans* isolate a15.

	Time minutes			
	T0	**T30**	**T60**	**T120**
RC1	$9.1x10^{5*}$	$6.8x10^{5}$	$4.2x10^{5}$	$9.8x10^{4}$
RC2	$6.7x10^{5}$	$4.4x10^{5}$	$2.7x10^{5}$	$3.6x10^{4}$
RC3	$7.3x10^{5}$	$6.3x10^{5}$	$3.9x10^{5}$	$5.5x10^{4}$
RC4	$6.9x10^{5}$	$3.9x10^{5}$	$1.9x10^{5}$	$8.6x10^{4}$
RC5	$8.2x10^{5}$	$5.7x10^{5}$	$3.7x10^{5}$	$1.5x10^{5}$

*Colony forming units/ml.

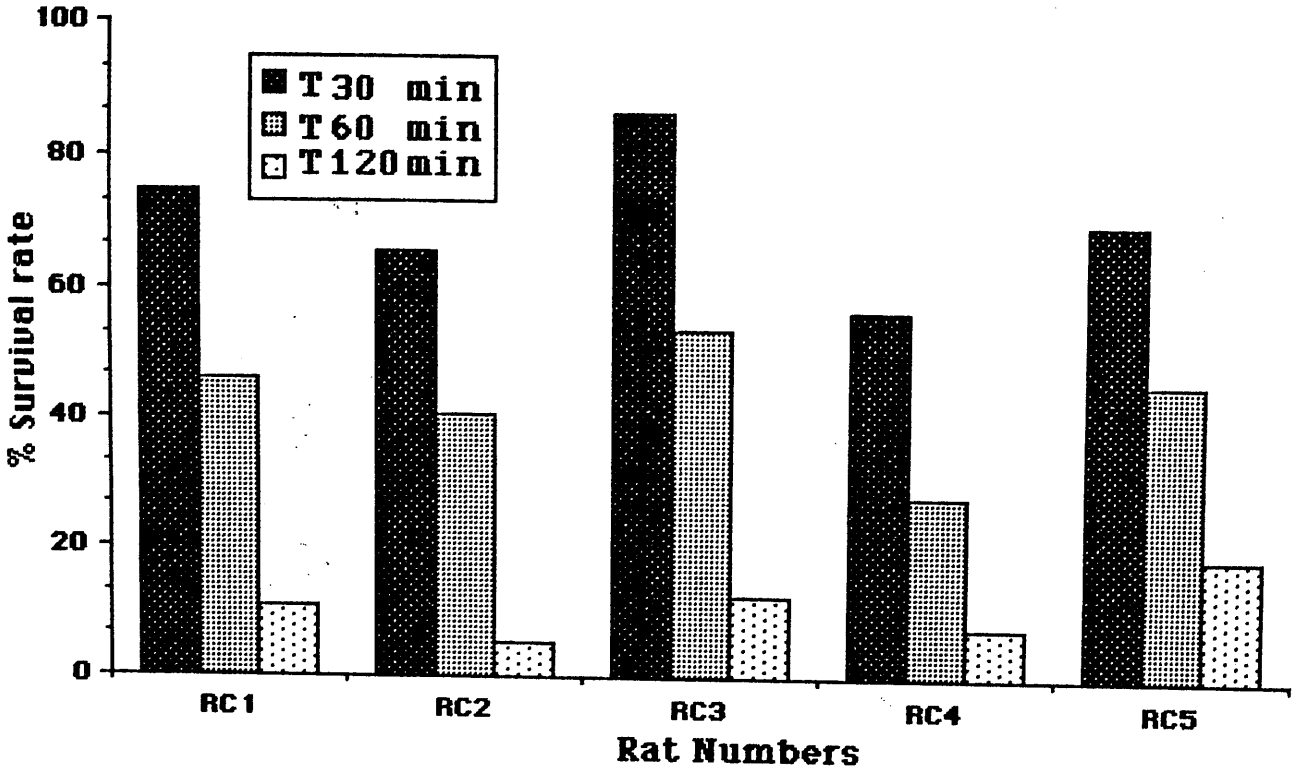


Figure 6.4: The mean percentage survival rate of *Candida albicans* strain a15 when exposed to bronchoalveolar lavage fluid (BLF) from 5-control rats RC1 to RC5 (see section 6.2.2).

Table 6.7: The candidacidal affect of bronchoalveolar lavage fluid of 7-test rats (see section 6.2.2) on *Candida albicans* strain a15.

Rat number	Time 0 (Minutes) 1	2	3	Time 30 (Minutes) 1	2	3
R1	$7.8x10^5$	$7.4x10^5$	$6.7x10^5$	$6.9x10^5$	$6.3x10^5$	$5.7x10^5$
R2	$8.9x10^5$	$8.2x10^5$	$6.9x10^5$	$6.1x10^5$	$4.9x10^5$	$4.6x10^5$
R3	$6.2x10^5$	$5.5x10^5$	$5.1x10^5$	$4.9x10^5$	$6.6x10^5$	$5.3x10^5$
R4	$7.4x10^5$	$6.8x10^5$	$5.9x10^5$	$7.4x10^5$	$6.8x10^5$	$5.9x10^5$
R5	$9.1x10^5$	$8.0x10^5$	$7.9x10^5$	$7.0x10^5$	$6.2x10^5$	$6.0x10^5$
R6	$9.0x10^5$	$8.2x10^5$	$7.1x10^5$	$6.0x10^5$	$5.5x10^5$	$5.0x10^5$
R7	$8.0x10^5$	$6.4x10^5$	$6.0x10^5$	$5.5x10^5$	$6.0x10^5$	$5.6x10^5$

‡Experiment number *Colony forming units/ml.

Table 6.7: (Continued).

Rat number	Time 60 (Minutes) 1	2	3	Time 120 (Minutes) 1	2	3
R1	$2.0x10^5$	$1.3x10^5$	$9.0x10^5$	$1.3x10^5$	$9.0x10^5$	$1.0x10^5$
R2	$4.0x10^5$	$4.4x10^5$	$3.6x10^5$	$2.1x10^4$	$1.9x10^4$	$2.3x10^4$
R3	$2.8x10^5$	$2.3x10^5$	$2.1x10^5$	$5.0x10^5$	$4.5x10^4$	$3.9x10^4$
R4	$7.6x10^5$	$6.1x10^5$	$5.2x10^5$	$2.9x10^5$	$4.1x10^5$	$3.8x10^5$
R5	$5.2x10^5$	$4.6x10^5$	$4.3x10^5$	$2.3x10^5$	$1.7x10^5$	$1.4x10^5$
R6	$5.8x10^5$	$4.7x10^5$	$3.9x10^5$	$2.1x10^5$	$1.6x10^5$	$1.1x10^5$
R7	$7.4x10^5$	$6.0x10^5$	$5.5x10^5$	$2.5x10^5$	$2.0x10^5$	$1.5x10^5$

‡Experiment number *Colony forming units/ml

Table 6.8: The mean candidacidal affect of bronchoalveolar lavage fluid of seven rats on *Candida albicans* strain a15.

	Time (Minutes)			
	T0	**T30**	**T60**	**T120**
R1	$7.3x10^5$	$6.3x10^5$	$1.4x10^5$	$1.1x10^5$
R2	$8.0x10^5$	$5.2x10^5$	$4.0x10^5$	$2.1x10^4$
R3	$5.6x10^5$	$5.6x10^5$	$2.4x10^5$	$4.5x10^4$
R4	$6.7x10^5$	$6.7x10^5$	$6.3x10^5$	$3.6x10^5$
R5	$8.3x10^5$	$6.4x10^5$	$4.7x10^5$	$1.8x10^5$
R6	$8.1x10^5$	$5.5x10^5$	$4.8x10^5$	$1.7x10^5$
R7	$6.8x10^5$	$5.7x10^5$	$6.3x10^5$	$2.0x10^5$

*Colony forming units/ml.

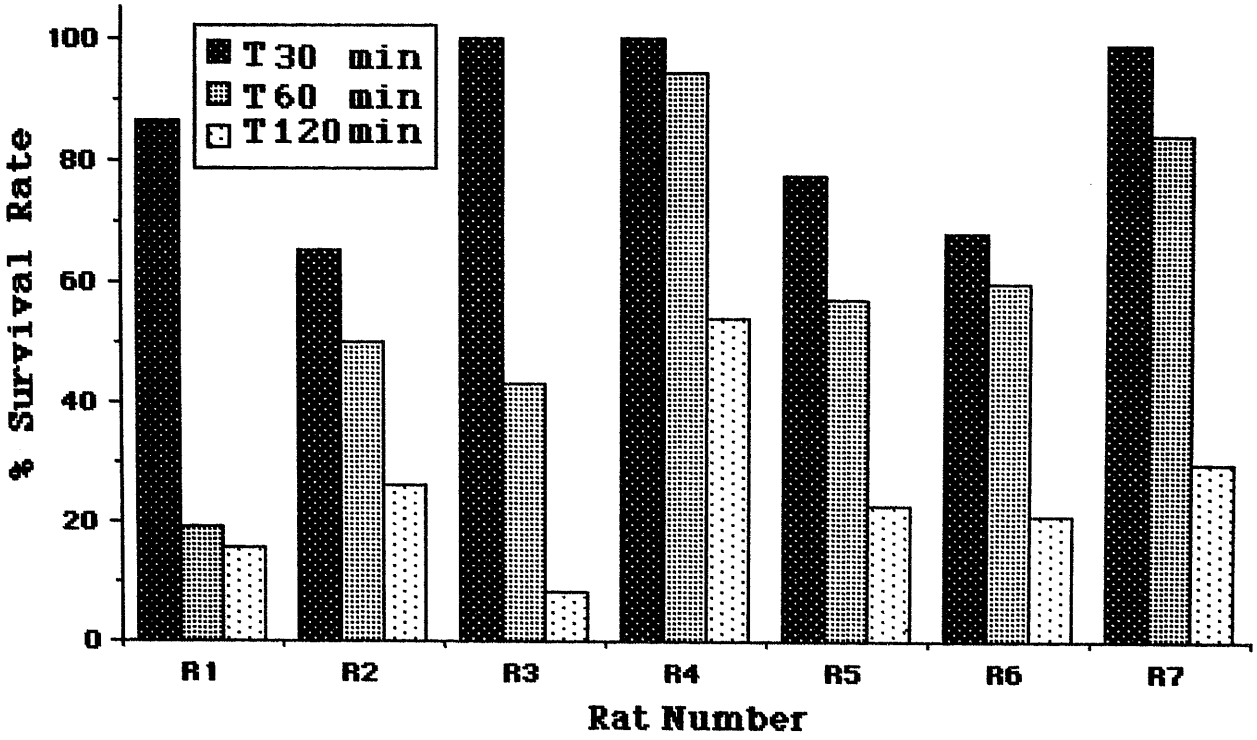


Figure 6.5: The mean percentage of survival rate of *Candida albicans* strain a15 when exposed to bronchoalveolar lavage fluid (BLF) from 7-test rats R1 to R7.

Table 6.9: The mean statistical significance of multiple comparisons of pair analysis of 5-control rats. ANOVA test was used.

Rat Rat Numbers	Pair analysis* Rat Numbers				
	RC1	**RC2**	**RC3**	**RC4**	**RC5**
RC1	-	NS	NS	NS	NS
RC2	NS	-	NS	NS	NS
RC3	NS	NS	-	NS	NS
RC4	NS	NS	NS	-	NS
RC5	NS	NS	NS	NS	-

NS*=Not-Significant

Table 6.10: The mean statistical significance of multiple comparisons of pair analysis of 7-test rats. ANOVA test was used.

Rat Rat Numbers	Pair analysis* Rat Numbers						
	R1	**R2**	**R3**	**R4‡**	**R5**	**R6**	**R7**
R1	-	NS	NS	S	NS	NS	NS
R2	NS	-	NS	S	S	NS	S
R3	NS	NS	-	S	S	NS	NS
R4	S	S	S	-	NS	S	NS
R5	NS	S	S	NS	-	NS	NS
R6	NS	NS	NS	S	NS	-	NS
R7	NS	S	NS	NS	NS	NS	-

*S=Significant NS=Not-Significant. ‡Note the significant difference of R4

Table 6.11: The mean statistical significance of multiple comparisons of pair analysis of 5-control (RC) and 7-test (R) rats. ANOVA test was used.

Rat Numbers	Pair analysis* Rat Numbers						
	R1	**R2**	**R3‡**	**R4‡**	**R5**	**R6**	**R7**
RC1	NS	NS	NS	S	NS	NS	NS
RC2	NS	NS	NS	S	S	S	S
RC3	NS	NS	NS	S	S	S	S
RC4	NS	NS	NS	S	NS	NS	S
RC5	NS	NS	NS	S	NS	NS	NS

*S=Significant NS=Not-Significant Note the significant difference of R3 and R4

Table 6.12: The mean* candidacidal activity of bronchoalveolar lavage fluid of seven rats on *Candida albicans* strain a15.

	Viable count at Time (minutes)				%
	0	30	60	120	120/0
Control (Rats)	$7.6x10^{5}$*	$5.4x10^{5}$	$3.3x10^{5}$	$8.5x10^{4}$	11.2
Test (Rats)	$7.3x10^{5}$	$5.9x10^{5}$	$4.3x10^{5}$	$1.6x10^{5}$	21.9
P ‡	NS	NS	NS	NS	

‡Probability (*t*-test) NS=Not Significant. *Mean of all results (Tables 13 and 15).

***Colony forming units**

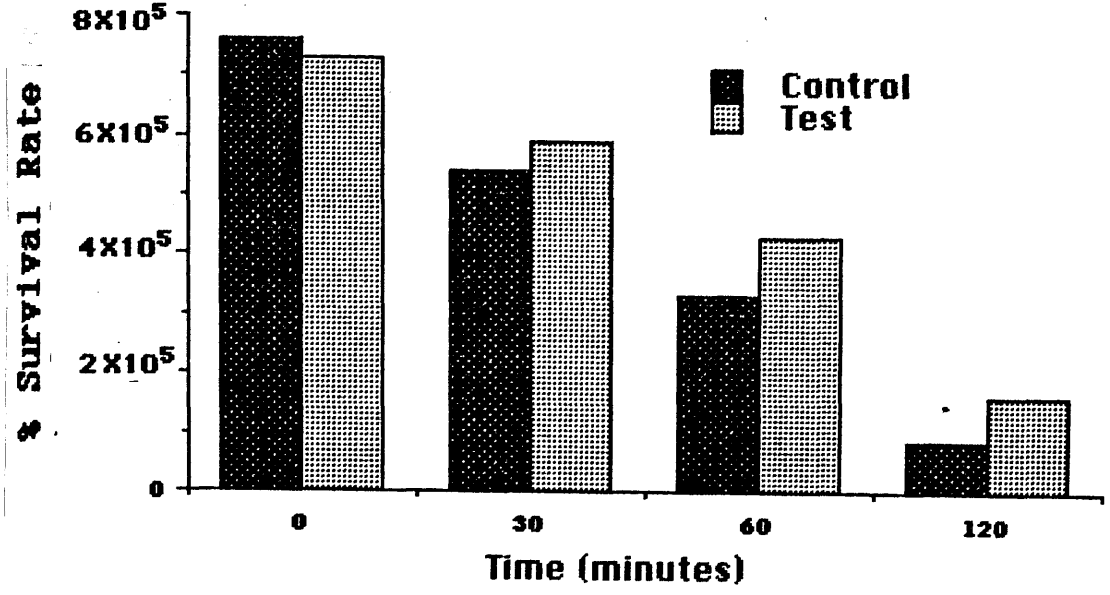


Figure 6.6: Comparison of the mean values of the bronchoalveolar lavage fluid activity from 5-control and 7-test rats against *Candida albicans* strain a15.

Table 6.13. The growth of *Candida albicans* (a2) when exposed to normal and heat-inactivated neat bronchoalveolar lavage fluid (BLF), from rat R2 at 0, 30, 60, 120 minutes.

Time	Normal BLF				Heat-inactivated BLF			
(Min.)	1‡	2	3	Mean	1	2	3	Mean
0	$1.5x10^{6*}$	$1.4x10^{6}$	$1.3x10^{6}$	$1.4x10^{6}$	$1.5x10^{6}$	$1.6x10^{6}$	$1.7x10^{6}$	$1.6x10^{6}$
30	$3.1x10^{5}$	$3.4x10^{5}$	$3.1x10^{5}$	$3.2x10^{5}$	$2.7x10^{5}$	$2.8x10^{5}$	$3.2x10^{5}$	$2.9x10^{5}$
60	$1.0x10^{5}$	$1.2x10^{5}$	$1.4x10^{5}$	$1.2x10^{5}$	$1.1x10^{5}$	$1.3x10^{5}$	$1.5x10^{5}$	$1.3x10^{5}$
120	$7.4x10^{4}$	$7.1x10^{4}$	$7.5x10^{4}$	$7.3x10^{4}$	$9.0x10^{4}$	$1.2x10^{5}$	$1.2x10^{5}$	$1.1x10^{5}$

‡Experiment number *Colony forming units/ml.

Table 6.14. The growth of *Candida albicans* (a11) when exposed to normal and heat-inactivated neat bronchoalveolar lavage fluid (BLF), from rat R2 at 0, 30, 60, 120 minutes.

Time	Normal BLF				Heat-inactivated BLF			
(Minutes)	1‡	2	3	Mean	1	2	3	Mean
0	$1.5x10^{6*}$	$1.4x10^{6}$	$1.6x10^{6}$	$1.5x10^{6}$	$1.5x10^{6}$	$1.7x10^{6}$	$1.3x10^{6}$	$1.5x10^{6}$
30	$2.7x10^{5}$	$3.0x10^{5}$	$3.1x10^{5}$	$2.9x10^{5}$	$3.5x10^{5}$	$3.6x10^{5}$	$4.0x\ 10^{5}$	$3.7x10^{5}$
60	$1.0x10^{5}$	$1.2x10^{5}$	$1.4x10^{5}$	$1.2x10^{5}$	$1.9x10^{5}$	$2.0x10^{5}$	$2.4x10^{5}$	$2.1x10^{5}$
120	$2.9x10^{4}$	$3.1x10^{4}$	$3.9x10^{4}$	$3.3x10^{4}$	$9.0x10^{4}$	$1.2x10^{5}$	$1.2x10^{5}$	$1.1x10^{5}$

‡Experiment number *Colony forming units/ml.

Table 6.15. The growth of *Candida albicans* (strain a15) when exposed to normal and heat-inactivated neat bronchoalveolar lavage fluid (BLF), from a rat R2 at 0, 30, 60, 120 minutes.

Time	Normal BLF				Heat-inactivated BLF			
(Minutes)	1‡	2	3	Mean	1	2	3	Mean
0	$1.7x10^{6*}$	$1.5x10^{6}$	$1.6x10^{6}$	$1.6x10^{6}$	$1.6x10^{6}$	$1.4x10^{6}$	$1.5x10^{6}$	$1.5x10^{6}$
30	$6.6x10^{5}$	$6.1x10^{5}$	$7.1x10^{5}$	$6.6x10^{5}$	$8.6x10^{5}$	$9.6x10^{5}$	$1.0x\ 10^{6}$	$9.4x10^{5}$
60	$3.1x10^{5}$	$2.9x10^{5}$	$3.3x10^{5}$	$3.1x10^{5}$	$4.5x10^{5}$	$4.7x10^{5}$	$4.9x10^{5}$	$4.7x10^{5}$
120	$9.9x10^{4}$	$9.5x10^{4}$	$1.0x\ 10^{5}$	$9.8x10^{4}$	$2.9x10^{5}$	$3.2x10^{5}$	$3.2x10^{5}$	$3.1x10^{5}$

‡Experiment number *Colony forming units/ml

Table 6.16. Statistical significance of the anti-candidal effect of normal and heat-inactivated bronchoalveolar lavage fluid (BLF) for 120 minutes using *Candida albicans* isolates a2, a11 and a15.

Multiple Comparison Pair analysis	99% Confidence Intervals *Candida albicans*		
	a2	a11	a15
PBS‡ (Control) V Normal BLF	S	S	S
PBS (Control) V Heat-inactivated BLF	S	S	S
Normal BLF V Heat-inactivated BLF	NS	NS	NS

‡PBS=Phosphate buffered saline control contains no BLF.

NS=No significant difference. S=Significant.

v = versus

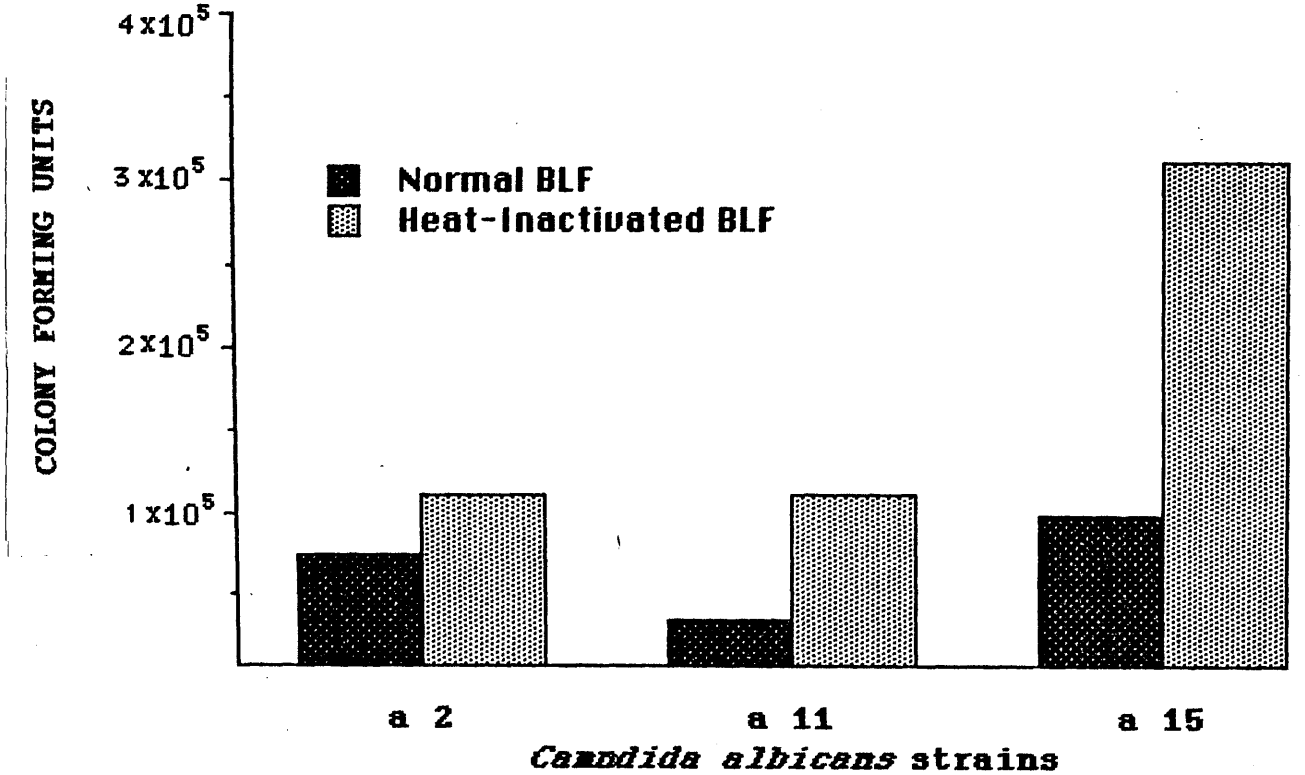


Figure 6.7. The reduction in CFU* of *Candida albicans* strains, a2, a11 and a15, when exposed to normal and heat-inactivated (56 °C for 30 min.) bronchoalveolar lavage fluid (BLF) from rat R2, at 120 minutes.

*Colony forming units

Table 6.17. The candidacidal effect of different concentrations of bronchoalveolar lavage fluid (BLF) from rat (R2) on *Candida albicans* strain a15

Concentration (Dilution) of BLF	Experiment Number 1	2	3	Mean	% Survival rate	95% C.I.'s#
(PBS) Control	$2.1x10^{6*}$	$25x10^{6}$	$26x10^{6}$	$2.4x10^{6}$	100	NS
1/1024	$2.2x10^{6}$	$2.3x10^{6}$	$2.4x10^{6}$	$2.3x10^{6}$	95.8	NS
1/512	$2.3x10^{6}$	$2.5x10^{6}$	$2.4x10^{6}$	$2.4x10^{6}$	100	NS
1/256	$1.9x10^{6}$	$2.3x10^{6}$	$2.4x10^{6}$	$2.2x10^{6}$	91.7	NS
1/128	$9.8x10^{5}$	$1.1x10^{6}$	$8.9x10^{5}$	$9.9x10^{5}$	41.3	NS
1/64	$2.0x10^{6}$	$2.1x10^{6}$	$1.9x10^{6}$	$2.0x10^{6}$	83.3	NS
1/32	$7.6x10^{5}$	$8.0x10^{5}$	$8.1x10^{5}$	$7.9x10^{5}$	32.9	S
1/16	$4.3x10^{5}$	$4.7x10^{5}$	$4.8x10^{5}$	$4.6x10^{5}$	19.2	S
1/8	$1.1x10^{5}$	$1.3x10^{5}$	$1.5x10^{5}$	$1.3x10^{5}$	5.4	S
1/4	$8.1x10^{4}$	$8.5x10^{4}$	$8.0x10^{4}$	$8.2x10^{4}$	3.4	S
1/2	$5.9x10^{4}$	$6.2x10^{4}$	$6.3x10^{4}$	$6.1x10^{4}$	2.5	S
1	$3.9x10^{4}$	$3.7x10^{4}$	$4.1x10^{4}$	$3.9x10^{4}$	1.6	S

#C.I.'s=Confidence intervals. *Colony forming units/ml.

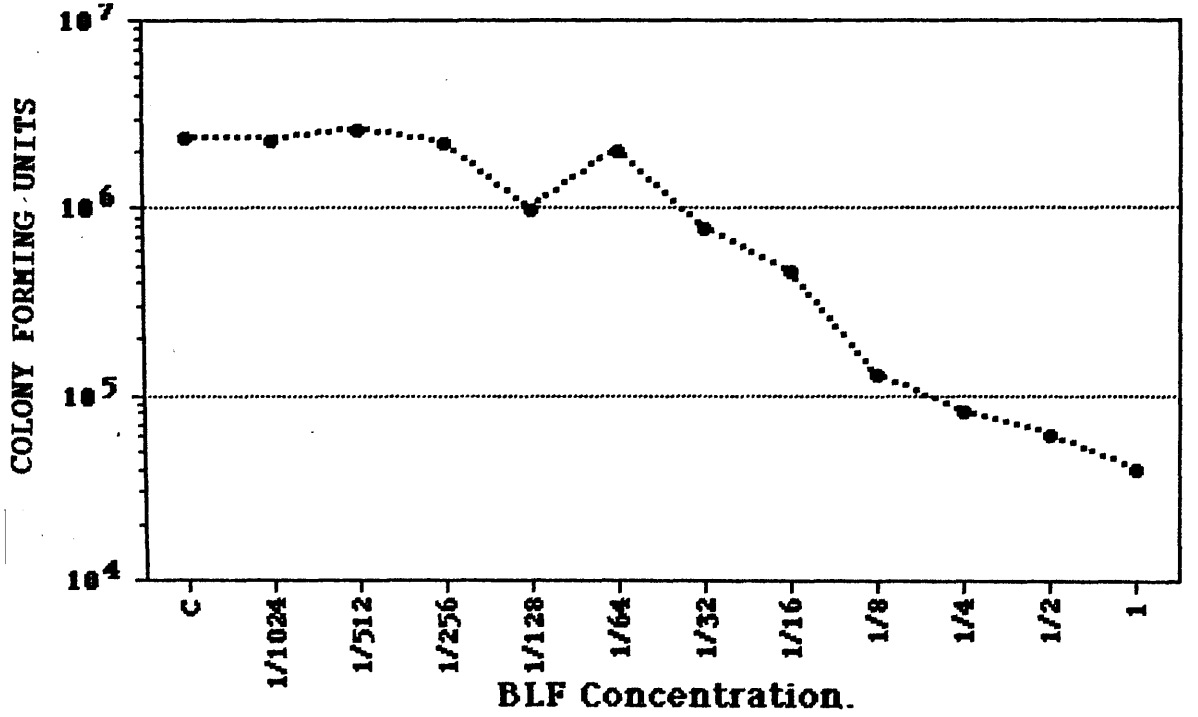


Figure 6.8. The dose response curve of bronchoalveolar lavage fluid (BLF) from rat R2 on *Candida albicans* strain a15. Each point represents the mean of 3 determinations.

different values in a pair analysis of the 22-*C. albicans* strains using 95 percent confidence intervals. For instance, the highly susceptible *C. albicans* strain a7 was significantly different from all other *C. albicans* strains tested, whereas the most resistant to the BLF from rat 3, was *C. albicans* a5 which did show significant differences with all other strains. Table, 6.20 and Figure 6.9 present the mean counts and percentage survival rate of the 22-yeast isolates when exposed to BLF from two rats (R3 and R4).

6.3.6 The effect of bronchoalveolar lavage fluid from rats (R3 and R4) on 27 isolates of five *Candida* species

The effect of BLF from the rats (R3 and R4) on different 27 isolates five *Candida* species are shown as follows; *C. krusei*, (Tables, 6.22, 6.23, 6.24), *C. tropicalis*, (Tables, 6.25, 6.26, 6.27), *C. parapsilosis*, (Tables, 6.28, 6.29, 6.30) *C. guilliermondii*, (Tables 6.31, 6.32, 6.33), and *C. glabrata*, (Tables 6.34, 6.35, 6.36). Figure 6.10 illustrates the relative effect of BLF from two rats on the above *Candida* species strains showing the marked variations in their response to BLF. Figure 6.11 shows the percentage and mean of servival rate of *Candida* species axposed to the candidacidal affect of BLF from two rats R3 and R4. Table, 6.37 shows the effect of BLF from rat 3 and rat 4 on *Candida* species when subjected to the fluid at 120 minutes compared with 0 time, and indicates that the susceptibility of the yeast species to BLF is remarkably constant irrespective of the source of BLF, with the exception of *C. krusei*, which shows

significant difference value when subjected to the BLF from rat 3 but not to the BLF from rat 4 (Table 6.37).

Candida albicans was the least susceptible to BLF, followed by *C. parapsilosis* and *C. tropicalis*. whereas, *C. krusei*, *C. guilliermondii* and *C. glabrata* were highly resistant to the BLF particularly from rat R4, with survival rates of 85.3, 92.9 and 93.7 percent respectively (Table, 6.38). Table, 6.38 shows the order of *Candida* species ranked according to their susceptibility to BLF from rats R3 and R4 with *C. albicans* being the most susceptible and *C. guilliermondii* the most resistant. Tables 6.39 and 6.40 indicate the mean susceptibility results of pair analysis of *Candida* species, to BLF from rats 3 and 4, respectively. *Candida albicans* was significantly more susceptible to BLF compared with all other species, irrespective of BLF source, except in the case of *C. parapsilosis* when exposed to BLF from rat 4 (Tables, 6.39 and 6.40). This atypical results might be partly caused by the odd results of strain a4 which demonstrates a very high survival rate in BLF compared with other strains of *C. albicans* (Figure, 6.11 and Table, 6.40).

Table 6.18 : The viable counts of 22-*Candida albicans* strains exposed to bronchoalveolar lavage fluid from rat R3.

Candida albicans	Time 0 minutes			Time 120 minutes		
	1‡	2	3	1	2	3
1	$1.0x10^6$*	$1.4x10^6$	$1.2x10^6$	$3.4x10^4$	$3.8x10^4$	$3.6x10^4$
2	$1.9x10^6$	$2.1x10^6$	$1.7x10^6$	$2.1x10^4$	$2.4x10^4$	$1.8x10^4$
3	$9.0x10^5$	$1.5x10^6$	$1.2x10^6$	$1.8x10^4$	$2.5x10^4$	$2.3x10^4$
4	$1.1x10^6$	$1.4x10^6$	$1.1x10^6$	$5.5x10^3$	$6.2x10^3$	$5.7x10^3$
5	$1.1x10^6$	$1.3x10^6$	$1.2x10^6$	$2.8x10^5$	$3.2x10^5$	$3.0x10^5$
6	$1.6x10^6$	$1.8x10^6$	$1.4x10^6$	$1.4x10^4$	$1.5x10^4$	$1.3x10^4$
7	$1.3x10^6$	$1.5x10^6$	$1.1x10^6$	$3.2x10^3$	$3.4x10^3$	$3.0x10^3$
8	$1.3x10^6$	$1.4x10^6$	$9.0x10^5$	$8.0x10^3$	$8.3x10^3$	$7.7x10^3$
9	$1.7x10^6$	$1.9x10^6$	$1.5x10^6$	$1.1x10^4$	$1.2x10^4$	$1.0x10^4$
10	$1.3x10^6$	$1.7x10^6$	$1.5x10^6$	$61.x10^4$	$6.6x10^4$	$6.2x10^4$
11	$1.6x10^6$	$2.0x10^6$	$1.8x10^6$	$8.9x10^3$	$9.3x10^3$	$9.1x10^3$

*Mean of duplicate determinations in colony forming units/ml. ‡Experiment number

Table 6.18: (Continued)

Candida albicans	Time 0 minutes			Time 120 minutes		
	1‡	2	3	1	2	3
12	$1.5x10^{6}$*	$2.1x10^{6}$	$1.8x10^{6}$	$9.5x10^{3}$	$1.0x10^{4}$	$9.9x10^{3}$
13	$1.4x10^{6}$	$1.2x10^{6}$	$1.0x10^{6}$	$1.2x10^{5}$	$1.6x10^{5}$	$1.1x10^{5}$
14	$1.2x10^{6}$	$1.6x10^{6}$	$1.4x10^{6}$	$2.4x10^{4}$	$2.8x10^{4}$	$2.6x10^{4}$
15	$1.4x10^{6}$	$1.7x10^{6}$	$1.4x10^{6}$	$6.9x10^{4}$	$7.2x10^{4}$	$6.6x10^{4}$
16	$1.2x10^{6}$	$1.4x10^{6}$	$1.3x10^{6}$	$2.6x10^{5}$	$3.2x10^{5}$	$2.9x10^{5}$
17	$1.6x10^{6}$	$1.4x10^{6}$	$1.2x10^{6}$	$7.1x10^{4}$	$6.6x10^{4}$	$6.4x10^{4}$
18	$1.5x10^{6}$	$1.3x10^{6}$	$1.1x10^{6}$	$1.4x10^{5}$	$1.2x10^{5}$	$1.0x10^{5}$
19	$1.4x10^{6}$	$1.7x10^{6}$	$1.4x10^{6}$	$1.4x10^{5}$	$1.6x10^{5}$	$1.2x10^{5}$
20	$1.3x10^{6}$	$1.5x10^{6}$	$1.1x10^{6}$	$1.2x10^{5}$	$1.4x10^{5}$	$1.0x10^{5}$
21	$2.8x10^{6}$	$3.2x10^{6}$	$3.0x10^{6}$	$2.7x10^{5}$	$3.1x10^{5}$	$2.9x10^{5}$
22	$1.6x10^{6}$	$1.8x10^{6}$	$1.4x10^{6}$	$3.6x10^{4}$	$3.8x10^{4}$	$3.4x10^{4}$

*Mean of duplicate determinations in colony forming units/ml. ‡Experiment number

Table 6.19: The viable counts of 22-*Candida albicans* strains exposed to bronchoalveolar lavage fluid from rat R4.

Candida albicans	Time 0 minute			Time 120 minutes		
	1‡	2	3	1	2	3
1	$1.4x10^6$*	$1.2x10^6$	$1.0x10^6$	$2.0x10^5$	$2.2x10^5$	$2.4x10^5$
2	$1.9x10^6$	$2.0x10^6$	$1.8x10^6$	$5.1x10^5$	$5.2x10^5$	$5.3x10^5$
3	$1.1x10^6$	$1.1x10^6$	$1.4x10^6$	$8.0x10^4$	$1.3x10^5$	$9.0x10^4$
4	$1.2x10^6$	$1.4x10^6$	$1.0x10^6$	$3.3x10^4$	$3.1x10^4$	$2.9x10^4$
5	$1.3x10^6$	$1.2x10^6$	$1.1x10^6$	$5.7x10^5$	$6.1x10^5$	$5.9x10^5$
6	$1.6x10^6$	$1.7x10^6$	$1.5x10^6$	$1.0x10^5$	$1.2x10^5$	$8.0x10^4$
7	$1.2x10^6$	$1.3x10^6$	$1.4x10^6$	$3.1x10^4$	$3.5x10^4$	$3.3x10^4$
8	$1.5x10^6$	$1.7x10^6$	$1.9x10^6$	$9.0x10^4$	$8.0x10^4$	$1.3x10^5$
9	$1.3x10^6$	$1.6x10^6$	$1.6x10^6$	$2.4x10^5$	$2.8x10^5$	$2.6x10^5$
10	$1.4x10^6$	$1.5x10^6$	$1.6x10^6$	$3.6x10^5$	$3.7x10^5$	$3.5x10^5$
11	$1.7x10^6$	$1.8x10^6$	$1.9x10^6$	$1.6x10^5$	$1.8x10^5$	$1.7x10^5$

*Mean of duplicate determinations in colony forming units/ml.

Table 6.19: (Continued)

Candida albicans	Time 0 minutes			Time 120 minutes		
	1‡	2	3	1	2	3
12	2.0×10^{6}*	1.6×10^{6}	1.8×10^{6}	1.9×10^{5}	1.7×10^{5}	1.5×10^{5}
13	1.5×10^{6}	1.3×10^{6}	1.1×10^{6}	2.1×10^{5}	2.5×10^{5}	2.3×10^{5}
14	1.4×10^{6}	1.2×10^{6}	1.6×10^{6}	2.7×10^{5}	3.1×10^{5}	2.9×10^{5}
15	1.5×10^{6}	1.3×10^{6}	1.2×10^{6}	1.4×10^{5}	1.6×10^{5}	1.5×10^{5}
16	1.3×10^{6}	1.0×10^{6}	1.3×10^{6}	2.7×10^{5}	2.5×10^{5}	2.9×10^{5}
17	1.4×10^{6}	1.5×10^{6}	1.6×10^{6}	2.8×10^{5}	2.6×10^{5}	3.0×10^{5}
18	1.1×10^{6}	1.6×10^{6}	1.2×10^{6}	1.6×10^{5}	1.5×10^{5}	1.7×10^{5}
19	1.4×10^{6}	1.4×10^{6}	1.7×10^{6}	1.9×10^{5}	1.8×10^{5}	2.3×10^{5}
20	1.2×10^{6}	1.2×10^{6}	1.5×10^{6}	2.6×10^{5}	2.8×10^{5}	3.0×10^{5}
22	3.1×10^{6}	3.5×10^{6}	3.0×10^{6}	4.0×10^{6}	3.8×10^{5}	4.2×10^{5}
21	1.3×10^{6}	1.4×10^{6}	1.5×10^{6}	2.8×10^{5}	2.9×10^{5}	3.0×10^{5}

*Mean of duplicate determinations in colony forming units/ml

‡Experiment number

Table 6.20: The means and percentages of survival rate of 22-*Candida albicans* strains exposed to bronchoalveolar lavage fluid from rats R3 and R4.

Candida albicans	Rat 3 T0	Rat 3 T120	% Survival rate	Rat 4 T0	Rat 4 T120	% Survival rate
a1	$1.2x10^{6}$*	$3.6x10^{4}$	3	$1.2x10^{6}$	$2.2x10^{5}$	18.3
a2	$1.9x10^{6}$	$2.1x10^{4}$	1.1	$1.9x10^{6}$	$5.2x10^{5}$	2.7
a3	$1.2x10^{6}$	$2.2x10^{4}$	1.8	$1.2x10^{6}$	$1.0x10^{5}$	8.0
a4	$1.2x10^{6}$	$5.8x10^{3}$	.5	$1.2x10^{6}$	$3.1x10^{4}$	2.6
a5	$1.2x10^{6}$	$3.0x10^{5}$	25	$1.2x10^{6}$	$5.9x10^{5}$	49.2
a6	$1.6x10^{6}$	$1.4x10^{4}$	.9	$1.6x10^{6}$	$1.0x10^{5}$	6.3
a7	$1.3x10^{6}$	$3.2x10^{3}$	.3	$1.3x10^{6}$	$3.3x10^{4}$	2.5
a8	$1.2x10^{6}$	$8.0x10^{3}$	.7	$1.7x10^{6}$	$1.0x10^{5}$	5.9
a9	$1.7x10^{6}$	$1.1x10^{4}$	.7	$1.5x10^{6}$	$2.6x10^{5}$	17.3
a10	$1.5x10^{6}$	$6.3x10^{4}$	4.2	$1.5x10^{6}$	$3.6x10^{5}$	24.4
a11	$1.8x10^{6}$	$9.1x10^{3}$	.5	$1.8x10^{6}$	$1.7x10^{5}$	9.4

*Colony forming units/ml.

Table 6.20: (Continued).

Candida albicans	Rat 3 T0	Rat 3 T120	% Survival rate	Rat 4 T0	Rat 4 T120	% Survival rate
a12	$1.8x10^{6*}$	$9.8x10^{3}$	.5	$1.8x10^{6}$	$1.7x10^{5}$	9.4
a13	$1.2x10^{6}$	$1.3x10^{5}$	10.1	$1.3x10^{6}$	$2.3x10^{5}$	18.0
a14	$1.4x10^{6}$	$2.6x10^{4}$	1.9	$1.4x10^{6}$	$2.9x10^{5}$	20.7
a15	$1.5x10^{6}$	$6.9x10^{4}$	4.6	$1.5x10^{6}$	$1.5x10^{5}$	10.0
a16	$1.3x10^{6}$	$2.9x10^{5}$	22.3	$1.2x10^{6}$	$2.7x10^{5}$	22.5
a17	$1.4x10^{6}$	$6.7x10^{4}$	4.8	$1.5x10^{6}$	$2.8x10^{5}$	11.2
a18	$1.3x10^{6}$	$1.2x10^{5}$	9.2	$1.3x10^{6}$	$1.6x10^{5}$	12.3
a19	$1.5x10^{6}$	$1.4x10^{5}$	9.3	$1.5x10^{6}$	$2.0x10^{5}$	13.3
a20	$1.3x10^{6}$	$1.2x10^{5}$	9.2	$1.3x10^{6}$	$2.8x10^{5}$	21.5
a21	$3.0x10^{6}$	$2.9x10^{5}$	9.7	$3.2x10^{6}$	$4.0x10^{5}$	12.5
a22	$1.6x10^{6}$	$3.6x10^{4}$	2.3	$1.4x10^{6}$	$2.9x10^{5}$	20.7

*Colony forming units/ml.

Table 6.21: Pair analysis of *Candida albicans* isolates when exposed to bronchoalveolar lavage fluid (neat) from rat 3 for 120 minutes. Significant comparison only shown.

Pair of *C.albicans*			Simultaneous 95% of Confidence Intervals
(a7*) $3.2x10^3$‡	V	(a17) $6.7x10^4$	S#
(a4) $5.8x10^3$	V	(a15) $6.9x10^4$	S
(a8) $8.0x10^3$	V	(a19) $8.2x10^4$	S
(a11) $9.1x10^3$	V	(a19) $8.2x10^4$	S
(a12) $9.8x10^3$	V	(a19) $8.2x10^4$	S
(a9) $1.1x10^4$	V	(a19) $8.2x10^4$	S
(a6) $1.4x10^4$	V	(a19) $8.2x10^4$	S
(a2) $2.1x10^4$	V	(a22) $1.2x10^5$	S
(a3) $2.2x10^4$	V	(a22) $1.2x10^5$	S

* *Candida albicans* code.‡=Survival rate.#S=Significant ANOVA test was used.

Table 6.21: (Continued).

Pair of *C.albicans*			Simultaneous 95% of Confidence Intervals
(a14) $2.6x10^4$	V	(a22) $1.2x10^5$	S
(a21) $3.6x10^4$	V	(a22) $1.2x10^5$	S
(a22*) $3.6x10^4$	V	(a20) $1.2x10^5$	S
(a10) $6.3x10^4$	V	(a13) $1.3x10^5$	S
(a17) $6.7x10^4$	V	(a13) $1.3x10^5$	S
(a15) $6.9x10^4$	V	(a19) $1.4x10^5$	S
(a20) $1.2x10^5$	V	(a16) $2.9x10^5$	S
(a18) $1.2x10^5$	V	(a16) $2.9x10^5$	S
(a13) $1.3x10^5$	V	(a16) $2.9x10^5$	S
(a19) $1.4x10^5$	V	(a16) $2.9x10^5$	S

**Candida albicans* code.‡=Survival rate.#S=Significant.
ANOVA test was used.

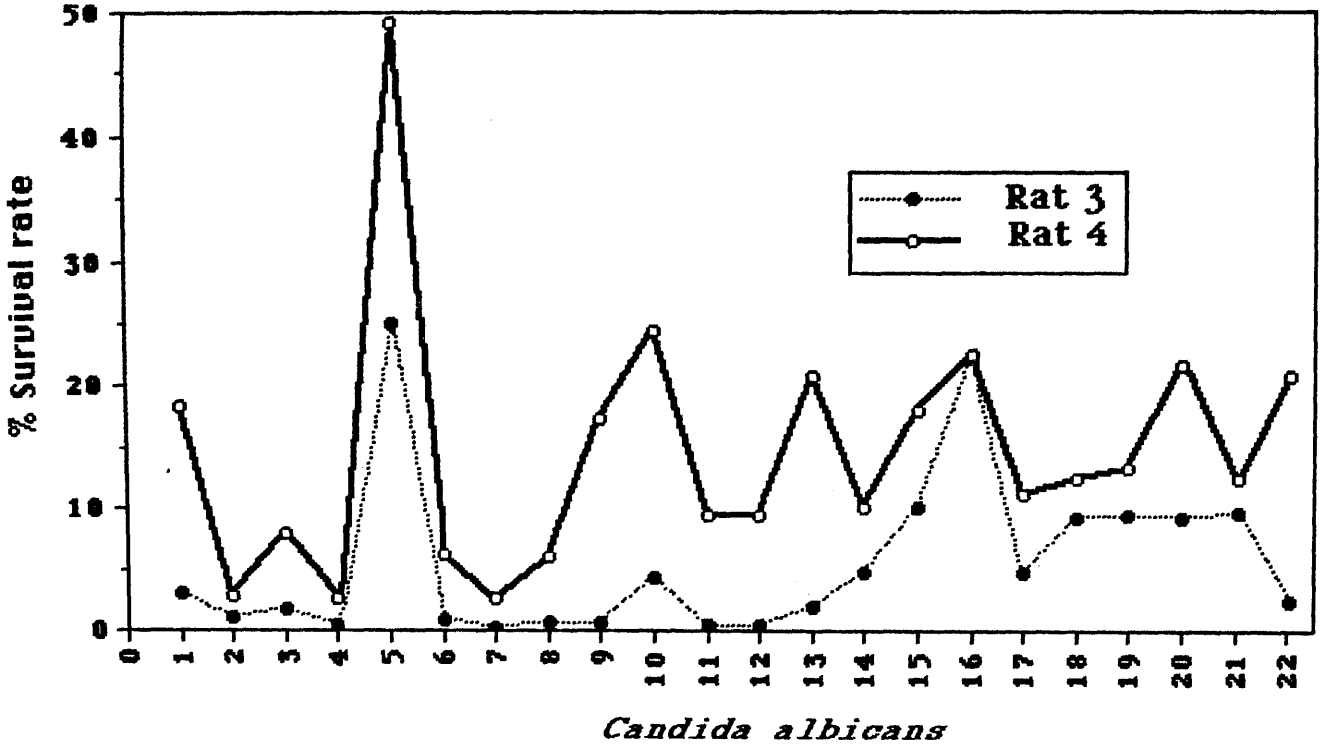


Figure 6.9: The percentage of survival rate of bronchoalveolar lavage fluid (neat) from rats R3 and R4 on 22-*Candida albicans* strains

Table 6.22: The viable counts of seven *Candida krusei* isolates exposed to bronchoalveolar lavage fluid from rat R3.

Candida	Time 0 minutes			Time 120 minutes		
krusei	1‡	2	3	1	2	3
k1	$1.8x10^{6}$*	$1.6x10^{6}$	$1.4x10^{6}$	$7.2x10^{5}$	$7.7x10^{5}$	$7.6x10^{5}$
k2	$1.6x10^{6}$	$1.7x10^{6}$	$1.5x10^{6}$	$8.1x10^{5}$	$8.7x10^{5}$	$8.4x10^{5}$
k3	$1.5x10^{6}$	$2.0x10^{6}$	$1.3x10^{6}$	$9.3x10^{5}$	$9.7x10^{5}$	$8.9x10^{5}$
k4	$1.5x10^{6}$	$1.8x10^{6}$	$1.2x10^{6}$	$9.9x10^{5}$	$1.0x10^{6}$	$9.5x10^{5}$
k5	$7.0x10^{5}$	$1.6x10^{6}$	$1.3x10^{6}$	$6.7x10^{5}$	$7.2x10^{5}$	$6.2x10^{5}$
k6	$1.0x10^{6}$	$1.8x10^{6}$	$1.4x10^{6}$	$9.2x10^{5}$	$1.0x10^{6}$	$9.7x10^{5}$
k7	$1.4x10^{6}$	$1.7x10^{6}$	$1.3x10^{6}$	$8.4x10^{5}$	$9.8x10^{5}$	$9.9x10^{5}$

*Mean of duplicate determinations of colony forming units/ml.

‡Experiment number

Table 6.23: The viable counts of seven *Candida krusei* isolates exposed to bronchoalveolar lavage fluid from rat R4.

Strain Number	Time 0 minutes			Time 120 minutes		
	1‡	2	3	1	2	3
k1	$1.3x10^6$*	$1.5x10^6$	$1.1x10^6$	$1.4x10^6$	$1.0x10^6$	$1.2x10^6$
k2	$1.8x10^6$	$2.0x10^6$	$1.6x10^6$	$1.0x10^6$	$1.5x10^6$	$1.4x10^5$
k3	$1.7x10^6$	$1.9x10^6$	$1.5x10^6$	$1.5x10^6$	$1.1x10^6$	$1.6x10^6$
k4	$1.9x10^6$	$2.1x10^6$	$1.7x10^6$	$1.7x10^6$	$1.8x10^6$	$1.3x10^6$
k5	$1.2x10^6$	$1.6x10^6$	$1.4x10^6$	$1.1x10^6$	$1.3x10^6$	$9.0x10^5$
k6	$1.3x10^6$	$1.8x10^6$	$1.4x10^6$	$1.4x10^6$	$1.7x10^6$	$1.1x10^6$
k7	$1.5x10^6$	$1.6x10^6$	$1.6x10^6$	$1.5x10^6$	$1.7x10^6$	$1.4x10^6$

*Mean of colony forming units/ml.

Table 6.24: The mean viable counts of seven *Candida krusei* isolates exposed to bronchoalveolar lavage fluid from two rats R3 and R4.

Strain Number	Rat 3 T0	Rat 3 T120	% Survival rate	Rat 4 T0	Rat 4 T120	% Survival rate
k1	$1.4x10^6$	$7.5x10^5$	53.6	$1.3x10^6$	$1.2x10^6$	92.3
k2	$1.6x10^6$	$8.4x10^5$	52.5	$1.8x10^6$	$1.3x10^6$	72.2
k3	$1.6x10^6$	$9.3x10^5$	58.1	$1.7x10^6$	$1.4x10^6$	82.4
k4	$1.5x10^6$	$9.8x10^5$	65.3	$1.9x10^6$	$1.6x10^6$	84.2
k5	$1.2x10^6$	$6.7x10^5$	55.8	$1.4x10^6$	$1.1x10^6$	78.6
k6	$1.4x10^6$	$9.7x10^5$	65.7	$1.5x10^6$	$1.4x10^6$	93.3
k7	$1.5x10^6$	$9.4x10^5$	62.7	$1.6x10^6$	$1.5x10^6$	93.8

*Mean of colony forming units/ml.

Table 6.25: The viable counts of seven *Candida tropicalis* isolates exposed to bronchoalveolar lavage fluid from rat R3.

Strain Number	Time 0 minutes			Time 120 minutes		
	1‡	2	3	1	2	3
t1	$9.0x10^5$	$1.3x10^6$	$1.1x10^6$	$5.3x10^5$	$6.5x10^5$	$5.6x10^5$
t2	$1.2x10^6$	$1.3x10^6$	$1.4x10^6$	$5.7x10^5$	$6.3x10^5$	$6.6x10^5$
t3	$1.7x10^6$	$2.2x10^6$	$1.8x10^6$	$8.6x10^5$	$9.2x10^5$	$8.9x10^5$
t4	$1.5x10^6$	$1.9x10^6$	$1.7x10^6$	$5.9x10^5$	$7.2x10^5$	$6.9x10^5$
t5	$1.4x10^6$	$1.7x10^6$	$1.8x10^6$	$6.7x10^5$	$8.1x10^5$	$7.4x10^5$
t6	$1.6x10^6$	$1.5x10^6$	$1.7x10^6$	$8.1x10^5$	$7.0x10^5$	$6.9x10^5$
t7	$1.6x10^6$	$1.6x10^6$	$1.5x10^6$	$7.6x10^5$	$6.8x10^5$	$5.4x10^5$

*Mean of duplicate determinations in Colony forming units/ml.

‡Experiment number

Table 6.26: The viable counts of seven *Candida tropicalis* isolates exposed to bronchoalveolar lavage fluid from rat R4.

Strain Number	Time 0 minutes			Time 120 minutes		
	1‡	2	3	1	2	3
t1	$8.0x10^5$	$1.2x10^6$	$1.0x10^6$	$6.9x10^5$	$7.6x10^5$	$6.2x10^5$
t2	$1.4x10^6$	$1.8x10^6$	$1.0x10^6$	$8.9x10^5$	$9.3x10^5$	$8.2x10^5$
t3	$1.7x10^6$	$1.8x10^6$	$1.3x10^6$	$9.9x10^5$	$1.1x10^6$	$1.0x10^6$
t4	$1.0x10^6$	$1.5x10^6$	$1.3x10^6$	$7.3x10^5$	$7.9x10^5$	$8.2x10^5$
t5	$1.3x10^6$	$1.7x10^6$	$1.5x10^6$	$9.1x10^5$	$9.5x10^5$	$8.5x10^5$
t6	$1.4x10^6$	$1.6x10^6$	$1.7x10^6$	$8.2x10^5$	$9.6x10^5$	$9.1x10^5$
t7	$1.7x10^6$	$1.7x10^6$	$1.5x10^6$	$9.1x10^5$	$7.7x10^5$	$8.3x10^5$

*Mean of duplicate determinations in colony forming units/ml.

‡Experiment number

Table 6.27: The mean viable counts of seven *Candida tropicalis* isolates exposed to bronchoalveolar lavage fluid from two rats R3 and R4.

Strain Number	Rat 3 T0	Rat 3 T120	% Survival rate	Rat 4 T0	Rat 4 T120	% Survival rate
t1	$1.1x10^6$	$5.8x10^5$	52.7	$1.0x10^6$	$6.9x10^5$	69.0
t2	$1.3x10^6$	$6.2x10^5$	49.6	$1.4x10^6$	$8.8x10^5$	62.9
t3	$1.9x10^6$	$8.9x10^5$	46.8	$1.6x10^6$	$1.0x10^6$	63.8
t4	$1.7x10^6$	6.7^x10^5	39.4	$1.3x10^6$	$7.8x10^5$	60.0
t5	$1.6x10^6$	7.4^x10^5	46.2	$1.5x10^6$	$9.0x10^5$	60.0
t6	$1.6x10^6$	$7.3x10^5$	45.6	$1.6x10^6$	$9.0x10^5$	56.3
t7	$1.6x10^6$	6.6^x10^5	41.3	$1.6x10^6$	$8.4x10^5$	52.5

*Mean colony forming units/ml.

Table 6.28: The viable counts of five *Candida parapsilosis* isolates exposed to bronchoalveolar lavage fluid from rat R3.

Strain Number	Time 0 minutes			Time 120 minutes		
	1‡	2	3	1	2	3
p1	$1.0x10^{6}$*	$1.4x10^{6}$	$1.2x10^{6}$	$5.0x10^{5}$	$5.7x10^{5}$	$5.2x10^{5}$
p2	$8.7x10^{5}$	$1.0x10^{6}$	$1.1x10^{6}$	$3.6x10^{5}$	$3.1x10^{5}$	$4.1x10^{5}$
p3	$1.3x10^{6}$	$9.0x10^{5}$	$1.1x10^{6}$	$3.9x10^{5}$	$3.3x10^{5}$	$3.6x10^{5}$
p4	$9.0x10^{5}$	$1.1x10^{6}$	$1.0x10^{6}$	$4.4x10^{5}$	$5.0x10^{5}$	$4.1x10^{5}$
p5	$1.1x10^{6}$	$1.2x10^{6}$	$1.3x10^{6}$	$3.7x10^{5}$	$4.5x10^{5}$	$3.9x10^{5}$

*Mean of duplicate determinations in colony forming units/ml.

‡Experiment number

Table 6.29: The viable counts of five *Candida parapsilosis* isolates exposed to bronchoalveolar lavage fluid from rat R4.

Strain Number	Time 0 minutes			Time 120 minutes		
	1‡	2	3	1	2	3
p1	$1.3x10^{6*}$	$1.1x10^{6}$	$9.0x10^{5}$	$6.4x10^{5}$	$6.1x10^{5}$	$5.5x10^{5}$
p2	$1.1x10^{6}$	$1.2x10^{6}$	$7.0x10^{5}$	$4.6x10^{5}$	$5.0x10^{5}$	$4.2x10^{5}$
p3	$1.1x10^{6}$	$1.3x10^{6}$	$1.2x10^{6}$	$3.4x10^{5}$	$3.9x10^{5}$	$2.9x10^{5}$
p4	$9.9x10^{5}$	$1.0x10^{6}$	$9.8x10^{5}$	$6.2x10^{5}$	$5.6x10^{5}$	$5.9x10^{5}$
p5	$1.2x10^{6}$	$1.3x10^{6}$	$1.1x10^{6}$	$5.4x10^{5}$	$6.8x10^{5}$	$6.3x10^{5}$

*Mean of colony forming units/ml.

Table 6.30: The mean viable counts of five *Candida parapsilosis* isolates exposed to bronchoalveolar lavage fluid from two rats R3 and R4.

Strain Number	Rat 3 T0	Rat 3 T120	% Survival rate	Rat 4 T0	Rat 4 T120	% Survival rate
p1	$1.2x10^{6*}$	$5.3x10^{5}$	44.2	$1.1x10^{6}$	$6.0x10^{5}$	54.6
p2	$9.9x10^{5}$	$3.6x10^{5}$	36.4	$1.0x10^{6}$	$4.6x10^{5}$	46.0
p3	$1.1x10^{6}$	$3.6x10^{5}$	32.7	$1.2x10^{6}$	$3.4x10^{5}$	41.2
p4	$1.0x10^{6}$	$4.5x10^{5}$	45.0	$9.9x10^{5}$	$5.9x10^{5}$	59.6
p5	$1.2x10^{6}$	$4.0x10^{5}$	33.3	$1.2x10^{6}$	$6.2x10^{5}$	51.7

*Mean of colony forming units/ml.

Table 6.31: The viable counts of five *Candida guilliermondii* isolates exposed to bronchoalveolar lavage fluid from rat R3.

Strain Number	Time 0 minutes			Time 120 minutes		
	1‡	2	3	1	2	3
gu1	$1.5x10^6$	$1.3x10^6$	$8.0x10^5$	$1.2x10^6$	$8.0x10^6$	$1.0x10^6$
gu2	$1.4x10^6$	$1.7x10^6$	$1.1x10^6$	$1.4x10^6$	$1.6x10^6$	$9.0x10^5$
gu3	$8.0x10^5$	$1.5x10^6$	$1.0x10^6$	$7.0x10^5$	$1.2x10^6$	$1.1x10^6$
gu4	$1.3x10^6$	$1.6x10^6$	$7.0x10^5$	$1.0x10^6$	$1.4x10^6$	$9.0x10^5$
gu5	$1.1x10^6$	$1.4x10^6$	$1.2x10^6$	$1.2x10^6$	$8.9x\ 10^5$	$1.1x10^6$

*Mean of duplicate determinations in colony forming units/ml.

‡Experiment number

Table 6.32: The viable counts of five *Candida guilliermondii* isolates exposed to bronchoalveolar lavage fluid from rat R4.

Strain Number	Time 0 minutes			Time 120 minutes		
	1‡	2	3	1	2	3
gu1	$1.2x10^6$	$1.4x10^6$	$1.0x10^6$	$1.2x10^6$	$1.3x10^6$	$9.0x10^5$
gu2	$1.8x10^6$	$2.0x10^6$	$1.6x10^6$	$1.6x10^6$	$2.0x10^6$	$1.5x10^6$
gu3	$1.1x10^6$	$1.5x10^6$	$1.0x10^6$	$1.1x10^6$	$1.3x10^6$	$9.0x10^5$
gu4	$1.0x10^6$	$1.2x10^6$	$8.0x10^5$	$9.6x10^5$	$1.0x10^6$	$8.9x10^5$
gu5	$1.2x10^6$	$1.3x10^6$	$1.1x10^6$	$1.1x10^6$	$9.1x10^5$	$1.2x10^6$

*Mean of colony forming units/ml.

Table 6.33: The mean viable counts of five *Candida guilliermondii* isolates exposed to bronchoalveolar lavage fluid from two rats R3 and R4.

Strain Number	Rat 3 T0	Rat 3 T120	% Survival rate	Rat 4 T0	Rat 4 T120	% Survival rate
gu1	$1.2x10^6$	$1.0x10^6$	83.3	$1.2x10^6$	$1.1x10^6$	91.7
gu2	$1.4x10^6$	$1.3x10^6$	92.8	$1.8x10^6$	$1.7x10^6$	94.4
gu3	$1.1x10^6$	$1.0x10^6$	90.9	$1.2x10^6$	$1.1x10^6$	91.7
gu4	$1.2x10^6$	$1.1x10^6$	91.6	$1.0x10^6$	$9.5x10^5$	95.0
gu5	$1.2x10^6$	$1.1x10^6$	90.7	$1.2x10^6$	$1.1x10^6$	91.7

*Mean of colony forming units/ml

Table 6.34: The viable counts of three *Candida glabrata* isolates exposed to bronchoalveolar lavage fluid from rat R3.

Strain Number	Time 0 minutes			Time 120 minutes		
	1‡	2	3	1	2	3
g1	$1.0x10^6$	$1.4x10^6$	$1.2x10^6$	$7.6x10^5$	$7.0x10^5$	$6.7x10^5$
g2	$1.0x10^6$	$1.1x10^6$	$9.0x10^5$	$6.5x10^5$	$7.1x10^5$	$6.2x10^5$
g3	$1.9x10^6$	$1.7x10^6$	$1.5x10^6$	$1.5x10^6$	$1.1x10^6$	$7.0x10^5$

*Mean of duplicate determinations in colony forming units/ml.

‡Experiment number

Table 6.35: The viable counts of three *Candida glabrata* isolates exposed to bronchoalveolar lavage fluid from rat R4.

Strain	Time 0 minutes			Time 120 minutes		
Number	1‡	2	3	1	2	3
g1	$1.1x10^6$	$1.5x10^6$	$1.3x10^6$	$1.0x10^6$	$1.5x10^6$	$1.1x10^6$
g2	$9.9x10^5$	$1.1x10^6$	$8.8x10^5$	$9.6x10^5$	$9.9x10^5$	$8.7x10^5$
g3	$1.6x10^6$	$1.9x10^6$	$1.3x10^6$	$1.6x10^6$	$1.8x10^6$	$1.1x10^6$

*Mean of duplicate determinations in colony forming units/ml.
‡Experiment number

Table 6.36: The viable counts of three *Candida glabrata* isolates exposed to bronchoalveolar lavage fluid from two rats R3 and R4.

Strain Number	Rat 3 T0	Rat 3 T120	% Survival rate	Rat 4 T0	Rat 4 T120	% Survival rate
g1	$1.2x10^6$	$7.1x10^5$	59.2	$1.3x10^6$	$1.2x10^6$	92.3
g2	$1.0x10^6$	$6.6x10^5$	66.0	$9.9x10^5$	$9.4x10^5$	95.0
g3	$1.7x10^6$	$1.1x10^6$	64.7	$1.6x10^6$	$1.5x10^6$	93.8

*Mean of colony forming units/ml

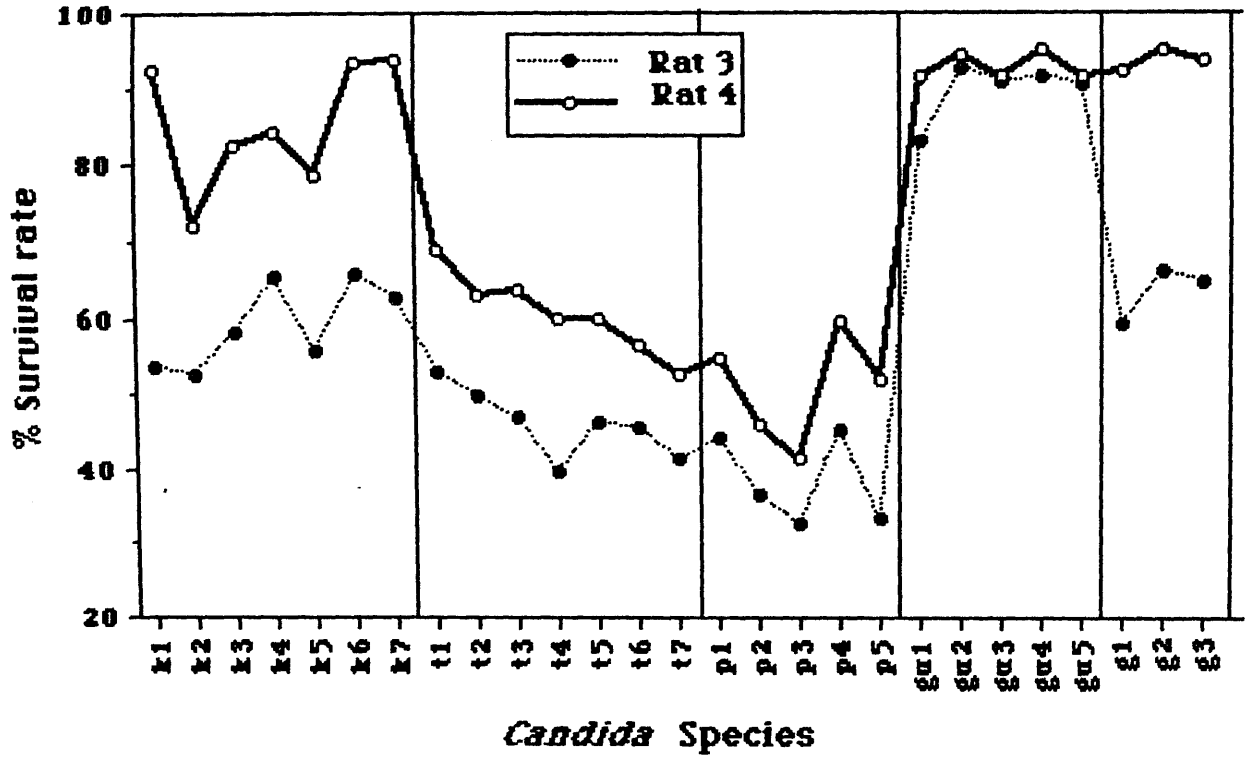


Figure 6.10: The percentage of survival rate of *Candida* species (other than *C. albicans*) when exposed to bronchoalveolar lavage fluid (neat) from rats R3 and R4.

k=*Candida krusei*, t=*Candida tropicalis*, p=*Candida parapsilosis*, g=*Candida glabrata* and gu=*Candida guilliermondii*.

Numbers=Numbers of *Candida* strains.

Table 6.37: A significant differences of viable counts at time 0 and time 120 minutes of six *Candida* species exposed to bronchoalveolar lavage fluid from R3 and R4.

Candida species	Rat 3 Time 0/Time 120 minutes	Rat 4 Time 0/Time 120 minutes
C. albicans	S	S
C. tropicalis	S	S
C. parapsilosis	S	S
C. krusei	S	NS
C. guilliermondii	NS	NS
C. glabrata	NS	NS

S= Susceptible* NS=Not Susceptible:

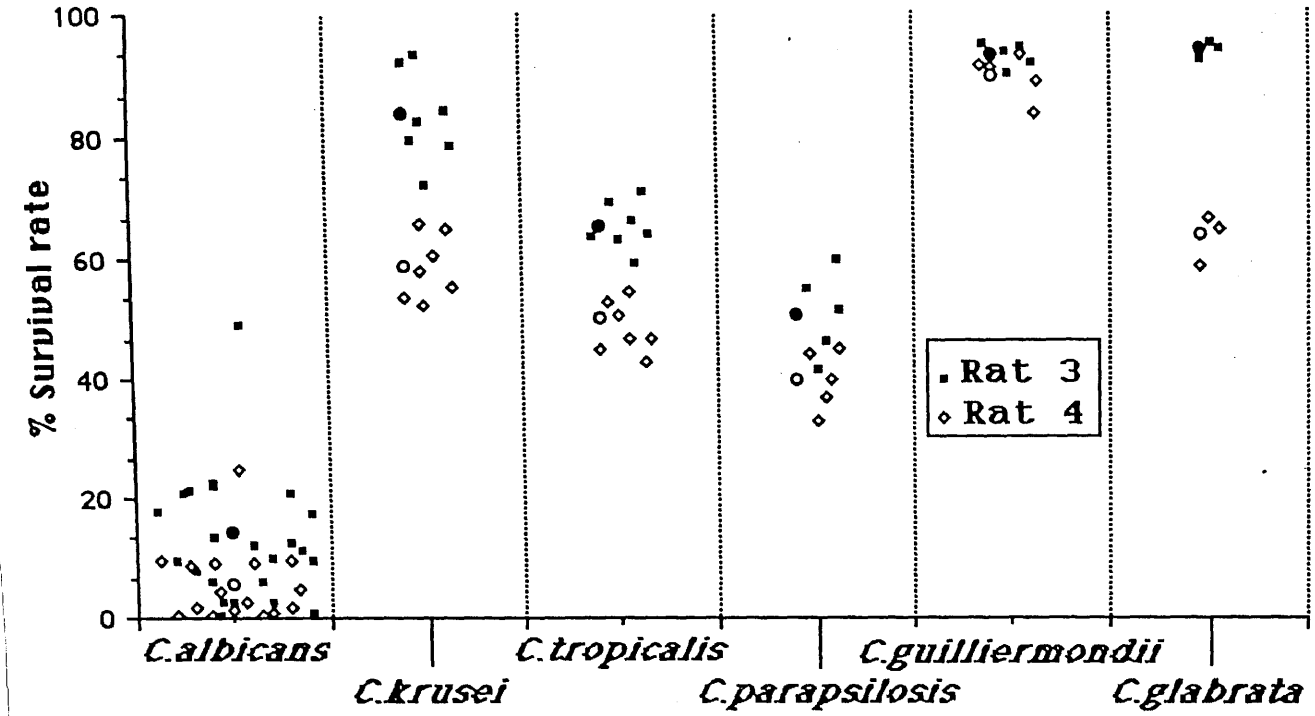


Figure 6.11: The Percentage and mean of survival rate of *Candida* species when exposed to bronchoalveolar lavage fluid from rat 3 and rat 4.

o =Mean percentage survival rate, BLF from R3.

• =Mean percentage survival rate, BLF from R4.

Table 6.38: Mean percentage of viable counts of six *Candida albicans*, when exposed to bronchoalveolar lavage fluid of two rats, R3 and R4.

Candida species	No. of isolates	Rat 3		Rat 4		Mean Rats 3 and 4	
		R.O.*	%‡	R O	%‡	R O	%
C. albicans	22	6	5.57	6	14.5	6	10.0
C. krusei	7	3	59.1	3	85.3	3	72.2
C. tropicalis	7	4	45.9	4	60.6	4	53.3
C. parapsilosis	5	5	38.3	5	50.4	5	44.4
C. guilliermondii	5	1	89.9	2	92.9	1	91.4
C. glabrata	3	2	63.3	1	93.7	2	78.5

*Rank order ‡Mean of viable counts at time 120/time 0 x 100

Table 6.39: A significant difference of six *Candida* species when exposed to bronchoalveolar lavage fluid from rat 3, for 120 minutes.

	Candida species					
	C.a #	*C.k*	*C.t*	*C.p*	*C.gu*	*C.g*
C.a	-	S	S	S	S	S
C.k	S	-	NS	S	S	NS
C. t	S	NS	-	S	S	NS
C.p	S	S	S	-	S	S
C.gu	S	S	S	S	-	S
C.g	S	NS	NS	S	S	-

S=Significant*NS=Not Significant .

ANOVA test was used, (95% confidence intervals).

#*C..a =C albicans, C. k=C. krusei, C.t=C. tropicalis, C.p=C. parapsilosis, C.gu=C. guilliermondii, C.g=C. glabrata .*

Table 6.40: A significant difference of six *Candida* species when exposed to bronchoalveolar lavage fluid from rat 4, for 120 minutes.

	Candida species					
	C.a	***C.k***	***C.t***	***C.p***	***C.gu***	***C.g***
C.a	–	S	S	NS	S	S
C.k	S	–	S	S	NS	NS
C. t	S	S	–	S	S	S
C.p	NS	S	S	–	S	S
C.gu	S	NS	S	S	–	NS
C.g	S	NS	S	S	NS	–

S=Significant* **NS=Not Significant**

ANOVA test was used, (95% confidence intervals).

[#] ***C.a =C albicans, C. k=C. krusei, C.t=C. tropicalis, C.p=C. parapsilosis, C.gu=C. guilliermondii, C.g=C. glabrata .***

6.4 Discussion

Pneumonia due to *Candida albicans* is very unusual, despite the wide use of antibiotics, immuno-suppressive drugs and cytotoxic therapy, and contrasts with the general increase in recent years of most other types of candidal infection.(Hurley *et al.*, 1986). This observation may indicate the presence of rather potent lung-defence mechanisms against *Candida*, because it is not uncommon to isolate this yeast from the upper respiratory tract.

Most secretions and fluids within the mammalian host contain examples of substances that act non-specifically to inhibit or kill microbial invaders (Mims 1987). For *Candida*, there are reports of inhibitory substances such as lysozyme (Chapter Five) in many body fluids (Kozinn Caroline and Taschdjian 1964; Aly *et al.*, 1972, 1975; Laforce Sharrar and Arai 1979). Although, non specific anti-microbial factors present in the respiratory tract may play a role in the host defence against candidal invasion, it is difficult to demonstrate this scientifically. Odds (1988) has enumerated a number of technical difficulties in proving the role of non-specific antifungal factors in body fluids against *Candida* species. Some of these problems include, clumping of viable candidal cells during growth or when exposed to an antagonistic substance which give, a false impression of a candidacidal action (Louria *et al.*, 1964, 1972). In 1955 Janke reported for the first time clumping of viable candidal cells by serum. A few years later Louria and Brayton (1964) described a heat-stable, dialysable component of serum and plasma which caused clumping and killing of *C. albicans*. However other *Candida* species such

as *C. tropicalis, C. parapsilosis* were not affected. Subsequently, Louria *et al.*, (1972) referred to this substance as a "*Candida* clumping factor". The wide variations in the methods used in different studies may explain the discrepancy in the results of the effect of biological fluids on *Candida*. Since agglutination of *Candida* by body fluids is one of the main technical difficulties in assessing non-specific anti-fungal systems, assay mixtures with lower *C. albicans* cell concentrations were used in this study to minimize any possible agglutination during the investigation. Furthermore, the anticandidal activity of BLF was compared with that of sterile horse serum. Generally, the candidacidal activity of both the serum ($p < 0.05$) and BLF ($p < 0.001$) were significantly different when compared with PBS control. However, highly significant differences did occur between serum and BLF ($p < 0.001$), indicating that the reduction in colony forming units of *C. albicans* after exposure to BLF is highly unlikely to be due to a clumping/agglutinating effect of *Candida* cells. This was further confirmed by microscopic observation.

In the present study, BLF was investigated for anti-candida activity and our results suggest that the fluid may well play an important role in the non-specific host defences of the lower respiratory tract against infection with *C. albicans*. Since heat-inactivation of BLF (56°C for 30 minutes) produced slight reduction in anti-candida activity when compared to normal BLF, it is possible that both heat-labile and heat-stable factors are involved.

When the relationship between the concentration of BLF and anti-candida activity was investigated, exposure to BLF had a significant growth inhibition and killing effect on *C. albicans* (a 15). For example, even a 32 fold dilution of BLF from rat 2 resulted in 66 percent reduction in CFU's.(Table, 6.17 and Figure, 6.8).

A series of experiments were carried out to evaluate the spectrum of candidacidal activity of BLF in 7 test and 5 control rats (see material and methods section 6.2.2). Although, the results revealed no significant differences between the two groups of rats significant variations between the individual rats within each group were observed. These findings suggest that the concentration of anti-candida factor in BLF may vary among rats and possibly could be a factor related to the resistance of individual animals to pulmonary candidosis.

The present results also show wide variation in sensitivity to BLF among different *Candida* species (Figure 6.11). Thus *C. albicans* is the most susceptible species and shows significant differences in susceptibility to BLF when compared with all other species (Table 6.39 and 6.40) with the exception of *C. parapsilosis* (using BLF from rat 4). *Candida guilliermondii*, proved to be the most resistant species. A number of interesting points can be drawn from these results. The more susceptible species are those which are regarded as more pathogenic, and since pulmonary candidosis is rare, the protective effect of BLF may play an important role in the defence of the pulmonary tract. Therefore, it seems possible that the BLF plays an important role

in the non-specific defence system against the more pathogenic *Candida* species.

Generally, little is known about the non-specific defence mechanisms which protect the lung. In a study using rabbit BLF, La Force Sharrar and Arai (1979) suggested that yeasts were agglutinated by the fluid, and that this facilitated non-phagocytic fungicidal mechanisms. Most studies of the mechanism of microbial killing in the pulmonary alveoli have been carried out using staphylococci, together with standard histologic techniques. The results indicated that if staphylococci gain access to the alveoli they are killed predominantly by macrophages (Green and Kass 1964 and Kim Goldstien Lewis Lippert and Warshaur 1976). However, alveolar macrophages often show poor chemotactic and phagocytic ability compared with peritoneal macrophages (Dauber and Daniels 1978) and have weak intracellular killing activity *in vitro* (Juers Rogers McCurdy and Cook 1976). Therefore, in the case of *Candida,* it is possible that the yeast cells may require to be killed extracellularly or coated by some as yet, unidentified factor(s) in BLF which would have the effect of assisting in the capture and ingestion of yeasts by the alveolar macrophages. In a search for extracellular bactericidal factors for pneumococci, Coonrod and Yoneda (1983) and Coonrod Lester and Hsu (1984) have demonstrated that the surfactant fraction (an exracellular product, which is haemolytic to certain bacteria) of leukocyte-free lung lavage of rats and other animals contains heat resistant factors that are rapidly bactericidal and lytic for pneumococci *in vitro.* Further studies are therefore,

required to clarify whether similar heat-resistant factors are involved in the candidacidal mechanisms of BLF.

Further, Nugent and Pesanti (1982) obtained evedence that inhaled staphylococci are killed outside alveolar macrophages by unknown mechanisms. Onfrio *et al.*, (1983) have reported that low doses of *Staphylococcus aureus* fail to evoke a PMN response. However, when a heavy inoculum of *Staph. aureus* was used a PMN response occurred, and a two step killing-system appeared to operate. If these results with bacteria are extrapolated to *Candida* it is possible that at low concentrations of yeast cells, non-specific factors in BLF, can kill and inhibit the growth of *Candida* which may then be phagocytosed by alveolar macrophages. However, if a heavy inoculum of *Candida* is present, two-stage killing system similar to that described for *Staph. aureus* may be required.

There is only one study in the literature which described the candidacidal effect of BLF. However, the latter group used BLF obtained from Swiss Webster mice as opposed to BLF obtained from Sprague-Dawley rats in this study. Nevertheless, data obtained from the current study is in close agreement with the findings of Nugent and Fick (1987), who reported that the blastospores of most of the *Candida* species which they tested (the number of isolates was not recorded) were inhibited by BLF for 120 minutes. They found that the mean viability of *C. albicans* isolate incubated with mice BLF for 120 minutes was reduced to 25 percent. This result compares well with the findings of the current study in which the percentage mean survival rate of *C.*

albicans isolate, a15 was 11.2 and 21.9 percent using BLF (exposure time, 120 minutes) from control and test rats respectively. The percentage mean survival rate of 22 isolates of *C. albicans* was 5.6 and 14.5 percent, with BLF from two rats (R3 and R4). Also Nugent and Fick (1987) reported that BLF from the mice killed a single isolate of *C. tropicalis, C. glabrata* and a second *C. albicans* isolate but did not kill one isolate of *C. parapsilosis,* one isolate of *C.krusei,* and a third isolate of *C. albicans.* The results of the current investigation indicate that different *Candida* strains and species are inhibited by BLF from different animals to varying degrees. For instance, the mean percentage survival rate of *C. krusei* was 56.1 and 85.3 percent when BLF from R3 and R4 respectively were used.

In conclusion, the results of these *in vitro* experiments suggest that the BLF possesses anti-candida activity which may protect the lower respiratory tract from colonization or infection by *C. albicans.* However, further experiments with BLF obtained from humans are required to ascertain if BLF is involved in protecting the human respiratory tract from candidosis.

CHAPTER SEVEN

CONCLUDING DISCUSSION

7.1. Pathogenic attributes of *Candida* species

Formerly, candidoses occurred quite rarely, but in recent years they have become much more common and of greater medical significance. The rise in the incidence of these opportunistic infections has paralleled the use of immune-disruptive procedures e.g., the use of antibiotics, cytotoxics, immuno-suppressive, drugs, and steroids, that result in lowered resistance of the host.

In the last decade, the number of studies dealing with *Candida* species has increased very rapidly and although, there is much more knowledge about specific pathogenic factors related to a relatively small number of yeast isolates, few workers have investigated in depth, a single or a range of pathogenic factors using many strains of *Candida* in the same study. In this investigation a large number of isolates (49) of six *Candida* species consisting of: *C. albicans*, (22), *C. tropicalis*, (7), *C. parapsilosis*, (5), *C. krusei*, (7), *C. guilliermondii* (5) and *C. glabrata*, (3), were used to study the following pathogenic parameters; adhesion to both BEC's and acrylic strips, production of phospholipase and proteinase, and the anti-candidal activity of non-specific host defence mechanisms (the candidacidal activity of lysozyme and bronchoalveolar lavage fluid).

The establishment of a candidal infection involves a complex series of interactions between the host, the yeast cell, and various environmental factors. Each of these factors consists of a number of pathogenic determinants which may be involved in the initiation or prevention of infection. Therefore, studying a single parameter *in vitro* may not give an accurate account of the factors activity *in vivo*. The one given aim of this study was to investigate a range of pathogenicity factors of *Candida* species *in vitro* and then select 'pathogenic' isolates on the basis of *in vitro* results, for use in an experimental rat model. However, due to lack of time the animal work was not completed, and therefore not reported in the thesis. A summary of the *in vitro* results obtained by this investigation are shown in Table 7.1 and 7.2, and in the next few pages the different factors will be discussed.

7.2. Adhesion.

The results of this study have established the presence of strain variation in the adhesion of *C. albicans* strains to BEC's as well as to acrylic strips. This variation may indicate that each individual isolate possess pathogenic features which are different from other isolates of *C. albicans* and may be related to virulence, The findings may also support recent reports concerning the so called clonality phenomenon, in which most natural microbial populations are believed to consist of several discrete clonal lineages, and diseases are caused by only a

small proportion of the total number of clones which occur in these species (Finlay and Falkow, 1989).

The hierarchy of adhesion of *C. albicans* to BEC's and acrylic surfaces is shown in Table 7.1. Interestingly, isolates from infected patients tended to give higher adhesion values to both surfaces, ranging from 850 to 1569 yeast cells/100 BEC's, and from 307 to 568 yeast cells/mm^2 of acrylic strips, compared with strains believed to be isolated from asymptomatic carriers which gave values ranging from 542 to 850 yeast cells/100 BEC's and from 180 to 298 yeast cells/mm2 of acrylic strips (see Chapter Three). The hierarchy of adhesion of *Candida* species to BEC's and acrylic surfaces, is shown in Table 7.2 and in general *C. albicans* strains adhered maximally to BEC's, whereas, *C. tropicalis* strains adhered in greatest numbers to acrylic strips.

Great care was taken to standardize the adhesion assay in this study since many factors have been shown to influence the adhesion of *C. albicans* to both biological and non-biological surfaces *in vitro* (Kennedy, 1987). Of those factors, growth conditions (especially, the presence of certain carbohydrates) are possibly the most important. The adhesiveness of *Candida* cells therefore, can be manipulated by the conditions under which the cells are cultured, and may depend as much on environmental factors present in the assay as on the ability of yeast. The adhesiveness of *Candida* cells therefore, can be manipulated by the conditions under which the cells are cultured, and may

Table 7.1. Hierarchy of potential pathogenic attributes of *Candida albicans* strains

Candida albicans	Adhesion to BEC's Yeast/100	Adhesion to Acrylic Yeast/mm^2	Phospholipase activity *Pa* *	Proteinase activity *Pr*a ‡	Lysozyme activity F_{1y}*	% of survival rate *C. albicans* (BLF from R3)
a1	883(15)	388(14)	46.6(3)	0.8(12)	5.022(1)	3.0(11)
a2	642(21)	207(20)	39.9(12)	0.0(18)	2.941(6)	1.1(15)
a3	754(19)	342(15)	39.4(13)	0.7(13)	3.201(4)	1.8(14)
a4	977(10)	492(6)	48.4(2)	1.0(6)	2.316(13)	0.5(19)
a5	542(22)	180(22)	21.3(22)	2.7(2)	3.272(2)	25.0(1)
a6	942(11)	391(12)	37.7(14)	0.0(19)	3.032(5)	0.9(16)
a7	1119(7)	554(3)	46.2(4)	0.3(16)	2.135(15)	0.3(22)
a8	1410(2)	557(2)	49.8(1)	2.7(3)	1.888(18)	0.7(17)
a9	913(14)	307(16)	43.0(8)	1.0(7)	1.332(21)	0.7(18)
a10	924(13)	432(10)	40.1(11)	0.0(20)	2.035(17)	4.2(10)
a11	1279(4)	477(7)	45.0(5)	0.0(21)	1.737(19)	0.5(20)

*F_{1y}=Lysozyme activity units. See Chapter Four. ()=rank order.

* *P*a = Proteinase activity units. See Chapter Five.

‡ *Pr*a = Phospholipase activity units. See Chapter Five.

Table 7.1. (Continued).

Candida albicans	Adhesion to BEC's Yeast/100	Adhesion to Acrylic Yeast/mm^2	Phospholipase activity *Pa* *	Proteinase activity *Pra* ‡	Lysozyme activity F_{ly}#	% of survival rate *C. albicans* (BLF from R3)
a12	1189(6)	471(8)	41.7(9)	1.0(8)	2.318(12)	0.5(21)
a13	791(17)	224(19)	28.4(19)	1.5(5)	2.493(9)	10.1(3)
a14	1051(8)	415(11)	32.2(17)	1.0(9)	2.046(16)	1.9(13)
a15	1569(1)	568(1)	40.1(10)	3.0(1)	2.437(10)	4.6(8)
a16	691(20)	190(21)	25.0(21)	0.5(15)	2.389(11)	22.3(2)
a17	816(16)	298(17)	36.3(16)	1.0(10)	1.151(22)	4.8(9)
a18	790(18)	288(18)	30.0(18)	0.0(22)	2.191(14)	9.2(6)
a19	1331(3)	540(4)	37.5(15)	2.7(4)	2.551(7)	9.3(5)
a20	990(9)	539(5)	28.2(20)	1.0(11)	2.547(8)	9.2(7)
a21	942(12)	391(13)	43.2(7)	0.7(14)	3.252(3)	9.7(4)
a22	1191(5)	444(9)	44.8(6)	0.3(17)	1.477(20)	2.3(12)

#F_{ly}=Lysozyme activity. See Chapter Four. ()=rank order.

* *Pa* = Proteinase activity. See Chapter Five.

‡ *Pra* = Phospholipase activity. See Chapter Five.

Table 7.2. Hierarchy of potential pathogenic attributes of *Candida* species studied

Candida species	Adhesion to BEC's Yeast/100	Adhesion to Acrylic Yeast/mm^2	PL* activity %	Proteinase‡ activity %	Lysozyme§ activity F_{1Y}	% Survival# rate (BLF-R3)
C. albicans	988(1)	395(2)	100(1)	68.2(3)	2.47(5)	5.57(6)
C. tropicalis	464(2)	669(1)	0.00	71.4(2)	3.27(3)	45.9(4)
C. parapsilosis	262(3)	375(3)	0.00	100(1)	3.34(2)	38.3(5)
C. glabrata	183(4)	340(4)	0.00	0.00	0.33(6)	63.3(2)
C. guilliermondii	171(5)	222(5)	0.00	0.00	2.89(4)	89.9(1)
C. krusei	89(6)	131(6)	0.00	0.00	4.54(1)	59.1(3)

*Percentage of phospholipase positive.strains. ()=rank order.

‡Percentage of proteinase positive.strains.

#The % mean survival rate of *Candida* species to BLF from rat 3.

§F_{1Y}=Lysozyme activity. See Chapter Four.

depend as much on environmental factors present in the assay as on the ability of yeast cells to produce an adhesin (Freter and Jones 1983; Kennedy and Sandin 1988). As noted by Odds Hall and Abbott (1978); and Kennedy and Sandin (1988) major differences in the chemical composition of a medium may occur from manufacturer to manufacturer, and from lot to lot from the same manufacturer The finding that sugars (e.g., sucrose) can promote synthesis of yeast adhesin(s) might partly explain the clinical observation that a carbohydrate-rich diet can predispose individuals to oral candidosis (Shuttleworth and Gibbs 1960, Samaranayake and MacFarlane 1985). Furthermore, since the results of the study suggests that the adhesion of different strains of *C. albicans* grown in sucrose, vary widely, it is possible that the effect of a high sucrose diet in the pathogenesis may be different among individual strains of *C. albicans* depending on the adherence ability of the infecting strain.

7.3. Phospholipase activity

Samaranayake Raeside and MacFarlane (1984) reported that phospholipase activity was present in most *C. albicans*, isolates but in none of the *C. parapsilosis* or *C. tropicalis* strains which they studied. This investigation also demonstrated that out of six *Candida* species tested only strains of *C. albicans* showed positive phospholipase activity. For the first time the phospholipase activities of *C. krusei* and *C. guilliermondii* were investigated and found to be uniformly negative. The only evidence which has linked the enzyme to *Candida* virulence was reported by

Barrett-Bee *et al.*, (1985) who demonstrated a correlation between phospholipase activity, LD_{50} results in mouse experiments and adhesion of the yeast to human buccal epithelial cells. As mentioned earlier phospholipase was produced by 100 per cent of the *C. albicans* isolates investigated in the present study, although, not all isolates of *C. albicans* produced the enzyme to the same extent. The variation in phospholipase production by the 22 *C. albicans* isolates tended to correspond with the adhesion values to BEC's (Figure 7.1). The phospholipase of three isolates (a2, a9 and a20) did not give similar pattern with their adhesion values to BEC's. These results tended to support those of Barrett-Bee *et al.*, (1985), who reported that four strains of *C. albicans* correlate with adhesion to BEC's. Production of phospholipase also, appeared to be related to the clinical source of the isolates, (Figure 7.2) since all strains isolated from candidosis cases produced more than 30 units of phospholipase activity, whereas those isolated from asymptomatic carriers produced less than 30 units of phospholipase activity. The one exception to this trend was isolate a17 (asymptomatic carrier strain) which produced 36.3 units of phospholipase activity. Hence, the overall increased levels this enzyme in the active pathogenic *Candida* strains compared to asymptomatic strains may support current perception that phospholipase plays a part of *Candida* pathogenicity.

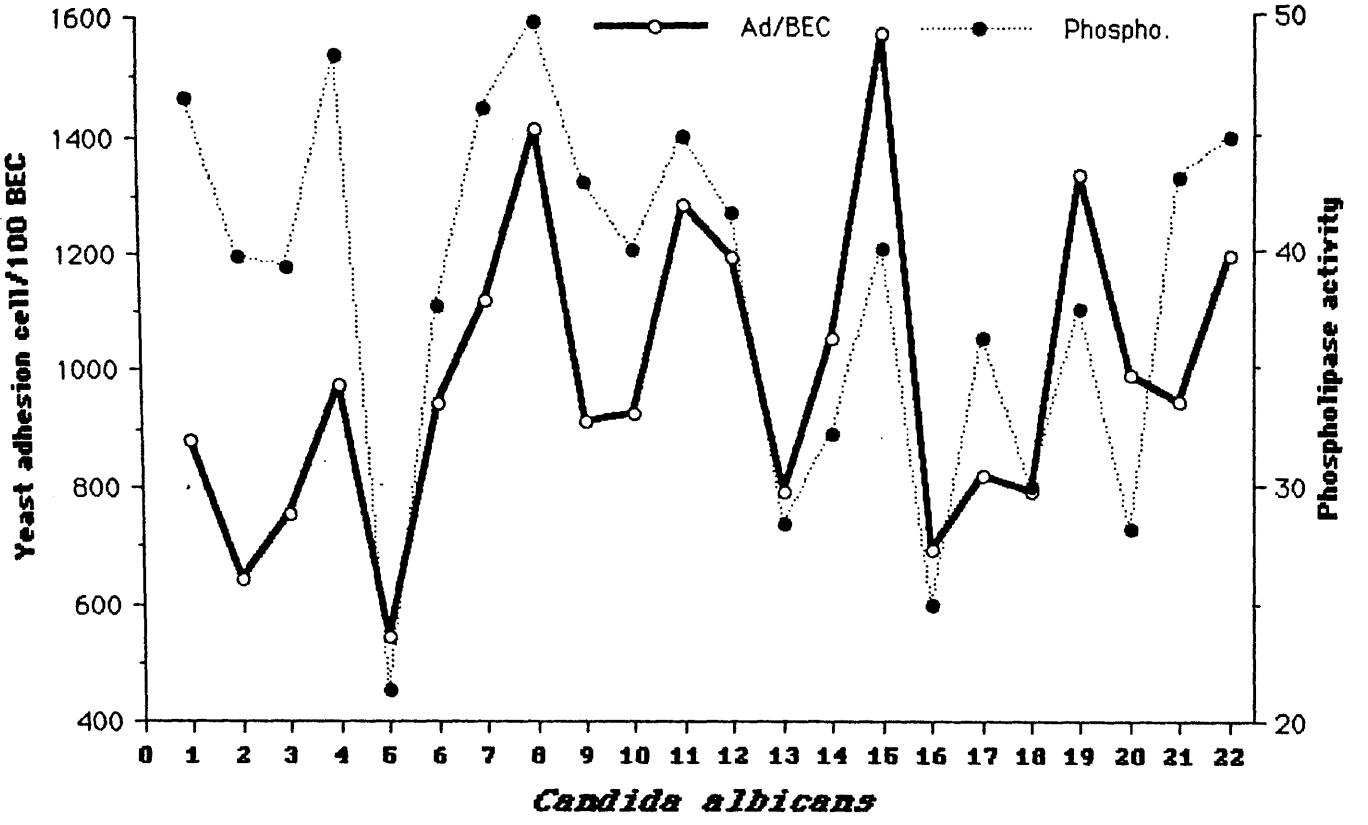


Figure 7.1. The adhesion of 22 *Candida albicans* isolates to buccal epithelial cells, compared with their phospholipase activity.

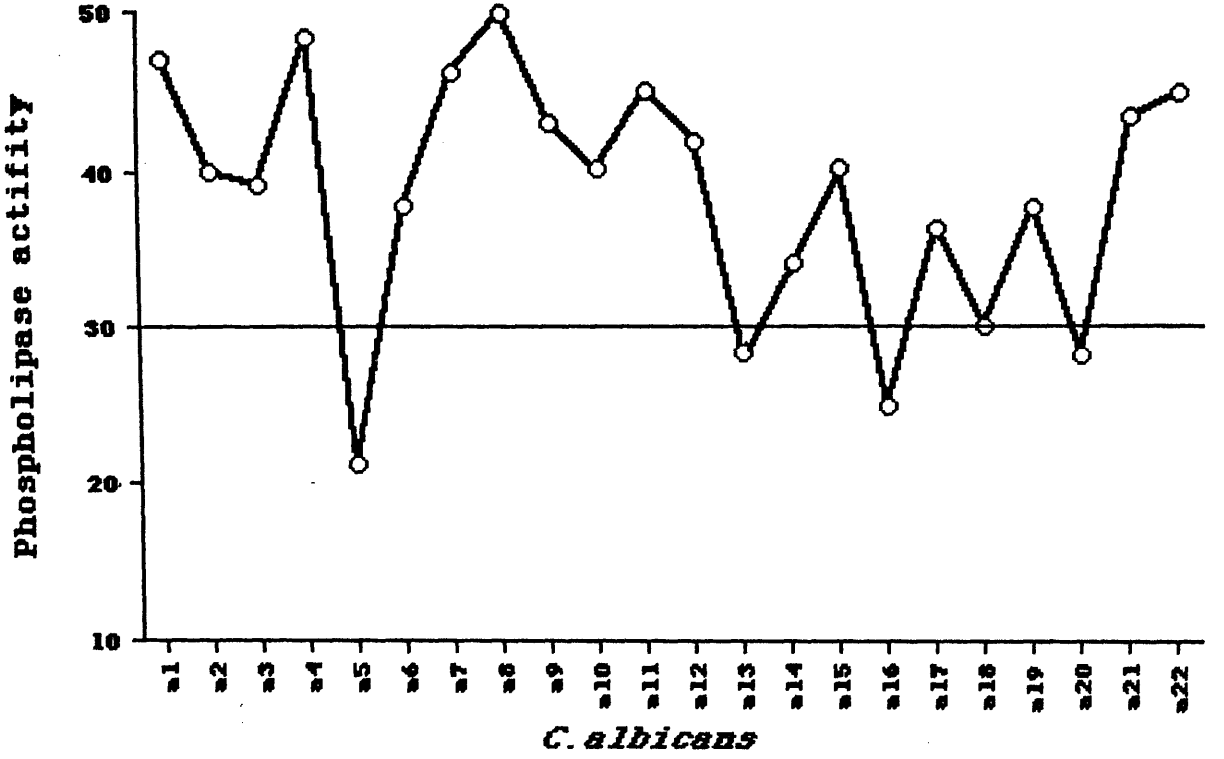


Figure 7.2. The phospholipase activity of *Candida albicans* related to the clinical source of the isolate. The isolates below the horizontal line were cultured from asymptomatic carriers, while those above the line were obtained from patients with candidosis.

7.4. Extracellular proteolytic activity

Proteolytic enzymes were produced by strains of *C. albicans, C. tropicalis* and *C. parapsilosis*, but not by other species as shown in Table, 7.2. Candida proteinases were first studied by Staib in 1965 (see Chapter Four) and subsequently, in 1968, Remold *et al.*, claimed that the enzyme was secreted only by strains of *Candida* species that were pathogenic for mice. This view was supported by others (Budtz-Jörgensen 1971 and Ghannoum and Abu-Elteen 1986) who found a correlations between proteinase activity and strain virulence. Antibodies to purified proteolytic enzyme from *C. albicans* have been demonstrated in patients with visceral candida infection and evidence was produced that the enzymes were synthesized by *Candida* in infected tissues *in vivo* (MacDonald and Odds 1980b). Recently, Rüchel *et al.*, (1986) have provided evidence that the *C. albicans* and *C. tropicalis* secretes enzymes *in vivo* unlike *C. parapsilosis* which produces the enzyme *in vitro* but not *in vivo*.

In this study no obvious relationships could be demonstrated between the proteinase activity of *Candida* species and adhesion to BEC's or acrylic surfaces or to their phospholipase activity or to source

7.5. Susceptibility to lysozyme

The host tissues protect themselves by producing broad spectrum anti-microbial substances, such as lysozyme. These substances are found in many body fluids (e.g., saliva and tears) as a part of the host defence system and are also present

in the lysozomes of human polymorphonuclear leukocytes (Franson *et al.*, 1977).

The effect of lysozyme on 22 isolates of *C. albicans* was examined in Chapter Five and the results suggested that due to its anti-candidal activity the enzyme could act as a potent inhibitor of candidal colonization of the oral cavity. A significant dose response relationship between lysozyme concentration and fungicidal activity was also found. However, overall the results show variations in the degree of susceptibility of different *C. albicans* isolates to lysozyme. Other *Candida* species also demonstrated wide variation in their susceptibility to the enzyme, (Table 7.2). For example, *C. glabrata* was most susceptible, whereas, *C. krusei* was the most resistant species, see Table, 7.2.

All phagocytic cells possess numerous degradation enzymes packaged in cytoplasmic granules, there also are present granule-associated enzymes, such as lysozyme (Bretz and Baggiolini 1974; and Franson *et al.*, 1977). However, the mechanisms of these reactive proteins in disruption of candidal cell wall are unknown. One speculation that it may form a complex with cell wall polymers, e.g., mannan, by electrostatic bond, initiating pore formation on the wall allowing the flow of external materials into the inner cell. However further studies are required to ascertain the role of the extracellular cell wall components in the susceptibility of *C. albicans* to lysozyme.

7.6. Bronchoalveolar lavage fluid

Bronchoalveolar lavage fluid, has non-specific anti-microbial factor which may play a part in the host defence of the pulmonary tree with regard to infection with *Candida* species. Although, rats are widely used in experimental candidosis models, the work presented in Chapter six, is the first investigation to study the anti-candidal activity of rat BLF. The results of this study suggest that BLF may well play an important role in the non-specific host defences of the lower respiratory tract against infection with *C. albicans*. When the susceptibility of 22 *C. albicans* isolates was investigated against the anti-candida activity of BLF, the fluid had significant growth inhibition and killing effects on *C. albicans*. Table 7.3 shows that 59.1 percent (13 strains) of 22 isolates of *C. albicans* produced <5 percent survival rate when incubated along with neat BLF for 120 minutes. The susceptibility and hierarchy of *Candida* species to BLF are shown in Table, 7.2, in which *C. albicans* appears as a highly susceptible species followed by *C. parapsilosis*, *C. tropicalis*, *C. krusei*, *C. glabrata* and *C. guilliermondii*. Inactivation of BLF by heat (56°C for 30 minutes) produced a slight reduction in anti-candida activity when compared to controls. This may indicate the presence of both heat-labile and heat-stable factors, but further work is required to clarify this area.

Interestingly, the susceptibility of most of the *C. albicans* strains to BLF was generally directly related to the susceptibility of the other non-specific anti-microbial agent,

tested i.e., lysozyme (Figure 7.3).

Microbes at the inflammatory site may be exposed, even before intercellular sequestration has taken place, to degradative non-specific factors released by phagocytes that apparently are functionally and structurally intact (Roitt 1980). Thus, for instance, *Candida* invading the lungs may be exposed to the cytolytic agents, such as BLF extracellularly and lysozyme within the phagocytes. This in part, may explain the relative absence of pulmonary candidosis in the population despite the presence of the organism in the mouth of approximately 51 percent of humans. However, must be emphasized that the other host defence mechanisms of the respiratory system such as the intact epithelium are important for the overall defence against candidal invasion.

7.7. Variations in the activity of different *Candida albicans* strains

The variation seen within each species, may indicate that each individual strain possess its own virulence factors which are different from other strains. Therefore, a candidal strain which may have the ability to colonize and infect the mucosa of one animal, may cause no infection in another animal, due to different immune-systems and environments, e.g., antibiotics, carbohydrates and bacteria . Hence, these two host factors may alter the potential of the yeast to colonize and cause infections.

7.8. Variations in the methods, growth conditions and stability of biological and non biological surfaces used in *Candida* adhesion

A range of different methods have been used to investigate the adherence of *Candida* species, and it is clear that the results obtained are often not comparable. There seem to be a need for an international agreement among scientists concerning the use of a standard method for studying adhesion. Although, the growth conditions, chemical compositions of media and the methodology used can all be more or less, regulated and standardized, the adhesion surfaces are the more difficult to stabilize and control. A recent suggestion by Kennedy (1987), that pooled epithelial cells from 10 or more healthy donors should be used as a standard biological surface has much to commend it. The cells appear to be unaffected by storage for days at 4°C when used in standard adhesion assays. However, there is no doubt that the adaption of a standard assay method would enable meaningful comparisons to be made between the work carried out in different research centres.

7.9. Concluding remarks

The work of this thesis supports and extends information about the pathogenic potential of *Candida*. In addition some original observations are presented.

1) Variations in adhesion were observed not only between members of the genus *Candida* but also between the isolates of

the same species. For instance, a strain of *Candida* species can adhere to different extents to the same epithelial cells in different assays. Also, different strains of the same *Candida* species, grown under the same conditions, adhere to different extents to the same BEC's. Further, the yeasts are susceptible to different degrees to the lytic action of lysozyme and BLF.

2) A relationship was found between two pathogenic parameters of *Candida* ie. adhesion and phospholipase activity. Also a relationship was shown between the susceptibility of *Candida* to non-specific cytolytic action of BLF and lysozyme.

3) *Candida albicans* strains are more susceptible to the lytic action of lysozyme and BLF than other *Candida* species. Thus it appears that the more pathogenic the yeast, the more susceptible it is, to non-specific antifungal factors.

4) The current study supports the existing data that the production of phospholipase is limited to strains of *C. albicans* only.

5) The results of the present study also supports the existing data that only strains of *Candida albicans*, *C. tropicalis* and *C. parapsilosis* produce proteinase *in vitro*.

6) These results give the impression that the pathogenicity of a *Candida* species is based on genetical factors, i.e., *C. albicans* is the only species which produces phospholipase activity and germ-tubes. Also the variations between the members of the same species may suggest that the virulence of a

particular strain is related to combined activity of pathogenic factors.

7) The current results concerning candidal adhesion, phospholipase and proteinase production, may indicate that not all *Candida* isolates have an equal chance of causing infection and that disease may be regulated by these and other factors.

8) The non-cytolytic host defence features, lysozyme and BLF results, may indicate that the initiation of candidosis does not depend solely on the organisms pathogenic features but also on the host and the environmental factors. For instance, adhesion requires the participation of two equally shared factors, a receptor on the host cell surface and adhesins on the candidal cell wall as well as environmental factors which may enhance or inhibit this process.

The results of this study expand and augment current knowledge and also cast new light on interrelationships between various pathogenic factors of *Candida* species which may be involved in causing of human candidoses. Clearly, much work remains to be done before the mechanism of candida pathogenicity is fully established.

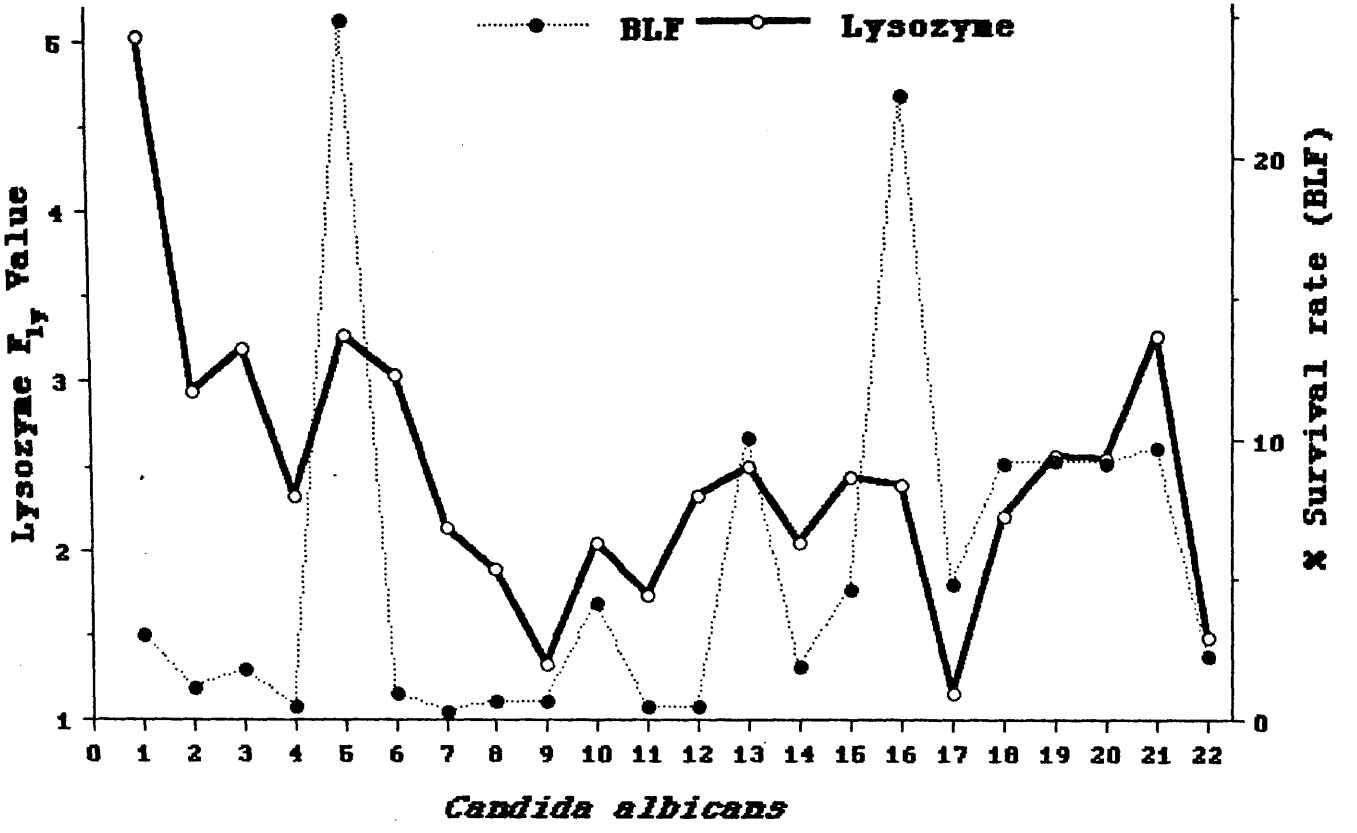


Figure 7.3. Interrelationship between the susceptibility of *Candida albicans* strains to lysozyme and the percentage of mean survival rate to bronchoalveolar lavage fluid.

Table 7.3. Distribution of the percentage survival of 22 isolates of *Candida albicans* when exposed to neat bronchoalveolar lavage fluid for two hours.

% survival	Frequency of isolates	% (of 22 isolates)
0-1	7	31.8
>1-5	6	27.3
>5-10	6	27.3
>10-50	3	13.6

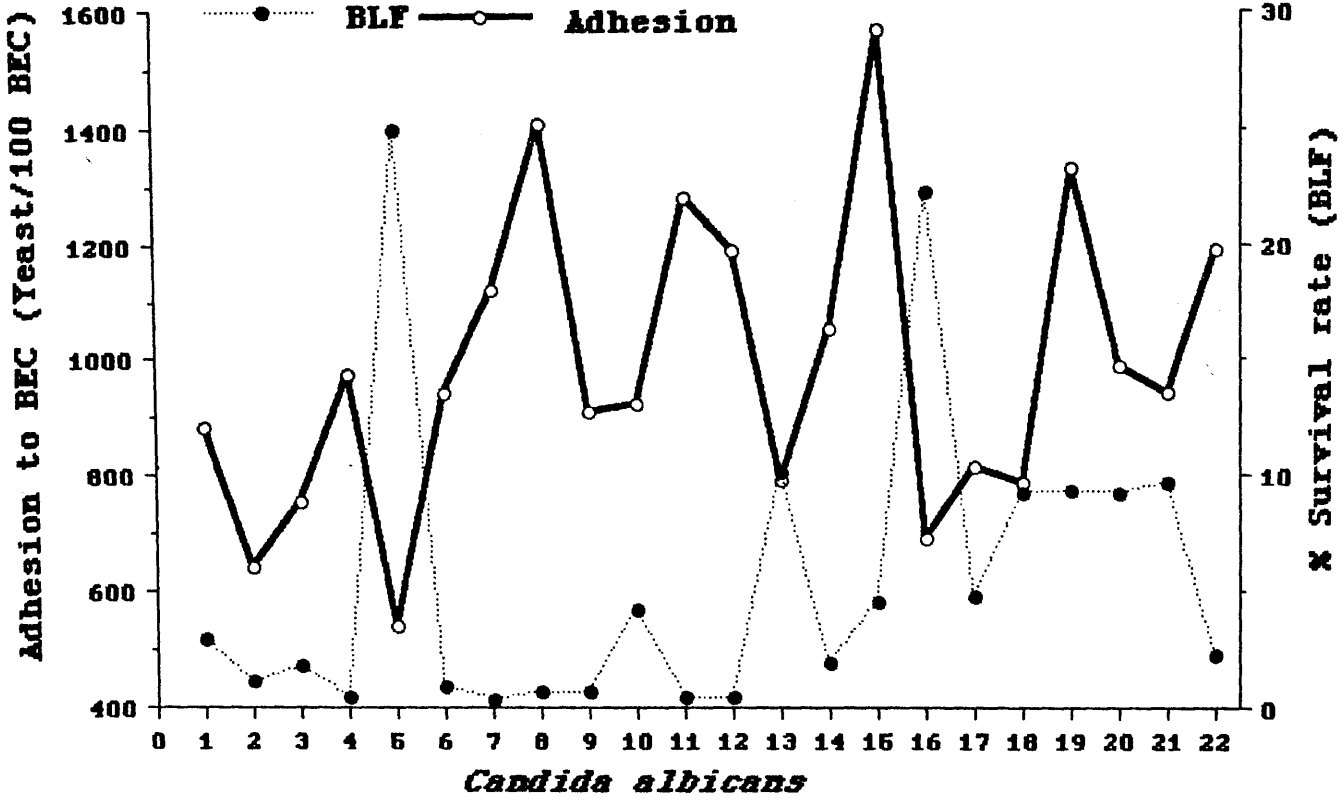


Figure 7.4. A comparison between adhesion of *Candida albicans* to buccal epithelial cells with the percentage of survival rate to bronchoalveolar lavag activity.

Appendix

I. Names and addresses of companies supplying apparatus and chemicals mentioned in the text

A Ridgeway-Watt A 400 multi-point inoculator	(Denley Instruments Ltd, Billinghurst, Sussex, England).
Colony counter	(Gallenkamp & Co. Ltd, P.O.Box 290, Technico House, Christopher St, London, EC2P ER.).
Haemocytometer counting chamber	(Hawksley, England).
Orbital incubator	(Gallenkamp, London, EC2P ER.).
Spiral plater system	(Spiral system Marketing; Baltimore, USA).
Zone reader	(Luckham Ltd, Victoria Gdns., Burgess Hill, West Sussex, UK.)
Millipore system	Millipore (UK) Ltd, 11-15 Peterborough Road, Harrow, Middx HA1 2YH
Freeze-drying	Edwards High Vacuum, Manor Royal, Crawley

II. Names and addresses of companies supplying chemicals and media mentioned in the text

Horse serum	(Gibco, Glasgow, UK)
IgA	(Sigma, London, Chemical Co., Fancy Road, Poole, Dorset, BH17 7NH)
Egg yolk	(Oxoid, Ltd, Basingstoke, England, UK).
Mycological Peptone Medium	(Oxoid).
Glucose	(British Drug House).

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