STUDIES ON CANDIDA-SALIVA INTERACTIONS

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DECLARATION

This thesis is the original work of the author

SUMMARY

Candida species are members of the normal oral flora in 40 to 60% of individuals and are nonpathogenic, but if certain environmental changes occur they can become harmful parasites and cause a variety of oral diseases. Although saliva is a major ecological factor of the oral environment, surprisingly, there is little published information about the interaction of Candida with the constituents of saliva.

One salivary factor that has been investigated in this study is the antifungal activity of lysozyme against C. albicans, and three other Candida species. In Chapter 2, the in-vitro antifungal activity of lysozyme for C. albicans was confirmed and a dose effect between lysozyme concentration and yeast inhibition was shown using a single strain of C. albicans. It is known that Candida can produce a variable layer of extracellular material around their cell wall, the thickness of which depends on the sugar utilized in growth. This layer can protect Candida from the lytic effect of Zymolase-5000, but there is no similar information concerning lysozyme. Since excess carbohydrate appears to be an important factor in Candida growth in saliva, the production of extracellular material in vivo could have an important inhibitory effect on antifungal activity of lysozyme. Therefore in Chapter 2 the antifungal activity of lysozyme on C. albicans GDH 1878 after culture in galactose, sucrose, glucose, maltose, lactose and xylitol was studied.

After growth to stationery phase in a defined medium containing 500mM concentrations of each sugar, the inhibitory effect of 20 µg/ml of hen egg white lysozyme was measured. The results showed that yeast cells previously cultured in sucrose and galactose were most resistant to the action of lysozyme, while those grown in maltose, xylitol, glucose and lactose were less sensitive to the inhibitory activity of lysozyme (20 µg/ml).

In order to determine if the protective effect conferred by dietary sugars against lysozyme was concentration dependent, C. albicans, C. tropicalis, C. glabrata, and C. krusei, were cultured in yeast nitrogen base containing three different concentrations of sucrose (0.5M, 0.125M, 0.03M) before exposure to 20 µg/ml lysozyme. Generally the results showed that pre-incubation of C. albicans and C. tropicalis in increasing concentrations of sucrose, produced increasing resistance to the antifungal action of lysozyme. The effect of different concentrations of sucrose on the viability of C. glabrata could not be clearly established. However the candidacidal effect of lysozyme on C. krusei decreased when pre-incubated with increasing concentrations of sucrose.

Few studies has been carried out to investigate the growth of Candida in saliva and to identify growth factors involved. In addition, data on growth, pH, acid production and protein degradation is poorly defined. There is some controversy regarding the ability of Candida to degrade salivary proteins and it has been suggested that protein loss may be related to adsorption to the yeast cells as well

as to enzyme degradation. Thus it was decided to investigate the ability of C. albicans GDH 1878, to grow in pooled parotid and pooled mixed saliva. The parameters measured were growth, pH, acid end products (using isotachophoresis) and measurements of salivary protein breakdown by biochemical assay and sodium dodecyl sulphate polyacrylamide gel electrophoresis.

When cultured in pooled parotid saliva, C. albicans GDH 1878 demonstrated moderate growth ($2.89x10^6$ to $6.90x10^7$), produced 6 different acid anions and 43% protein degradation while the pH remained alkaline throughout the 72 hours of the experimental period (8.90 to 7.76). This protein breakdown could be due to the alkaline proteases of C. albicans GDH 1878. Since very little is known about the alkaline protease of Candida species no definite conclusions can be drawn from the present finding. However, C. albicans GDH 1878 cultured in pooled whole saliva demonstrated more profuse growth ($5.06x10^6$ to $1.39x10^8$), produced 6 different acid anions, 83% protein degradation and an average reduction of 4 pH units. Thus pooled whole saliva was judged more suitable for growth experiments than pooled parotid saliva, and was used in subsequent experiments.

Although there is little information regarding the growth of C. albicans and C. glabrata in pooled mixed saliva no previous work has been carried out with the other species of Candida. Therefore in addition to C. albicans, experiments were carried out with C. tropicalis GDH 1009, C. glabrata GDH 1397 and C. krusei NCPF 3165 using pooled mixed saliva. All 4 Candida species, demonstrated growth

in pooled whole saliva, produced a range of acid anions (the predominant ones being pyruvate and acetate), degraded salivary proteins and lowered the pH of saliva by an average of 4 pH units. The statistical analysis of the 4 growth parameters indicate that there are significant differences in the 4 Candida species in growth, pH, acid anion production and protein degradation. It was also revealed that these differences occur at the later hours of the incubation period i.e., 48 and 72 hours.

In conclusion, this study has demonstrated that the presence of a dietary carbohydrate such as sucrose may possibly protect C. albicans and C. tropicalis from the fungicidal activity of lysozyme but not C. glabrata and C. krusei. This study has also demonstrated that the addition of glucose to saliva and the inhibition of the related microflora results in the growth of the four Candida species.

ABBREVIATIONS

A	Actinomyces
BA	blood agar
BCA	bicinchoninic acid
C	Candida
Ca^{++}	calcium ions
CO_2	carbon dioxide
cfu	colony forming units
cfu/ml	colony forming units per millilitre
cfu/50µl	colony forming units per 50 microlitres
cm	centimetre
cm^2	square centimetre
conc.	concentration
DNase	Deoxyribonuclease
eg	for example
g	gravitational force
g/l	grams per litre
H_2O_2	hydrogen peroxide
HRP	histidine rich proteins
ie	that is to say
Ig	immunoglobulin
K	1000
K^+	potassium ions
L	Lactobacillus
$\log_e$	natural logarithm
ma	milliamperes
Mg^{++}	magnesium ions

mg	milligrams
mg/l	milligrams per litre
ml	millilitres
ml/min	millilitres per minute
ml/min/ gland	millilitres per minute per gland
mM	millimoles
mmol/l	millimoles per litre
/ua	microamperes
/ug	micrograms
/ul	microlitres
MPO	myeloperoxidase
Mr	relative molecular weight
MW	molecular weight
Na^+	sodium ions
ND	not detected
nm	nano moles
pH	negative decimal log of molar hydrogen ion concentration
PO_4^{--}	phosphate ions
PPS	pooled parotid saliva
PWS	pooled whole saliva
ppm	parts per million
PRP	proline rich proteins
rpm	rotations per minute
PMN	polymorphoneuclear leukocyte
RNase	Ribonuclease
S	Streptococcus

SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SDA	Sabouraud's dexrose agar
sIgA	secretory immunoglobulin A
UV	ultraviolet
V	volts
v/v	volume per volume
w/v	weight per volume
YNB	yeast nitrogen base
Zn^{++}	zinc ions
&	and
^{o}C	degrees Celsius
%	percent
<	less than
>	greater than
+ve	positive
-ve	negative

CHAPTER 1

THE ECOLOGY OF THE HUMAN MOUTH

1.1. Introduction

The oral cavity consists of four main components namely the oral mucosa, teeth, saliva and the commensal microflora. These factors normally interact to produce a state which is recognized as health. In this section each of these four factors will be reviewed briefly.

1.1.1. The oral mucosa

The oral mucosa is covered by stratified squamous epithelium, which is modified in certain regions of the oral cavity according to function (Lavelle, 1975). Thus, it shows a wide variation in thickness and in its pattern of keratinization. It can be classified into three functional types, namely masticatory mucosa (palate, gingivae) lining mucosa (lips, cheeks, ventral surface of the tongue and floor of the mouth) and specialized mucosa (dorsum of the tongue, and the vermillion border and transitional zone of the lips) (Meyer et al., 1984).

The crevicular epithelium which lines the gingival crevice is thin and non-keratinized. It is often inflamed due to the action of subgingival plaque bacteria. However, the close contact between the crevicular epithelium and the tooth surface does minimize the

penetration of microorganisms and saliva into the sulcus region. The oral mucosa which lines the mouth protects the underlying tissues from mechanical insult such as the trauma of mastication and the penetration of toxins (Meyer et al., 1984).

The most superficial cells of the oral epithelium are continously sloughed off into the saliva, where they are ultimately swallowed. The desquamated epithelial cells carry variable numbers of bacteria and yeasts, and this helps to remove microorganisms from the oral cavity and thus keep the microbial flora in balance.

1.1.2. Teeth

Teeth are the most calcified tissues in the body and are mainly used for the mastication of food. The tooth is composed of four tissues: pulp, dentine, cementum and enamel, the latter being continually exposed to the oral environment (Renner, 1985). Cementum, is not normally exposed to the oral environment but in the elderly due to the natural recession of the gingivae this does occur.

The primary dentition is complete by the age of three years and around six years the permanant teeth begin to erupt. During these periods of change the local ecological conditions of the mouth will vary. Once a tooth begins to function in the mouth, its developmental cuticle or pellicle is rapidly worn away and replaced by a constantly replenished film, the acquired pellicle which is mainly derived from saliva (Kleinberg et al., 1979). It has been shown that

lysozyme, amylase, albumin, glucosyltransferase and immunoglobulins may also be present in the pellicle (Eggen and Rolla, 1984). Glucose may also be present in small amounts in salivary glycoproteins and may be derived from remnants of bacterial extracellular polysaccharides (Sonju et al., 1974). This selectively adsorbed coat of proteins provides both a protective barrier and reduces excessive wear by acting as a lubricant. It may also act as a diffusion barrier against acid penetration and limits mineral egress (Slomiany et al., 1986).

1.1.3. Oral fluid

The major secretion of the oral cavity is saliva derived from the salivary glands. Salivary glands are generally divided into major and minor glands. The major salivary glands are the parotid, the submandibular (also called submaxillary) and the sublingual glands. They are situated some way from the oral cavity and their secretions are discharged into the mouth via a distinct ductal system. Minor salivary glands consist of small clumps of secretory cells distributed in the mucosa of the cheek, lips, hard and soft palates and tongue. The minor glands mainly produce mucoprotein secretions and major salivary glands produce serous-mucous secretions (Ferguson, 1988).

When teeth are present, and gingivitis or periodontitis exist, the oral fluid will be augmented by gingival crevicular fluid which is a transudate of serum and the main source of immune activity in the gingival and approximal regions (Cimasoni, 1983). It

contains IgG, IgM, IgA, polymorphonuclear leucocytes, macrophages, T and B-lymphocytes, components of the complement system and other antibacterial products liberated from phagocytic cells (for example, lysozyme, lactoferrin, myeloperoxidase) (Cimasoni, 1983).

The main functions of saliva are listed in Table 1.1. The mechanical washing activity of saliva plays an important role in defence mechanisms of the mouth. A lack of saliva in the oral cavity leads to a symptom called xerostomia, and in severe forms of this disease, the secretions from all salivary glands are reduced. The oral mucosa becomes dry and red due to inflammation and the salivary pH falls markedly. The mucosa is often covered by a tenacious film of mucus that can be scraped off with difficulty. Reduced salivary secretion results in delayed removal of carbohydrate from the teeth and soft tissues by the washing action of saliva (Sundstrom, 1979; MacFarlane, 1984). As a result, these patients experience severe dental caries and are predisposed to candidosis.

1.1.4. Microorganisms

The normal oral flora of the mouth is complex, consisting of a wide variety of microorganisms. These microorganisms are mainly found as plaque on teeth (both supragingivally and subgingivally), but also are present on mucosal surfaces as well as in saliva. In addition plaque forms on prostheses such as acrylic dentures.

Function	Factors involved
Lubrication	Mucin, basic proline-rich glycoprotein
Remineralization	Statherin, acidic proline-rich proteins
Anti-bacterial	sIgA, lysozyme, lactoferrin, lactoperoxidase
Anti-fungal	Histidine-rich proteins
Digestion	Food breakdown (amylase, DNase, RNase, proteases, food bolus formation (mucin, fluid) gustation (fluid, i.e., solvent delivery)
Buffering	Bicarbonate, phosphate
Maintenance of tooth structures, remineralization, activation of enzymes	Calcium
Oxidation of peroxidase (host defence)	Thiocyanate ion (SCN^-)

Table 1.1. Some major functions of saliva (Adapted from Baum, 1987 and Fejerskov, 1986).

Bacteria are the predominant organisms of the mouth and the major groups of microorganisms found in the oral cavity are shown in Table 1.2. The oral flora also consists of a range of other microorganisms including yeasts especially C. albicans, mycoplasma, and protozoa. The distribution of these microorganisms varies at different sites in the oral cavity, and it is likely that this high species diversity may be due to the different habitats in the mouth and the range of possible nutrient limitations.

During plaque formation salivary proteins become adsorbed on to the tooth surface and form the acquired pellicle. The adsorbed material includes a variety of glycoproteins and antibodies (see Section 1.4.). When glycoproteins of the saliva are adsorbed on to the enamel during the formation of the pellicle it may selectively promote adhesion of Gram positive cocci in the early stages of plaque formation (Liljemark et al., 1986). This accumulation is called dental plaque and a number of bacterial genera and species e.g., streptococcus, lactobacillus, actinomyces, neisseria and veillonella are involved in its formation (Nyvad and Fejerskov, 1986). In the normal mouth, plaque is seen particularly in the protected regions of the tooth surface such as fissures, approximal areas and the gingival crevice. Plaque is also formed subgingivally and this may result in the development of gingivitis and later periodontitis.

It is generally accepted that the composition of the oral flora is controlled by complex interactions among the oral microorganisms themselves, the host tissues, and the mechanical

Major groups of microorganisms	Main Genera/Species
Gram + cocci	Streptococci: *S. mutans* *S. oralis* *S. mitis* *S. sanguis*
Gram - cocci	Neisseria Veillonella
Gram + rods	Actinomyces Lactobacillus
Gram - rods	Haemophilus Bacteroides: *B. gingivalis* *B. intermedius* *B. oralis* Fusobacteria
Spiral organisms	Treponema
Mycoplasma	*Mycoplasma salivarius*
Candida	*C. albicans* *C. glabrata*
Protozoa	*Entamoeba gingivalis*

Table 1.2. The major groups of microorganisms in the oral cavity

washing action and antimicrobial activity of saliva (Marsh and Martin, 1984). Interactions may occur between bacteria and Candida, which may promote or retard oral candidal colonisation. For instance, Kinght and Fletcher (1971), reported that *in vitro* yeast growth only occurred when glucose was added to mixed saliva. This supports the concept that when carbohydrate is not in excess the bacteria present in mixed saliva compete successfully with yeasts for available nutrients and thus depress yeast numbers. It has also been shown that some oral streptococci can slightly impair candidal adherence to epithelial cells (Samaranayake and MacFarlane, 1982a), while *E. coli* and *K. aerogenes* actually assists the yeast to bind to epithelia (Mackrides and MacFarlane, 1982). The most commonly encountered interactions among microorganisms involve the competition for essential or growth promoting nutrients (Saunders, 1969), alteration of environmental pH (Hu and Sandham, 1972; Donoghe and Tyler, 1975), generation of toxic end-products (Holmberg and Killander, 1971), production of antibiotic like substances (Weerkamp et al., 1977 and Hillman et al., 1984), and competition for attachment receptor sites (Davidson and Hirsh, 1975).

Therfore Candida, usually live in a hostile environment competing with bacteria for their survival. However, when the numbers of commensal bacteria are reduced, for example during prolonged antimicrobial therapy, Candida may proliferate and cause diseases such as acute atrophic candidosis (Samaranayake, 1990). This is a good example of the beneficial effect of oral bacteria in maintaining the health of the oral cavity.

1.2. THE PREVALENCE OF CANDIDA IN THE MOUTH

1.2.1. Historical aspects

Hippocrates, described two cases of oral aphthae, associated with severe underlying diseases, in his book Epidemics (Hippocrates, 460-377 BC). These were probably cases of thrush, although not recognized as such at that time. The thrush organism was discovered in 1839 by Langenbeck (Langenbeck, 1839), but Berg (1846) was the earliest to describe a relationship between the thrush fungus and mouth lesions (Berg, 1846). A century since its discovery several generic and species names were attributed to the thrush fungus i.e., Oidium albicans, Syringospora robinii and Saccharomyces albicans. The dimorphic nature of the fungus, and the production of chlamydospores contributed much to this confused taxonomy. In 1887, Plaut isolated a yeast-like fungus from rotting wood, with which he produced lesions resembling oral thrush in the throats of chickens (Plaut, 1887). Plaut considered that his isolate resembled Monilia candida and Zopf, in (1890), named the thrush fungus Monilia albicans (Zopf, 1890). This gave rise to the name "moniliasis" for infections due to the thrush fungus.

In the nineteenth century it was suggested that oral thrush was a local manifestation of underlying disease in humans (Trousseau, 1869) and Parrot who noted the almost invariable association of thrush with underlying debility, stated that "thrush is always the consequence of a pre-existing morbid state" (Parrot, 1877). The

first description of the yeasts currently known as C. guilliermondii, C. kefyr, C. krusei and C. tropicalis was made by Castellani early in this century (Castellani, 1912). The taxonomic position of these yeasts were subsequently classified by Berkhout in 1923 and he proposed the generic name "Candida" for the thrush yeast (Berkhout, 1923). Although the terms candidosis and candidiasis are both used for the diseases which they cause, in this thesis the term candidosis is favoured.

1.2.2. The oral carriage of Candida

A number of studies have been carried out to investigate the oral carriage of Candida in healthy individuals and in those who show signs and symptoms of oral candidosis (see Section 1.3.2). These studies have been extensively reviewed by Odds (1988). The following is a brief survey of this topic.

A. In health

The percentage prevalence of Candida in the oral cavities of normal healthy individuals and patients without overt oral candidosis vary from 2.0 to 71.3% (Odds, 1988). Lehner (1967) reported a prevalence rate of 50% for adults with natural teeth and without any overt sign of infection. The prevalence in a group of 140 healthy children, 3 to 12 years of age, examined by Berdicevsky et al. (1984) was 45% for the 3 to 5 year olds and 65% for those between the ages of 6 to 12. Young et al. (1951) cultured yeasts from the saliva of

584 healthy college students and reported positive results in 48.6% of volunteers, with C. albicans being the most commonly isolated species (93.8%). A much higher prevalence of Candida carriage (71.3%) was found in a study conducted by Martin and Wilkinson (1983) in 10 year old school children. The disparity in the results obtained in the Young et al. (1951) and Martin and Wilkinson (1983) studies may be explained by the different methods of sampling and incubation period employed by these workers. For example Young et al., used saliva samples and primary cultures were incubated for up to 24 days before being discarded as negative, while in the Martin and Wilkinson study samples were collected using cotton wool swabs from different oral sites and cultures were incubated for up to 6 weeks before a negative result was recorded.

Other epidemiological and clinical studies however have quoted a much lower incidence of candidal carriage in a healthy population. Clayton and Noble (quoted by Drake and Maibach (1973) isolated Candida from fewer than 10% of healthy children and adults, and a prevalence of 22.2 % of oral samples was obtained from 54 healthy children in a survey performed by Basu et al. (1961).

The concentration of Candida in the oral cavity of healthy individuals and the distribution of the yeast in different oral sites have also been studied by a number of workers. Epstein et al. (1980) have shown that the number of Candida cells per ml of mixed saliva in normal carriers is about 200 to 500 cfu/ml and McKendrick et al. (1967) have reported values of 600 cfu/ml when samples were collected

by an oral rinse technique. Although low counts are often recorded, values as high as 10^4 cfu/ml mixed saliva have been reported for normal Candida carriers (Berdicevsky et al., 1984).

Research carried out by Arendorf and Walker (1980) using an imprint technique in a healthy dentate adult population, showed that Candida is not uniformly distributed throughout the mouth; the tongue appeared to be the primary oral reservoir with other parts of the oral mucosa, dental plaque and saliva becoming secondarily colonised in a proportion of carriers (Arendorf and Walker, 1980).

B. Diurnal variation and daily variation

Only a few studies have been carried out to investigate the daily variation in the numbers of Candida in saliva. Such diurnal variation may be due to a number of factors which can produce change in the oral environment, for example; food, eating and drinking habits, oral hygiene, denture wearing and the relative inactivity of the mouth during sleep (Williamson, 1972 a,b). Williamson, (1972 a, b) have reported that there is a fairly consistent pattern in the daily variation of C. albicans in saliva among dentate subjects. Gergly and Uri (1966) suggested that diurnal variation in yeast concentrations occur in plaque too. However, Russel (1987) did not find significant variation in the number of Candida in the saliva of 10 children sampled on 9 occasions over 3 consecutive days. Therefore more studies are required to clarify this area.

C. Disease

Generally a correlation has been found between the signs and symptoms of candidosis and colony counts greater than 400 cfu/ml of saliva (Epstein et al., 1980). According to these workers symptomless carriers of Candida usually have colony counts of less than 400 cfu/ml of saliva although exceptions do occur. Therefore, quantitative assessment of cultures of saliva may help in the diagnosis of oral candidosis when considered as part of an overall clinical and laboratory assessment of each individual. The increased intraoral counts of yeasts in diseased individuals agree with the findings of Arendorf and Walker, (1979) who found a significantly higher density and frequency of candidal colonisation at all sites sampled, compared with that of healthy denture wearing and dentate subjects. However, earlier workers (McKendrick et al., 1967) have reported that the isolation of C. albicans from the oral cavity in large numbers does not necessarily correlate with increased frequency of infection.

D. Candida species

The most common species of Candida isolated from the mouth in both health and disease is C. albicans. The prevalence of C. albicans in relation to other species from the mouth varies from 47% (Wright et al., 1985) to 84% (Shipman, 1979) with a mean of about 70% to 75%. While studies on the multiple carriage of Candida species in

the oral cavity have not been properly evaluated (Odds, 1988) the most common yeast combinations isolated from oral samples consist of C. albicans with one or more of the following: C. glabrata, C. tropicalis and C. krusei. A study by Samaranayake et al. (1987) using isolates from patients in a dental hospital have shown that combinations of C. albicans and C. tropicalis accounted for about 7% of oral isolates while C. albicans was also found in combination with C. krusei, C. parapsilosis, and C. guilliermondii. Similarly Yamane and Saitoh, (1985) reported mixed yeast isolates from 9% pharyngeal specimens, with C. albicans and C. glabrata as the most common combination. The above studies were performed using Sabouraud's medium in combination with the Pagano-Levin medium. The latter greatly assists differentiation of multiple yeasts present in oral samples.

E. Sampling Methods

The large variation in the carriage rate of yeasts obtained in the studies mentioned in Section 1.2.2. ispartly dueto the variation in the populations selected and partly due to the various sampling and laboratory techniques used, in estimating the results (Odds, 1988). The sensitivity of the detection methods also influence the results of these estimations. For instance, Lilienthal (1950) obtained a 10 % increase in yield by using salivary samples compared with mucosal swabs. Subsequent workers (Lehner 1966, 1967; Budtz-Jorgensen 1974 and Sharon et al., 1977) largely relied upon mixed saliva samples as the best available indicator of the presence of intra oral yeasts. However, the imprint culture technique of

Arendorf and Walker, (1979) has proved to be a better method for in-depth-studies, involving different specific intraoral sites. In a study carried out by Arendorf and Walker in 1980 the effect of different methods used for detecting Candida was clearly shown. While they obtained a carrier rate of 44.4% as determined by imprint culture, salivary samples and impression cultures yeilded only 39.6% and 13% carriage respectively. However, the higher sensitivity of imprint cultures over saliva samples is most clearly evident with healthy subjects, where the numbers of oral yeasts are relatively low (Odds, 1988).

One explanation for the high recovery rates of yeasts in the study carried out by Martin and Wilkinson (1983) quoted in Section 1.2.2 (A), is the incubation time of the primary culture plates. While previous investigators (Arendorf and Walker 1979; Barlow and Chattaway, 1969; Basu et al., 1961; Budtz-jorgensen et al., 1975; Clayton and Noble, 1966; and Mackenzie, 1962) cultured plates for only 48 hours, Martin and Wikinson (1983) continued incubation for up to 6 weeks.

An alternative method which is useful for screening large numbers of individuals for the carriage of Candida, is the concentrated oral rinse method described by Samaranayake et al. (1986a). The results showed that the oral rinse technique was as sensitive as the imprint culture method both in healthy and diseased population groups.

F. Media

The laboratory techniques used to isolate, count and identify Candida species may also affect the reported carriage rate. Most research workers have used Sabouraud's agar medium for the isolation and enumeration of Candida. Bertholf and Stafford, (1983) used dip slides with saliva and Schonheyder et al. (1984) used an enriched culture procedure prior to isolation. The possibility of isolating multiple species of Candida from oral samples must be borne in mind, and appropriate media used, for example, Sabouraud's agar medium together with Pagano-Levin medium as described by Samaranayake et al. (1987).

G. Intra-oral-appliances

The overall prevalence of Candida is generally higher in individuals who wear an intra-oral appliance. Several authors have detected a higher oral yeast prevalence among denture wearers of various types than among non-wearers (Berdicevsky et al., 1977, 1980; Budtz-Jorgensen et al., 1975; Mitchell, 1982; Peters et al., 1966; Tapper-Jones et al., 1981 and Vandenbussche and Swinne, 1984). Similar considerations apply to removable and fixed orthodontic appliances (Addy et al., 1982; Arendorf and Addy, 1985). Increase in the carriage rate of Candida has also been found in children who consistently suck dummies/pacifiers (Sio et al., 1987).

One reason for this phenomenon may be the ability of the organisms to grow better under the fitting surface of the upper denture than the adjacent mucosa (Davenport, 1970) due to the absence of salivary flushing action. Furthermore, yeasts can adhere to denture surfaces particularly in the presence of dietary carbohydrates such as sucrose (Samaranayake and MacFarlane, 1980). The concentration of yeasts associated with the fitting surface of palatal denture prosthesis usually exceeds that of other sites in edentulous subjects. Arendorf and Walker (1979) have shown that there is a maximum mean count of 49 cfu/cm^2 on dentures compared with a maximum count of 30 cfu/cm^2 on the mucosa. Furthermore, impression cultures and other quantitative sampling methods have revealed yeast densities in the order of $10^6 cfu/cm^2$ on some denture fitting surfaces (Budtz-Jorgensen and Theilade, 1983; Budtz-Jorgensen et al., 1981 and Gusberti et al., 1985).

It has been suggested that non-carriers of Candida can be converted to carriers by the insertion of a prosthesis or appliance (Arendorf and Addy, 1985). However, it is possible that the main effect of dentures is to increase localised yeast concentrations rather than to establish yeast populations in a mouth that did not previously carry them (Gusberti et al., 1985; Mitchell, 1982 and Tapper-Jones et al., 1981). It has been proposed that poor denture cleanliness might predispose to infection of the palatal mucosa (Fisher and Rashid, 1952; Love et al., 1967 and McKendrick, 1968). Nevertheless, Nyquist, 1952, could not produce evidence for such a correlation. Budtz-Jorgensen and Bertram, (1970) however found a

significant relationship between poor denture hygiene and enhanced palatal inflammation. A similar but less significant relationship between the number of candidal cells recovered from the mucosa and palatal inflammation was also reported.

H. Infancy

Infants acquire an oral yeast flora in the first year of their life. Russell and Lay, (1973) who monitored babies in a neonatal intensive care unit, found a peak of oral yeast colonisation in babies around 1 to 2 months old. C. albicans was present in 82% of the babies declining to 50% in older infants. However, only 2% of the babies showed clinical signs of infection.

It has been suggested that the main source of yeasts, which colonise the mouth of infants within the first few days of life, is the mothers birth canal (Kozinn, Taschdjian and Wiener, 1958; Taschdjian and Kozinn, 1957). However other avenues of infection have also been suggested for example, droplet spread from mother and nursery staff (MacFarlane, 1990), and contamination of feeding bottle teats by Candida (Cremer and De Groot, 1967). The reason why very young children are more susceptible to colonisation and infection by Candida, may be the immaturity of their specific and in some instances their non-specific antimicrobial defences e.g., a poorly developed oral microflora (MacFarlane, 1990).

I. Conclusion

From the above literature survey it can be seen that large variations in yeast carriage are found among normal human populations, who harbour C. albicans intraorally, without overt signs or symptoms of candidosis. Furthermore, Candida species are found intra-orally in almost all age groups, in healthy and diseased subjects and in dentulous and edentulous subjects.

A large number of diseases, disorders, and iatrogenic situations predispose humans to candidosis and few reports dealing with yeast carriage rates, define adequately the subjects and the technical parameters of their investigations. It is also important to remember that samples from hospitalized patients do not truly reflect the status of a normal population. Variations in the methods used to isolate and identify Candida can further contribute to differences in the frequencies of yeast carriage rate. The general conclusion is that about (30 to 50%) of healthy people harbour Candida in their mouth, while an even higher percentage about (27 to 60%) occurs in those receiving medical attention (Odds, 1988).

1.3. Oral candidosis

1.3.1. Introduction

C. albicans is an opportunistic pathogen which normally lives in balance with its host, but can change from commensalism to parasitism, usually as a result of changes in the host rather than in alterations in its own biochemistry (Winner, 1969). A number of local and general changes in the host may contribute to a diseased state in the oral cavity and cause oral candidosis (Samaranayake and Lamey, 1988). These host changes are not always easy to identify, but factors such as the frequency and type of food ingested, (particularly dietary carbohydrates), insertion of dentures due to loss of the permanant dentition, are two local factors which appear to be important in the onset of disease (Budtz-Jorgensen, 1974). Systemic factors including iron deficiency and malnutrition may also lead to oral candidosis (Jenkins et al.,1977). Salivary factors that may predispose the host to oral candidosis such as pH, increased dietary carbohydrates and decreased salivary flow, will be discussed later in Section 1.5. In general terms, the severity and extent of Candida infections tends to increase with the number and severity of predisposing factors that operate in a given case (Odds, 1988). A diagram of the host factors which may increase the risk of oral candidosis is given in Figure 1.1. It is clear that this topic is complex but since it is not directly relevant to the work presented in this thesis it will not be discussed further.

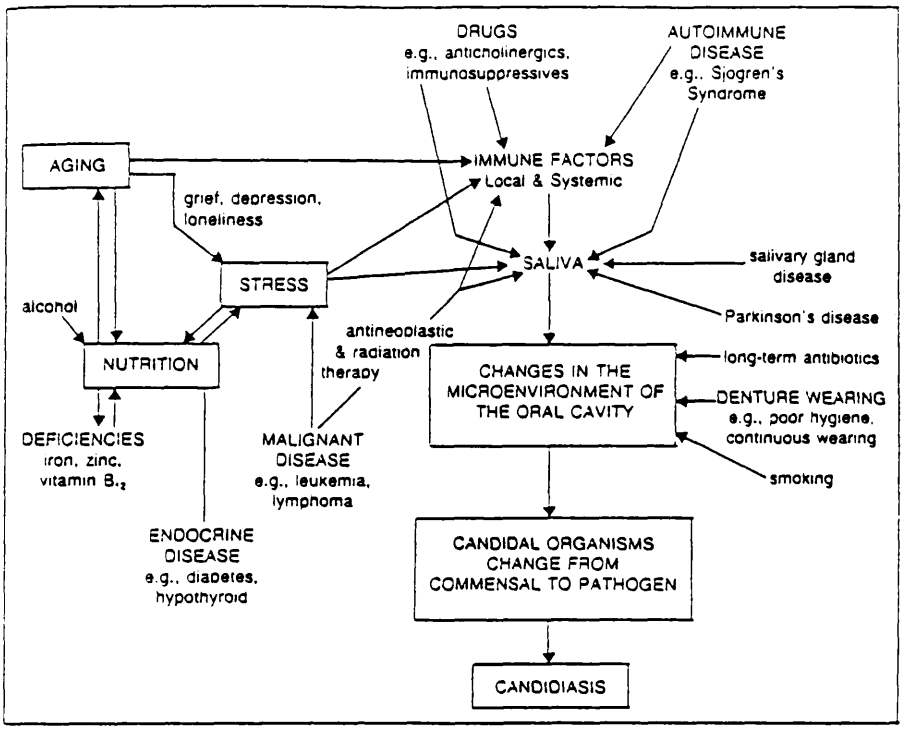

Figure 1.1. Localised and systemic factors that predispose humans to candidosis (Ettinger, R. L., 1987)

1.3.2 Candida infections of the mouth

There are a number of localised oral diseases caused by Candida. These are classified as follows (MacFarlane and Samaranayake 1989):

A. Acute pseudomembraneous candidosis
B. Acute atrophic candidosis
C. Chronic atrophic candidosis
D. Chronic hyperplastic candidosis
E. Angular chelitis

A. Acute pseudomembraneous candidosis (Thrush)

Acute pseudomembraneous candidosis mainly occurs in the very young, very old and the very sick (Odds, 1988). In neonates, the immature host defenses probably mediate the disease while in the debilitated it could be related to one or more of the predisposing factors mentioned earlier (Figure 1.1.). Characteristically, candidal growth appears as white curd like patches on the tongue, cheeks, palate and lips. These pseudomembraneous patches can be removed easily, leaving an erythematous base. The disease may spread to the pharnyx and oesophagus causing feeding difficulties especially in infants, sore throat or dysphagia. Candidal hyphae invade only the superficial epithelial layer, and keratin, fibrin, necrotic tissue and bacteria matted together by fungal hyphae are the main constituents of the pseudomembrane. Thrush is one of the earliest

oral manifestations of acquired immunodeficiency syndrome (AIDS) (Klein et al., 1984 and Holmstrup and Samaranayake, 1990).

B. Acute atrophic candidosis (Candida glossitis/ glossodynia)

Acute atrophic candidosis usually arises in association with local or systemic broad-spectrum antibiotic therapy, for example tetracycline (Lehner, 1966). This can affect any part of the oral mucosa, including the buccal mucosa, palate and tongue. When the tongue is affected it shows marked depapillation and the affected areas become fiery red, shiny and painful. It is perhaps the only form of candidosis which is consistently painful. This condition is thought to be caused by the increased growth of commensal Candida due to a reduction of commensal oral bacteria, which are normally antagonistic to yeasts, as a result of broad spectrum antibiotic therapy (Samaranayake, 1990).

C. Chronic atrophic candidosis (denture induced stomatitis)

Chronic atrophic candidosis is regarded as the commonest form of oral candidosis in the Western world. It is generally seen as an erythematous area on the palate directly related to the extent of the upper denture bearing mucosa. Both full and partial denture wearers are affected, and lower dentures are very rarely involved (Budtz-Jorgensen, 1974). The main reservoir of infection is the fitting surface of the upper denture (Budtz-Jorgensen, 1990).

D. Chronic hyperplastic candidosis (Candida leukoplakia)

Chronic hyperplastic candidosis usually occurs as a single lesion and affects the commissural region of the buccal mucosa. These chronic lesions of the oral mucosa persist as white patches (leukoplakia) and are indistinguishable from leukoplakias due to other causes (Samaranayake and Lamey, 1988). The white patches cannot be removed by scraping and the Candida cells invade the superficial layers of the oral mucosa (Walker and Arendorf, 1990).

E. Angular Cheilitis

Angular cheilitis usually occurs in association with other forms of oral candidosis, mainly denture stomatitis. The lesions of angular cheilitis are inflamed with or without ulceration and crusted fissures. The laxity of the facial muscles, partly due to the loss of teeth and partly due to anatomical peculiarities, allows the mucocutaneous junction at the angles of the mouth to be constantly moist. This is a major reason for fungal and bacterial growth which may either initiate or aggravate the cheilitis. Iron deficiency, anaemia and vitamin B 12 deficiency also may be contributary factors (Samaranayake, 1985 and MacFarlane and Helnarska, 1976).

1.4. Composition of saliva

1.4.1. Introduction

Saliva is produced by three pairs of major glands, and numerous smaller glands distributed throughout the oral mucosa (labial, lingual, buccal and palatal) (Ferguson, 1988). The sublingual, submandibular and the minor salivary glands produce a mucous secretion (Seifert et al., 1986), while a less viscous secretion which is rich in digestive enzymes such as alpha-amylase, is produced mainly by the parotid gland (Mason and Chisholm, 1975). These secretions together with the gingival crevicular fluid, is known as whole saliva or mixed saliva. Approximately 1 to 1.5 litres of saliva are produced by the salivary glands per day (Fejerskov, 1986). The constituents of saliva are extremely complex and their concentrations are altered due to several variables i.e., age, general health of the subject, seasonal, diurnal and medication (Fejerskov, 1986) and are also related to the type of salivary stimulus involved (mechanical, chemical, psychological) (Rolla et al., 1983). These and the other factors that affect the composition of whole saliva will be described in the next Section (1.4.2.).

1.4.2. Factors affecting the composition of saliva

A continous resting secretion of saliva occurs without any external stimulus. The percentage contribution of individual salivary glands towards the resting salivary secretion is as follows: parotid

gland (25%), submandibular gland (71%), sublingual gland (3-4%) and minor glands (trace amounts), (Seifert et al., 1986). Virtually no measurable secretion occurs during sleep (Schneyer et al., 1956). While there are no universally accepted standards for the normal flow of whole or gland derived saliva, a range of values have been reported by a few workers (Sreebny and Broich, 1987, Mason and Chisolm, 1975) (Table 1.3.).

There are only a few reports that deal with the salivary flow from minor salivary glands. Dawes and Wood (1973) estimated that the minor salivary glands contributed 7 to 8% of the total amount of saliva secreted daily, (a total volume of about 50 ml) Schneyer et al. (1956), demonstrated that 0.002 to 0.008 g of labial gland saliva was secreted over a 10 to 20 minute interval. More recent estimates of the rate of flow of saliva from the minor salivary glands demonstrates that the mean rate of flow of stimulated saliva obtained from the lower lip, was 0.0021 ml/min (Gandara et al., 1985).

The rate of flow and composition of the secretions from salivary glands varies greatly in response to stimuli eg., chewing, taste and smell and this in turn alters the composition of whole saliva (Mason and Chisholm, 1975). Other factors which influence the salivary flow rate are for example, time of day, climate, ingestion of food, the effect of light, drug therapy, age, sex, physical activity and also genetic differences (Ferguson et al., 1973; Jenkins, 1978 and Seifert et al., 1986). The contribution of the submandibular

Source	Unstimulated saliva	Stimulated saliva (2% citric acid)
Whole saliva	0.3 to 0.5 (ml/min)	1.0 to 3.0 (ml/min)
Parotid	0.04 (ml/min/gland)	0.70 (ml/min/gland)
SM/SL	0.15 (ml/min/gland)	0.60 (ml/min/gland)

Table 1.3. **Normal flow rates of whole and gland-derived saliva (Sreebny & Broich 1987).**

SM = submandibular; SL = sublingual

and parotid salivary glands to mixed saliva under varying degrees of stimulation is as shown in Table 1.4.

The variations in the composition of saliva samples from different individuals are a result of changes in body rhythms. This type of circadian variation may produce marked differences in the concentration of some but not all salivary components. For example potassium levels appear to vary by relatively small amounts while protein concentrations can vary considerably (Ferguson and Botchway, 1980). The presence of circadian rhythms in salivary flow rate and composition influence the concept of normal values in any study using saliva, since the time of day when sampling occurs could have a significant bearing on the results (Ferguson, 1981).

Thus mixed saliva consists of a complex mixture of secretions derived from both major and minor salivary glands, factors from the crevicular fluid (see Section 1.4.5. for more detail) desquamated epithelial cells, microorganisms, and food debris. Since there are a large number of variables that can influence salivary flow rate and thus it's composition, accurate, biochemical analysis of saliva can be problematical.

1.4.3. The organic components

Saliva contains a range of organic constituents and although the different pure secretions contain generally the same organic components, the proportions present are different e.g., parotid and

Stimulation	Submandibular gland (%)	Parotid gland (%)
Minimal	66.6	33.3
Half maximal	50.0	50.0
Maximal	33.3	66.6

Table 1.4. Contribution of submandibular and parotid gland saliva to the entire secretion under varying degrees of stimulation (Seifert et al., 1986). The contribution of sublingual and minor salivary gland secretion was not studied.

submandibular secretions contain 2.3g/l and 1.1g/l protein respectively (Ferguson, 1988). The organic substances found in these secretions contain substantial amounts of protein, traces of free carbohydrate, and some lipids (Kleinberg et al., 1979; Fejerskov, 1986). The concentration of some of the major organic components found in saliva are shown in Table 1.5.

Proteins and glycoproteins constitute the bulk of the organic constituents of saliva. Glycoproteins have molecular weights ranging between 10^5 and 10^7 daltons (Nugent and O'Connor 1984), and contain considerable amounts of the amino acids, serine, threonine, aspartic and proline (Pigman, 1977). The carbohydrates that are added to the protein backbone of the glycoproteins are N-acetyl-galactosamine, N-acetyl-glucosamine, galactose, mannose, sialic acid and fucose in different amounts (Fejerskov, 1986).

Proteins with well known functions are secretory IgA, lactoperoxidase, lactoferrin, proline and histidine rich proteins and statherin. An account of the biological significance of some of these proteins and their relationship with Candida is given in Section 1.5. A wide variety of enzymes are found in saliva, i.e., alpha-amylase, beta-glucuronidase, lysozyme, acid phosphatase, alkaline phosphatase, lipase and peroxidase. Salivary amylase is the only enzyme of digestive importance in the mouth (Ferguson, 1988) and will be discussed in detail in Section 1.5.5. Some of the enzymes are derived solely from salivary secretions others from the crevicular fluid and even more from bacteria and host cells (Schuster, 1988).

Component	Mixed Saliva Mean	Parotid Saliva Mean
Organic		
Protein (g/l)	1.75	2.3
Amylase (g/l)	0.45	0.8
Lysozyme(g/l)	0.14	0.2
Carbohydrate (g/l)	0.65	0.45
Glucose (mg/l)	0.75	0.03
Amino acids (mg/l)	40.0	10.0
Serum albumin (mg/l)	25.0	10.0
Mucoproteins (g/l)	0.45	0.8
Inorganic		
Potassium (mmol/l)	20.0	21.0
Sodium "	6.0	36.0
Chloride "	14.0	28.0
phosphate "	5.0	4.0
Bicarbonate "	1.0	30.0
Calcium "	1.5	1.6
Magnesium "	0.1	0.12

Table 1.5. The organic and inorganic components of human saliva. Adapted from Fejerskov (1986) and Ferguson (1988).

1.4.4. The inorganic components

While water is the main inorganic constituent of saliva, the concentration of the other major inorganic components are shown in Table 1.5. The main electrolytes of saliva are potassium, sodium, calcium, chloride, bicarbonate and inorganic phosphate. Other electrolytes that are present in concentrations less than 1mM include; fluoride, sulphate, thiocyanate, iodide and magnesium (Nikifourk, 1985 a).

About 85% of the total buffering capacity of saliva comes from the bicarbonate system (Wah Leung, 1951). Phosphate and the amino acid arginine in saliva constitute the other buffer system, but their contribution is thought not to be significant (Kleinberg, et al., 1983). Since an acidic environment will initiate the proteolytic activity of the Candida proteinase and thereby encourage the growth and colonisation of this organism, the buffering capacity of saliva is important and is discussed in Section 1.5.

The activities of the other inorganic ions such as Fe^{++}, Cl^{-}, SCN^{-}, $OSCN^{-}$ are discussed later in Section 1. 5.

1.4.5. Crevicular fluid

The crevicular fluid is a transudate of serum which leaks into the gingival crevice in very small amounts in health (Hatting and Ho 1980). The transudate contains desquamated cells, leukocytes

and bacteria and can be regarded as mechanically cleansing the gingival crevice (Table 1.6.). In periodontal disease, it has been shown that the absolute number of leukocytes increases with the intensity of the inflammatory process while the proportion of polymorphonuclear to mononuclear leucocytes is independant of the degree of inflammation present (Cimasoni, 1983).

The crevicular fluid probably represents an importantsource of immunoglobulins in the oral cavity (Challacombe and Russell, 1978). Holmberg and Killander (1971), confirmed by radial immunodiffusion that IgG, IgA, and IgM are present in crevicular fluid in concentrations comparable to that of serum. They claimed that in contrast to salivary IgA no secretory piece is attached to IgA in crevicular fluid and this finding was confirmed by Goldberg (1972). The intact molecules of IgG, IgA and IgM can pass from plasma to the oral cavity via the gingival crevice (Challacombe and Russell, 1978). Whether derived from plasma or synthesized locally, the immunoglobulins of crevicular fluid might significantly contribute to the oral defence mechanisms, particularly in the crevicular domain (Challacombe and Russell, 1978). A number of enzymes have been shown to be present in the gingival crevice for example lysozyme, acid phosphatase, lactic dehydrogenase (see Table 1.6.). However many if not all of the enzymes in the fluid could be produced both by the cells of the host and by bacteria (Cimasoni, 1983).

An estimated volume of 0.5 to 2.4ml of crevicular fluid is secreted into the mouth per day (Challacombe,1980). However, most

Proteins
Albumin
Gamma-globulin
ENZYMES Acid phosphatase Alkaline phosphatase beta-Glucuronidase Lysozyme Hyaluronidase Cathepsin D Elastase Cathepsin G Collagenase Lactic dehydrogenase
Inorganic
Na^{+}
K^{+}
Ca^{++}
Mg^{++}
PO_4^{--}

Table 1.6. Constituents of crevicular fluid. (Adapted from Lavelle 1975 and Cimasoni, 1983).

investigators agree that the flow of crevicular fluid increases several days prior to detectable gingival inflammation (Cimasoni, 1983). Considering the high concentration of nutrients that are present in the crevicular fluid it is probable that this fluid is capable of supporting the growth of many oral microorganisms, including Candida.

1.5. THE INTERACTIONS OF CANDIDA AND SALIVA

1.5.1. Introduction

The oral cavity, can be compared to a reservoir where Candida survive in an environment bathed in saliva, and may proliferate and invade host tissues if certain ecological changes occur. Although saliva has a major role to play in determining intra-oral environmental conditions and is often thought of as an uniform composite of all salivary secretions, there are nevertheless, marked environmental differences in different sites within the oral cavity, for example the dorsum of the tongue and the buccal mucosa (McNamura et al., 1979). These environmental variations partly arise from differences in the accessibility of saliva to various sites, and this may be reflected in the proximity of particular sites to the major salivary glands, and the capacity of saliva to provide a continual source of nutrients to support the growth of Candida.

Populations within a microbial community are dependent solely on the habitat for the nutrients essential for growth.

Therefore the association of Candida with the oral cavity is direct evidence that all of the necessary nutrients are present. However, since the composition of saliva is very complex as explained in Section 1.4., it is not surprising that a number of difficulties exist when attempts are made to study the interactions of Candida and saliva. The most important problem is the uncertainty of the number and identity of the factors that might interact with Candida species in the mouth. Another is the difficulty in isolating, characterising and testing the individual components of saliva that might interact with Candida in vivo. While saliva is a source of carbohydrate and nitrogenous compounds both of which are necessary for candidal growth, it also contains a number of antimicrobial factors such as lysozyme, lactoferrin, lactoperoxidase, secretory immunoglobulin and histidine rich proteins. Thus, in the following section the more important of these salivary factors will be discussed in relation to the oral ecology of Candida.

1.5.2. Carbohydrates

Literature on the growth of Candida in saliva is very scarce. It is generally accepted that neither whole saliva nor parotid saliva supports the growth of this organism. This has been shown by Germaine Tellefson and Johnson, (1978) who carried out experiments with whole mixed saliva and parotid saliva collected from healthy individuals. However, Knight and Fletcher, (1971) and Germaine et al. (1978) were able to induce candidal growth in mixed saliva with added nutrient supplements such as glucose. In addition,

Samaranayake et al. (1986b) conducted *in vitro* experiments similar to those of Germaine and Tellefson, (1981) using mixed saliva, supplemented with glucose and antibiotics to suppress bacterial growth. Samaranayake et al. (1986b), reported substantial growth of *C. albicans* associated with a rapid decline of pH from 7.5 to 3.2 over a 48 hour period. In comparison little growth occurred in glucose free salivary controls. The predominant anions which lowered the pH were identified as pyruvate and acetate.

C. albicans must survive the flushing actions of saliva if the fungi are to colonise and ultimately infect the oral mucosa. Therefore for successful colonisation and infection candidal adherence to host mucosal surfaces is an essential prerequisite (Gibbons and van Houte, 1975). Studies carried out by Douglas et al. (1981) indicate that adherence of *C. albicans* to human buccal epithelial cells was promoted by growth in medium with a high content of certain sugars, for example, galactose and sucrose. Adherence of *C. albicans* to acrylic surfaces *in vitro* was also affected in a similar way (Samaranayake and MacFarlane, 1980; Douglas and McCourtie, 1981; McCourtie and Douglas, 1981). Therefore, in the mouth, where high concentrations of sucrose and other dietary sugars are commonly found, these changes which occur in yeast cell surface composition, may well increase candidal adherence, colonisation and perhaps subsequent infection.

Variations in the adherence capabilities of *Candida* species might, in part explain why some species are found to colonise oral

surfaces more frequently than others. Differences in adherence to epithelial cells *in vitro* are closely paralleled by differences in virulence. *C. albicans* attaches to exfoliated vaginal or buccal cells in much greater numbers than *C. tropicalis*, and *C. parapsilosis*, while *C. pseudotropicalis*, *C. krusei* and *C. guilliermondii* show little or no adhesion (King et al., 1980).

The investigations mentioned so far, suggest that the availability of carbohydrate i.e., glucose is an important factor in determining candidal growth in saliva, and thus the presence of carbohydrate in the oral cavity could play a role in the pathogenesis of oral candidosis. Knight and Fletcher, (1971) found elevated levels of salivary glucose in patients receiving broad spectrum antibiotics and, saliva collected from these patients were able to support well the growth of endogenous or artificially added Candida cells compared with saliva from normal controls. A possible explanation for these results is that the addition of antibiotics killed the rapidly growing bacteria and therefore made available the salivary glucose and nutrients for the more slow growing Candida. For a review of how antibacterial antibiotics may lead to yeast overgrowth and subsequent infection see Odds, (1988).

Gentles and La Touche (1969) listed a carbohydrate-rich diet as a factor predisposing to candidosis. Since then there have been a number of *in vivo* clinical studies which support this relationship. Olsen and Birkeland in 1975 reported that oral sucrose rinses aggravated palatal candidosis in patients with denture

stomatitis, while Stafford and Russel, (1971) found that denture adhesives mixed with sucrose support C. albicans growth in vitro. This observation, suggesting a local effect for sugar, accords with experiments performed with monkeys whose oral yeast concentrations rose in association with a high-sugar diet fed by mouth and fell when the same diet was given via an intragastric tube (Bowen, 1974; Bowen and Cornick, 1970). Finally a high carbohydrate intake was presumed to be the direct cause of oral candidosis in 4 denture wearers (Shuttleworth and Gibbons 1960).

It is generally believed that patients with diabetes mellitus have increased oral carrier rates and higher candidal loads than normal individuals. This is thought to be due to an increase in the glucose concentration of mixed saliva in diabetics compared with non-diabetics (Faulconbridge et al., 1981 and Harrison and Bowen, 1987). However, Sharon et al. (1985) could not find a corresponding increase of glucose in whole saliva in diabetics although the glucose content in parotid saliva was increased.

The raised level of glucose in the saliva of diabetics may predispose them to oral carriage and infection with Candida species. Several investigators have reported higher than normal frequencies of oral carriage of yeasts among diabetic patients (Tapper-Jones et al., 1981; Odds et al., 1987 and Lamey et al., 1988). In addition two studies have shown a significantly elevated yeast concentration in oral samples from diabetics (Peters et al., 1966; Tapper-Jones et al., 1981). Furthermore Odds et al. (1987) found an association

between oral yeast carriage, and the glucose levels in both the blood and urine of diabetic patients. The results of Tapper-Jones et al. (1981), indicate that Candida becomes more easily established in the mouths of diabetics than in healthy subjects. However they found that in dentate diabetic patients the number of yeasts on the oral mucosa remains within the normal range. Fisher et al. (1987) demonstrated the degree of glycaemic control in diabetics does not significantly affect oral candida populations although diabetic denture wearers have increased colony counts when compared to diabetic dentate patients.

1.5.3. pH

The pH of unstimulated saliva is usually between 6.0 and 6.5, but it can rise to 8.0 at high flow rates mainly because of the increased secretion of bicarbonate (Seifert et al., 1986), which is the main buffer of saliva (Jenkins, 1979). Bicarbonate ions are incorporated into the saliva due to cellular metabolism of the salivary glands and its concentration in saliva may rise to as high as 60mmol/l as the flow rate increases.

The normal range of pH in the human mouth is 5.6 to 7.6 (Seifert et al., 1986). There are a number of factors which affect the pH of saliva. A low pH may be associated with the production of acidic by-products when dietary carbohydrates are utilized by the commensal microorganisms especially if the flow rate of saliva is very low. The bacteria in dental plaque are capable of fermenting

different carbohydrates such as starch, dissacharides and monosaccharides as substrates. Some streptococci and lactobacilli ferment sugars producing 90% or more lactic acid (Nikiforuk, 1985a). However this effect depends on the concentration of cariogenic bacteria, density of plaque and the flushing action of saliva (Nikiforuk, 1985a). It has also been shown that parotid and mandibular (submandibular and sublingual) saliva has different effects on plaque pH (Kleinberg and Jenkins, 1964). An alkaline salivary pH may be related to the production of ammonia, amines and other basic components by bacterial degradation of proteins, peptides, urea and other nitrogenous compounds (Kleinberg and Jenkins, 1964).

When saliva with its high content of both dissolved CO_2 and hydrogen carbonate is exposed to air it loses CO_2 and becomes more alkaline (Ferguson, 1988). Therefore a wide spectrum of pH is found naturally in the human oral environment which is susceptible to constant pH changes due to a variety of factors.

Candida species are acidogenic and aciduric by nature and a low salivary pH may enhance their growth. However, they can withstand a wide range of pH values (2.5 to 7.5) (Odds, 1988). Astruc reported an association between oral thrush and acid salivas as early as 1747 and subsequently, a relationship between low pH and increased yeast isolation has been described (Young et al., 1951; and Shipman, 1979). In a group of 154 individuals Young et al. (1951) isolated Candida from saliva samples with a pH range of 5.0 to 7.5 and they demonstrated a direct relationship between the degree of acidity of

saliva and the prevalence of C. albicans.

The precise reason why a low salivary pH favours candidal carriage is still unclear. There is evidence however that pH may directly affect the adhesion of Candida to host cells. The optimum pH for adherence is reported to be around pH 6 to 8 (King et al., 1980; Sobel et al., 1981). However, in an in-vitro technique used to investigate the adhesion of C. albicans to HeLa cells Samaranayake and MacFarlane (1982a), found that the maximal adherence occurred at pH 3. These results imply that pH may play a role in adhesion and hence the survival of yeasts in the oral cavity.

The results obtained from a clinical evaluation of oral candidosis infection in cancer patients receiving chemotherapy (Shipman, 1979) show that as salivary pH becomes more acidic, there is an increase in the numbers of Candida species isolated. This finding correlates with that of Young et al. (1951) who showed a direct relationship between the acidity of saliva and the number of Candida carriers in a group of healthy adults. Arendorf and Walker (1980) also observed that candidal carriers had a lower salivary pH on the lingual surface than non-carriers.

In addition to the above effects the pH can also affect the morphological form of C. albicans. The hyphal form is more likely to develop under acidic conditions that are usually found in host tissues in vivo (Odds, 1988). However, the demonstration of candidal blastospores as the sole morphological form in 2 denture stomatitis

patients and hyphae in 9 healthy carriers in studies carried out by Arendorf and Walker (1980) suggests that hyphae are not necessary for infection. Many other factors are involved in the blastospore to hyphal change, for example the carbon source used to grow the yeast, serum and divalent ions especially Mg^{++} and Zn^{++}, (see review by Odds, 1988).

1.5.4. Proteins

The complexity,of the composition and functions of saliva were described earlier in Section 1.4. Proteins constitute a large percentage of the organic content of saliva as described in Section 1.4.3. The total protein concentration of whole saliva is about 2.2g/l, at low flow rates (Ferguson 1988). However, the concentration of total protein in the saliva also depends on the type of subject investigated and the methods of analysis used (Seifert et al., 1986).

Qualitative variation in salivary proteins in different glands among individuals have been comprehensively reviewed by Ellison (1979). A minimum of 100 different protein components have been resolved from whole saliva using two-dimensional electrophoresis (Giometti and Anderson, 1979). Some of these proteins are secreted by acinar or ductal cells while others represent serum proteins from the gingival exudate see Table 1.7.

Salivary proteins include digestive enzymes, the most important being alpha-amylase. Another group of proteins, the glycoproteins, are responsible for the viscosity of saliva. Proteins

Protein	Production by	
	Parotid gland	Submandibular gland
alpha-Amylase	High	Low
Proline-rich proteins		
Acidic	+	+
Basic	+	+
Glycosylated	+	+
Lactoperoxidase	+	+
Lactoferrin	+	+
Secretory component	+	+
Cysteine-containing phosphoproteins	Low	High
Histidine-rich proteins	+	+
Gustin	+	-
Mucins		
High molecular weight	-	+
Low molecular weight	-	+
Blood group substance	-	+

Table 1.7. **Proteins produced by parotid and submandibular acinar cells (modified after Mandel (1983). + = present; - = absent**

such as lysozyme, lactoferrin and immunoglobulin play a role in defence against infections. A detailed discussion of all the known salivary protein is beyond the scope of this thesis. However, a brief description of the proteins in saliva which have been shown to interact with Candida will be discussed later in this section.

A. Proteolytic activity of Candida in saliva

The proteolytic activity of C. albicans in the presence of human salivary proteins was first examined by Germaine et al. (1978). They reported that the growth of C. albicans at neutral pH in whole salivary supernatants or parotid fluid was not accompanied by degradation of any of the salivary proteins. Incubation of crude extracellular proteinase preparations of C. albicans with human parotid proteins, at pH 4 to 5 did however result in the degradation of several proteins. These results were confirmed by polyacrylamide gel electrophoretic analysis. Subsequent studies of the proteolytic activity of C. albicans (Germaine and Tellefson, 1981) confirmed that whole salivary supernatant was a potent inhibitor of proteinase synthesis. Samaranayake et al. (1983a) carried out similar experiments to those described by Germaine and Tellefson, (1981) with two oral strains of C. albicans and mixed whole saliva supplemented with glucose. The proteolytic activity of C. albicans supernatants was confirmed by isoelectric focusing (IEF), and also by the spectrophotmetric method of Bradford (1976). The pH of the test saliva samples declined from 7.5 to 3.5 within 48 hours and was related to proliferation of Candida.

These results indicate that C. albicans is proteolytic under acidic pH conditions in vitro but whether proteolytic activity occurs in vivo needs to be ascertained. Furthermore the biological significance of the proteolytic activity of Candida in saliva is not known but it is possible that proteinases may help in depressing non-specific and specific defence mechanisms such as degradation of IgA. More work is therefore needed to clarify these points.

B. Characteristics of C. albicans proteinase

C. albicans and other Candida species (i.e., C. tropicalis, C. parapsilosis) secrete a carboxyl proteinase when they are grown in media containing proteins as the sole nitrogen source (MacDonald and Odds, 1980a; Ruchel et al., 1983). There are at least three proteinase isoenzymes, secreted by different C. albicans strains (Odds, 1985). The pH optima for the enzymes range from 2.2. to 3.2 (Remold et al., 1968; Ruchel et al., 1983). These proteinases are denatured irreversibly above pH 8 (Ruchel, 1981; Ruchel et al., 1983) and their molecular weights are in the range 40,000 to 45,000 (Remold et al., 1968; Ruchel, 1981). However, the carboxyl proteinase of C. parapsilosis is distinguished by their lower molecular weight (approximately 33,000) (Ruchel et al., 1986).

C. Proteolytic activity and pathogenicity

Evidence of a role for Candida proteinase in the pathogenesis of candidosis has been reviewed by Odds, (1985). The

association between strain proteolytic activity and pathogenicity was suggested by Staib in 1969 and was supported by others who found correlations between total proteinase activity and strain virulence (Budtz-Jorgensen, 1971; Ghannoum and Abu Elteen, 1986). The significance of the proteolytic activity of Candida strains as causative agents of severe candidosis (i.e. thrush, and septicaemia) has been discussed by Budtz-Jorgensen (1971); Germaine et al. (1978). Remold et al. (1968) claimed that the enzyme was secreted by C. albicans strains that were pathogenic for mice but was absent from non-pathogenic strains. The strongest evidence to date that the inducible acid proteinase of C. albicans may be related to virulence comes from two studies which showed that mutant strains which were defficient in proteinase activity were significantly less virulent in a mouse experimental model compared with the original strains (Kwon-Chung et al., 1985). Furthermore they showed, that C. albicans revertants with restored proteolytic activity, also regained their virulence. However, another study which attempted to show a relationship between proteolysis and virulence, by comparing C. albicans isolates from active infections and carriers (Schreiber et al., 1985) failed to find any significant correlation between proteinase production and active infection.

Ruchel (1983) and Odds (1985) have studied the ability of different Candida species to produce proteinases, and have reported that this varies among different Candida species; for example C. tropicalis produces the enzyme to a lesser degree than C. albicans and C. parapsilosis forms the enzyme only occassionally. It is very

rare or absent in other species such as C. krusei and C. glabrata (Ruchel et al., 1983; Odds, 1985). Borg and Ruchel, (1988) in their study of 400 patients found that 76% carried C. albicans, 14% carried C. tropicalis and 0.75% carried C. parapsilosis. In a previous study Ruchel et al. (1986) showed that in contrast to C. albicans, phagocytosis of C. parapsilosis was not accompanied by secretion of fungal proteinase. This failure to induce the enzyme under conditions of infection may account for the low virulence of most isolates of C. parapsilosis. Thus the distribution of extracellular Candida proteinases appears to reflect the order of virulence of these opportunistic fungi, and suggests that proteinase production contributes to the virulence of Candida species.

There is also indirect evidence that suggests a role for proteinase in fungal virulence. For instance, the synthesis of proteinase by C. albicans has been shown by immunofluorescence in histological sections from patients with candidosis. (MacDonald and Odds, 1980 (b); Ruchel, 1983). Furthermore there are data that suggest that proteolytic strains of C. albicans and C. tropicalis secrete their enzymes during infection in humans (Borg and Ruchel, 1988). In addition, high titres of specific antibodies to proteinases can be demonstrated in the sera of patients suffering from candidosis (MacDonald and Odds, 1980b) and proteinase-related-antigens can be detected in infected tissues (Zimmermann, 1986).

MacDonald and Odds, (1983) suggested that proteinase might be involved in fungal resistance to phagocytosis, but Walther et al.

(1986) have shown that proteinase-positive Candida cells are killed more readily by polymorphs than proteinase-negative cells. It is thought that the proteinases of C. albicans may activate the proteins associated with lysosomal membranes thus leading to an increase in intracellular killing.

Borg and Ruchel, (1988) have recently observed a possible relationship between fungal adherence to buccal mucosa and the activity of extracellular fungal proteinase. The latter is inhibited by a proteinase inhibitor, pepstatin, and there is evidence that the formation of corneocyte cavitation around adherent blastoconidia, on the skin, is inhibited when treated with pepstatin (Ray & Payne, 1988).

1.5.5. Alpha-amylase

Alpha-amylase is a major protein component in human parotid saliva and is present in a concentration 1.0 g/l. Submandibular saliva contains much less amylase (0.8 g/l) and sublingual saliva contains very little, while saliva from the labial accessary glands appears to have no amylase activity (Ferguson, 1988).

Human salivary alpha-amylase (alpha-1,4-glucan 4-glucanohydrolase) has been studied using a number of different electrophoretic methods (Karn et al., 1973) and it has been reported that salivary amylase isozymes may be separated into two families: one which has a high molecular weight and slow electrophoretic mobility,

and the other with a lower molecular weight but faster electrophoretic mobility.

When parotid saliva is run on SDS-PAGE alpha-amylase appears as 2 distinct blue bands. It exists in 2 forms, glycosylated (Mr: 63,000) and non-glycosylated (Mr: 59,000). It can be separated into a series of isozymes by isoelectric focusing and familial inheritance of the various phenotypes has been shown (Eckersall et al., 1981).

Salivary alpha-amylase is a digestive enzyme which helps in the digestion of starch in the mouth and later in the digestive tract. Starch consists of two polysaccharides; amylose, an unbranched chain made up of alpha 1,4 linked glucose residues and the branched glucose polymer, amylopectin (Jenkins, 1978).

There is increasing evidence that this enzyme may play an antibacterial role. When Streptococcus sanquis is incubated with clarified saliva, alpha-amylase is seen to bind to bacteriaand this may inhibit bacterial adhesion in the oral cavity (Douglas, 1983). In addition the growth of Neisseria gonorrhoeae and Legionella pneumophila is inhibited by salivary amylase (Bortner et al., 1983). Although there seems to be some interaction with alpha-amylase and certain bacteria so far there is no evidence of any interaction between the enzyme and Candida.

1.5.6. Lysozyme

Lysozyme, also known as muraminadase is widely distributed in human tissues and secretions and was discovered by Fleming, more than six decades ago (Fleming, 1922). It is a basic protein (Petit and Jolles, 1963) consisting of single polypeptide chains of molecular weight 15,000 (Balekjian et al. 1969). It is present in saliva, the gingival tissues and gingival crevicular fluid (Gibbons, Stoppelar and Harden, 1965). The concentration of lysozyme from the minor salivary glands is much higher than the concentrations of lysozyme from submandibular or parotid saliva (Stuchell et al., 1980). Lysozyme is also found in association with both azurophilic and specific granules of human polymorphonuclear leukocytes (Bretz and Baggiolini, 1974; Elsbach, 1980).

Lysozyme has been shown to have antimicrobial activity, and a few investigations have been carried out using a range of oral microorganisms. Some strains of Streptococcus mutans are susceptible to the lytic activity of the enzyme while the growth of Actinomyces viscosus was inhibited in the presence of 160 µg/ml lysozyme (Iacono et al., 1980). Furthermore Actinobacillus actinomycetemcomitans and Capnocytophaga gingivalis demonstrated remarkable sensitivity to lysozyme (Iacono et al., 1983; Iacono et al., 1985).

Some data regarding the antimicrobial action of lysozyme is available. Since lysozyme is a glucosidase, it hydrolyses the

glycosidic bond between C-1 of N-acetylmuramate (NAM) and C-4 of N-acetyl glucosamine (NAG) of the bacterial cell wall polysaccharide. This splitting of the cell wall causes the disintegration and death of some bacterial cells. The sensitivity of microorganisms to lysis is dependent upon the susceptibility as well as the accessibility of the cell wall peptidoglycan to the enzyme (Iacono et al., 1982). If these polysaccharides are absent from a species of bacteria, then it is not destroyed by lysozyme.

In addition to the bactericidal activity of lysozyme, it has also been shown to inhibit bacterial growth (Pollock et al., 1976) and also cause bacterial aggregation. For example, it has been observed that lysozyme binds to and aggregates the various serotypes of S. mutans. However, serotype a and b strains of S. mutans bind more lysozyme and aggregate to a greater extent than the other serotypes (Pollock et al., 1976; Iacono et al., 1982).

Although the sensitivity of a range of bacterial species to lysozyme has been investigated, few studies have investigated it's action on Candida species. Kamaya (1970) was the first to demonstrate that several human isolates of Candida i.e. C. albicans, C. tropicalis, C. stellatoidea, C. pseudotropicalis, C. krusei, C. parapsilosis, and C. guilliermondii were all sensitive to the killing effect of lysozyme. He used different concentrations of lysozyme in a number of glucose-water solutions. Thereafter Collins and Papagianis, (1974) found that the growth inhibition of C. albicans by amphotericin B was enhanced by the incorporation of 10 Aug of lysozyme.

Both Marquis et al. (1982) and Tobgi et al.(1987) found a significant dose response relationship between lysozyme concentration and fungicidal activity. When different species of Candida were tested, C. krusei and C. parapsilosis were found to be most sensitive to lysozyme and C. albicans and C. glabrata the least sensitive. However, there were differences in susceptibility to lysozyme among different strains within the same species (Tobgi et al., 1987).

The precise mechanism by which lysozyme lyses the fungal cell wall is not clear. However, two distinct complementory mechanisms have been suggested. Firstly the enzymatic hydrolysis of N-glycosidic bonds that link polysaccharides and structural proteins of the yeast cell wall may result in subsequent injury to the cytoplasmic membrane and secondly damage to the plasmalemma of C. albicans cells, is followed by activation of mannan synthetase, chitin synthetase and probably other enzymes that code for the yeast cell wall constituents (Scherwitz et al., 1978). Therefore damage to the plasmalemma of C. albicans by lysozyme may also result in periplasmic accumulation of wall-like material (Marquis et al., 1982; Tobgi et al., 1987).

Thus, lysozyme seems to be an important factor in the non-specific host defence system and may take part in the regulation of the microbial flora of the oral cavity and other mucosal surfaces due to its microbicidal properties. However, more information is required to ascertain whether salivary lysozyme levels, in vivo regulate the oral carriage of Candida.

1.5.7. Lactoferrin

Lactoferrin is an iron-binding glycoprotein with a molecular weight of 76,000 which binds two atoms of iron per molecule, and has a high affinity even at low pH (Malamud, 1985). This enzyme is secreted in saliva (Caselitz et al., 1981; Moro et al., 1984), is present in the gingival crevicular fluid (Friedman et al., 1983) and is also contained in the cytoplasmic granules of human polymorphonuclear leukocytes (Green et al., 1971; Masson et al., 1969). Leffell and Spitznasel, (1972) have shown that both lactoferrin and lysozyme are found in a group of lysosomal granules within the polymorphonuclear leukocytes.

Both bacteriostatic (Weinberg, 1978) and bactericidal activity (Arnold, Brewer and Ganthier 1980) has been demonstrated against members of the oral flora such as S. salivarius, S. mitis and C. albicans. In recent years the complexity of the interaction between S. mutans and lactoferrin have been described (Lassiter et al., 1987). In addition lactoferrin has been shown to have an active antifungal effect on yeasts when they are cultured in medium with a low concentration of iron to which lactoferrin is added (Kirkpatrick et al., 1971; Arnold et al., 1980; Valenti et al., 1986). This inhibitory effect is lost in an iron saturated environment. Therefore it may have a role to play in the protection of mucosal surfaces against infection by denying a supply of essential iron to potentially pathogenic microorganisms such as Candida species. However, there is no firm evidence regarding the role of salivary

lactoferrin in regulating Candida in the oral cavity.

1.5.8. Salivary immunoglobulins

The main immunoglobulin in saliva is IgA and it is produced almost entirely by the salivary glands. The IgA found in saliva is dimeric or polymeric in nature (dIgA or pIgA) and is associated with a glycoprotein called secretory component. Therefore antibodies raised to 'secretory IgA' (sIgA) distinguishes IgA found in secretions from that found in serum (Butler, 1985). The secretory component is important in the protection of secretory IgA against proteolysis (Morris et al., 1981). The IgG, IgM and IgA content of saliva is relatively small, and the major source of these is probably the gingival exudate (Schenkein & Genco, 1977). In whole saliva the contributions of sIgA, IgG and IgM are approximately 20, 0.1 and 0.1mg per 100ml, respectively (Challacombe, 1990). Minor salivary glands contribute only about 10% of the total volume of saliva, but as the secretory IgA content is much greater than that found in the main salivary glands, the contribution of the minor salivary glands to the total salivary IgA could be as great as 25% (Challacombe, 1990).

Several workers have sought a relationship between salivary immunoglobulin and oral disease. It has been demonstrated that secretory immunoglobulin A inhibits the adhesion of some bacteria to mucosal surfaces (Williams and Gibbons, 1975). Also there is evidence that salivary IgA antibodies may prevent Streptococcus mutans from adhering directly to pellicle, or glucan coated tooth surfaces.

It is now generally agreed that secretory IgA, may contribute to the decreased adherence of microorganisms on teeth and oral mucosal surfaces by aggregating the bacteria which are then removed by swallowing (Hoppenbrouwers et al.,1984). Interestingly specific IgA antibodies to C. albicans have been shown to inhibit the adherence of yeast to buccal epithelial cells. (Epstein et al., 1982; Vudhichamnong, Walker and Ryley, 1982).

A common feature of Candida proteinase is its ability to cleave various human immunoglobulins in vitro. It has been found that C. albicans as well as C. tropicalis is able to degrade salivary immunoglobulins (Ruchel, 1986); these include IgG1, IgG4, IgA1, IgA2 and IgD classes as well as secretory IgA and isolated secretory component (Ruchel, 1986). The presence of IgA-coated Candida cells in salivary sediments in vivo has been shown by Epstein et al. (1982). The extent to which this occurs in vivo and the specific conditions required for these interactions, are not known. In clinical terms patients with oral candidosis have increased levels of salivary secretory IgA to C. albicans (Lehner, 1966) suggesting that antibodies may play a role in defence against infection. Since functional sIgA appears to prevent the attachment of C. albicans to mucosal epithelium (Vudhichamnong et al.,1982) cleavage of salivary IgA is likely to facilitate the adherence of yeasts. Individuals with lowered levels of sIgA are more often afflicted by mucosal candidosis than those with normal levels (Vudhichamnong, Walker and Ryley, 1982). Furthermore, the ability of yeast species (C. albicans and C. tropicalis) to hydrolyse immunoglobulins corresponds with their

ability to colonise the mucosa and to cause thrush (Reinholdt et al., 1987).

1.5.9. Histidine-rich proteins

Human parotid and submandibular glands secrete at least 40 electrophoretically identifiable proteins and peptides although relatively few of these major salivary constituents have been isolated and fully characterized. These include a group of polypeptides which is reported to have an unusually high content of histidine (MacKay et al., 1984). All these proteins display unusual amino acid compositions and to date the complete amino acid sequence of only one histidine-rich polypeptide from human saliva has been established (Oppenheim et al., 1986).

The antimicrobial activity of 'histidine-rich' polypeptides (HRP) has been recently studied by several groups of investigators. Partially purified mixtures of these proteins has been shown to exhibit antibacterial and antifungal activity (MacKay et al., 1984; Pollock et al., 1984). In this dual capacity, salivary HRP join a select-group of naturally occurring antimicrobial agents such as lactoferrin, lysozyme and myeloperoxidase. The anticandidal nature of histidine-rich polypeptides was first reported by Pollock et al. (1984) who found that purified salivary HRP exerted antifungal activity against two different strains of C. albicans at concentrations similar to the known antifungal activity of the imidazole antibiotics such as ketoconazole. Candidastatic and candidacidal activities of HRP's have also been observed (MacKay et

al., 1984; and Pollock et al., 1984). Furthermore, Oppenheim et al. (1986) has reported that HRP is capable of inhibiting the germination of C. albicans in vitro, without killing taking place. This is significant because germination appears to optimize the adherence of C. albicans to oral mucous membranes (Kimura and Pearsall, 1978; Sobel et al., 1981). Consequently, inhibition of germination could reduce Candida colonisation in the mouth.

1.5.10. Proline-rich proteins

In human parotid saliva, 75 to 80% of the total protein is characterized as a group of proteins which are composed primarily of proline, glycine and glutamic acid and are thus designated as proline-rich proteins (Bennick, 1982). Since the proline-rich proteins form a major fraction of the total protein of saliva, they may be expected to play a significant role in the mouth. These proteins have been related to several functions in the oral cavity, especially in maintaining the concentrations of ionic calcium in saliva. Moreover they inhibit the formation of hydroxyapatite whereby crystal growth of calcium phosphate salts on the tooth surface in vivo may be avoided (Hay, 1983). However, it is unknown whether proline rich proteins have any antifungal/antibacterial activity in the mouth.

1.5.11. The salivary peroxidase system

The salivary peroxidase system is one of the non-immunoglobulin defence factors which appears to be involved in

regulating the quantity and species distribution of oral microorganisms (Tenovuo and Pruitt, 1984). In the human mouth, peroxidase activity is derived either from the acinar secretions of the salivary glands (secretory peroxidase) or from leukocytes entering the oral cavity through the gingival crevicular epithelium (myeloperoxidase) (Makinen and Tenovuo, 1976, Kowolik et al., 1981). The interaction of dental plaque and PMN's is necessary for the release of myeloperoxidase from PMN's (Taichman et al., 1977). In addition to secretory peroxidase the salivary glands secrete thiocyanate ions (SCN^-) (Tenovuo and Pruitt, 1984), and hypothiocyanite ($OSCN^-$) has been demonstrated in human mixed (Thomas, Bates and Jefferson, 1980) and parotid saliva, (Pruitt, Mansson-Rahemtulla and Tenovuo, 1983).

Salivary peroxidase catalyses the oxidation of SCN^- by hyrogen peroxide thus producing the hypothiocyanate ion which is toxic and antimicrobial. This is the principal antimicrobial product generated by the salivary peroxidase system at neutral pH, (Tenovuo and Pruitt, 1984). The net reaction of this complex system is:

$$H_2O_2 + SCN^- \xrightarrow{\text{peroxidase}} OSCN^- + H_2O$$

Therefore all the ingredients required for the activation of the salivary peroxidase system (namely, the peroxidase enzyme, hydrogen peroxide, and the thiocyanate ion (SCN^-) are present in saliva.

Numerous species of oral bacteria are inhibited by the oxidation products of the salivary peroxidase system. Among these are clinically important species such as lactobacilli (Klebnoff and Luebke, 1965), streptococci (Thomas et al., 1983) and actinomyces (Germaine and Tellefson, 1982). Although there is evidence for the inhibitory activity of salivary peroxidase system on the above species of bacteria, there is little information regarding its antifungal activity.

Diamond and Krzesicki (1978), and Diamnond et al. (1978) established that C.albicans could be damaged and probably killed by human neutrophils in the absence of complete ingestion and they attributed this to the products of oxidative metabolism of neutrophils. Subsequent in vitro studies have shown that the myeloperoxidase system is important in the candidacidal activity of leukocytes (Wagner et al., 1986; Diamond et al., 1980). Although myeloperoxidase-deficient individuals do not seem to have much difficulty in coping with other microorganisms they may develop infections with C. albicans (Lehrer and Cline, 1969). All these experiments support the concept of human neutrophil myeloperoxidase-mediated candidacidal activity.

Myeloperoxidase appears to have two unique properties essential for its candidacidal activity. First, this enzyme can use hydrogen peroxide (H_2O_2) as a substrate to oxidise chloride ions, yielding hypochlorus acid (HOCl). The latter has excellent antimicrobial activity and could be used directly by neutrophils to

kill microorganisms. Secondly, myeloperoxidase is able to bind to fungal cell walls by specific interactions with cell-wall-associated mannan (Wright and Nelson, 1988; Wright et al., 1983). Mannan is readily available in Candida cell walls as a target for binding of myeloperoxidase as this polysaccharide may compose 30 to 50% of the dry weight of the fungal cell wall (Chattaway et al., 1968). The binding of myeloperoxidase to the fungal cell surface carbohydrates may provide a mechanism for the generation of hypochlorus acid in close proximity to vital components of the cells. However, adsorption of the enzyme to Candida cell wall-associated mannans may occur irrespective of the viability of the yeast (Wright and Nelson, 1988). Consequently the enzyme may not be able to dissociate from dead yeasts and thus not be available for binding to live fungal cells (Wright and Nelson, 1988).

1.5.12. Salivary flow rate

The importance of saliva for oral health is well known (Baum, 1987) and normal salivary flow rates vary widely among individuals. As with normal flow rates, a spectrum of diminishing flow must accompany the transition from the normal functional state to dry mouth. Salivary flow rates may fall due to a number of reasons, i.e., rheumatoid arthritis (Syrjanen, 1982); malfunction of salivary glands, for example Sjörgren's syndrome (Stuchell et al., 1984) and irradiation (Main et al., 1984).

One disease that leads to severe xerostomia is Sjörgren's syndrome, which affects the lacrimal and salivary glands leading to dryness of the eye and mouth (Mason and Chisholm, 1975). In some cases, lacrimal and salivary enlargement may also be present. Sjörgren's syndrome causes progressive destruction of the salivary glands, and in addition to progressive and severe dryness of the lips and the oral mucosa, the salivary secretions become viscous. Microbiological assessment of the oral flora of patients with Sjörgren's syndrome has shown that an increase in the number of C. albicans and Staphylococcus aureus and coliform bacilli when compared with normal control subjects (MacFarlane, 1984).

Patients receiving cytotoxic drug therapy and radiotherapy have been found to develop oral problems as a direct consequence of treatment (Samaranayake et al., 1984, Martin et al., 1981). Main et al. (1984) concluded that cytotoxic chemotherapy prduces a decrease in salivary flow, a reduction in salivary IgA and an increase in the oral carriage of Candida species, particularly in the case of C. albicans and C. glabrata.

Diabetes mellitus has been associated with altered salivary secretion and mouth dryness, with some investigators reporting a reduced salivary flow rate in diabetics (Marder et al., 1975; Conner et al., 1970). In other studies, no change in the salivary flow rate has been found either in resting or in stimulated whole saliva (Sharon et al., 1985; Tenovuo et al., 1986). However none of these studies investigated changes in the microflora of these patients.

One of the most important functions of mixed saliva with respect to oral candidosis may be in the clearance of carbohydrate from the mouth. In a theoretical study of sugar clearance, Dawes, (1983) concluded that the most important factors related to this were the unstimulated salivary flow rate, and the volume of saliva in the mouth before and after swallowing. Hence the reduced salivary flow rate seen in patients with dry mouth would retard sugar clearance and presumably, contribute to the increased incidence of candidosis in such subjects.

Therefore there is evidence that both the quantity and the quality of saliva is important for the persistence of Candida in the oral cavity. However, only a few studies have investigated the interaction of saliva and Candida and further information is required.

1.6. Aims of the study

Since Candida blastospores are usually in intimate contact with saliva when colonising the oral mucosa both in health and disease, it is important to determine how salivary constituents and Candida interact. From a review of the literature, it is clear that there is little information about this important topic and therefore it was decided to carry out a series of experiments to help clarify the situation.

While the antifungal effect of lysozyme on Candida has been investigated to some extent little is known about the factors involved in the interaction of Candida and lysozyme. Preliminary *in vitro* experiments were carried out to confirm the dose effect of lysozyme on Candidaas described by previous workers using *C. albicans* GDH 1878. Extracellular polymeric material is produced around the rigid cell wall of *C. albicans* when it is grown in media containing a high concentration of sugars such as galactose and sucrose, and this layer appears to protect the fungal cells from the lytic effect of Zymolase-5000. Hence, in theory, it is possible that *Candida* species may protect themselves from the antifungal activity of lysozyme in a similar way. Therefore, it was decided to investigate the action of lysozyme on *Candida* species *in vitro*, by pre-incubating *C. albicans* GDH 1878 in media containing six different carbohydrate sources and subsequently determining the fungicidal effect of this enzyme. Further, the effect of growing four species of Candida in 3 different concentrations of sucrose on their subsequent sensitivity to lysozyme, was studied.

Only a few studies have been carried out to determine how Candida utilizes salivary constituents. Thus it was decided to study the growth, pH changes, acid anion production and protein degradation, which occurred when *C. albicans* GDH 1878 was cultured in pooled parotid saliva. Since data in the literature suggests that pooled parotid saliva may be an unsatisfactory growth medium for *C. albicans*, a comparison with pooled whole saliva was also performed. Acid anion analysis was carried out using isotachophoresis, and

protein degradation analysed using a biochemical method (bicinchoninic acid protein assay) and an electrophoretic technique (sodium dodecyl sulphate polyacrylamide gel electrophoresis). Although there is some data concerning the growth of C. albicans in saliva there is little or no information about other Candida species. Therefore it was decided to investigate further the growth, pH, acid anion production and protein degradation produced by 3 strains of C. tropicalis, C. glabrata and C. krusei, when grown in either parotid or mixed saliva, depending on the results of the preliminary experiments.

Finally, as there is some confusion in the literature concerning the ability of C. albicans to hydrolyse salivary proteins, experiments were devised to detremine if loss of protein from cultures was due solely to proteolysis or a combination of proteolysis and adsorption of salivary proteins to the surfaces of yeast cells.

CHAPTER 2

THE SUSCEPTIBILITY OF CANDIDA SPECIES TO LYSOZYME IN VITRO

2.1. INTRODUCTION

Lysozyme is a constituent of several biological secretions which are responsible for the protection of mucosal surfaces (Fleming, 1922; Hankiewicz and Swiercze, 1974). It is an important antimicrobial agent in the non-specific host immune system and plays a part in the regulation of the microbial flora in the oral cavity (Iacono et al., 1982).

The *in vitro* antifungal and the lytic effect of the enzyme was first recognised by Kamaya (1969 and 1970) but since then Collins and Papagianis (1974) have investigated the *in vitro* fungicidal activity of lysozyme in the presence of the polyene antibiotic Amphotericin B. Marquis et al. (1982), subsequently demonstrated that *C. albicans* when exposed to lysozyme produced ultrastructural damage which was concentration dependent. More recently, Tobgi et al. (1987) have reported the *in vitro* sensitivity of 68 oral and vaginal isolates of *Candida* species to lysozyme and showed that its fungicidal activity was proportional to the concentration of the enzyme in the medium and to the period of exposure. The species of Candida examined by Tobgi et al. (1987) proved to be susceptible to lysozyme in the following order: *C. krusei* (most susceptible), *C. parapsilosis*, *C. tropicalis*, *C. guilliermondii*, *C. albicans* and *C. glabrata* (least susceptible).

It is known that C. albicans when incubated in media containing high concentrations of glucose, galactose and sucrose produces an extracellular polymeric material around its rigid cell wall which confers increased resistance to spheroplast formation with Zymolase-5000 (McCourtie and Douglas, 1981; McCourtie and Douglas, 1985). Therefore, it is possible that Candida species could become less susceptible to the antifungal effect of lysozyme if cultured under conditions which favour the production of extracellular material. Since there is no data available concerning this topic, experiments were carried out to investigate the possible inhibition of the candidacidal activity of lysozyme by yeast cells pre-incubated in relatively high concentrations of carbohydrate (glucose, galactose, sucrose, maltose, xylitol and lactose) for 24 hours before subsequent exposure to lysozyme. However, initially experiments were carried out to determine the dose dependent effect of lysozyme on yeast viability when the yeast cells were cultured on Sabouraud's dextrose agar plates.

2.2. MATERIALS AND METHODS

2.2.1. Microorganisms

A total of 12 Candida isolates were used: C. albicans (GDH 1878, GDH 1786, GDH 0019), C. tropicalis (GDH 1009, GDH 0998, GDH 0057), C. glabrata (GDH 1337, GDH 1397, GDH 0081) and C. krusei (NCPF 3165, GDH 1742, GDH 1331). The GDH strains were obtained from patients with oral infections attending the Oral Medicine Clinic at Glasgow Dental Hospital and School. The NCPF strain 3165 was obtained from the National Collection of Pathogenic Fungi, Colindale, U.K. (Table 2.1.).

2.2.2. Identification of microorganisms

The yeast isolates were identified using API 20C test kits (API Laboratory Products Limited., Grafton Way, Basingstoke, Hants, RG 22 6H9) and the germ tube test (Mackenzie, 1962). To carry out these identification tests the yeasts were first grown on Sabouraud's dextrose agar (SDA; Gibco Limited., Paisley, Scotland), in an aerobic incubator (Gallenkamp U.K.) at 37^0C for 24 hours.

API 20C (Analytical Profile Index)

A colony grown on SDA was touched lightly with a sterile wire loop and dispersed in 2ml of sterile distilled water to make a light yeast suspension. The API 20C Auxanogram consists of a strip of

Candida Species	Source	Clinical Description
Candida albicans		
GDH-1878	Oral isolate	No information
GDH-1786	"	"
GDH-0019	"	Patient with CAC
Candida tropicalis		
GDH-1009	"	Patient with SS
GDH-0998	"	Patient with myeloma
GDH-0057	"	Patient with angular chelitis
Candida glabrata		
GDH-1337	"	Patient with CAC
GDH-1397	"	Patient SS
GDH-0081	"	Patient with CAC
Candida krusei		
NCPF-3165	Sputum	No information
GDH-1742	Oral isolate	Patient with SM
GDH-1331	" "	No information

Table 2.1. The source of the Candida species used in various experiments reported in this thesis.

CAC - Chronic atrophic candidosis
SS - Sjörgen's syndrome
SM - Sore mouth
GDH - Glasgow Dental Hospital and School
NCPF - National Collection of Pathogenic Fungi, Collindale, U.K.

20 microtubes containing dehydrated substrates in which nineteen different assimilation tests are performed. The API 20C Auxanogram test procedure was carried out by inoculating 100 µl of the yeast suspension into an ampoule of API 20C medium using an Eppendorf pipette. The yeast suspension was gently mixed with a sterile Pasteur pipette taking care not to incorporate air bubbles. The cupules were filled with the yeast suspension using a sterile Pasteur pipette and the strip placed on an incubation tray, covered with a lid and placed in the incubator at 30^0C for 48-72 hours (Gallenkamp U.K.). The first cupule '0' serves as the negative control. A positive reaction is one observed as being more turbid than the control and the results from all tests are recorded on a data sheet. The results are separated into groups of three, with each test being assigned a value of 1, 2 or 4 if positive. By adding together the numbers within each group of three tests, a seven digit numerical profile is obtained. The coded numbers were entered into an Opus Computer (Complete Computer Centre, Ltd., Glasgow) supplied with API identification software (API) which printed out the identity of the yeast with confidence limits.

Germ tube test

A yeast colony grown on SDA was lightly touched with a sterile wire loop and dispersed in 0.5ml of horse serum (Gibco; Paisley, Scotland) and incubated aerobically at 37^0C for a minimum of 4 hours. A drop of this culture was placed on a glass slide, covered with a coverslip and examined for the presence of germ tube formation

at 400 times magnification using an Olympus Microscope (Olympus BH2, Japan). Cylindrical cytoplasmic extensions which were longer than the diameter of the blastospore were recorded as germ tubes.

After completion of the identification tests the yeasts were maintained on Sabouraud's dextrose agar slants at 4^0C. Every month fresh maintenance cultures were prepared using freeze-dried samples of the yeasts (see Section 2.2.3.).

2.2.3. Storage of Yeasts

The yeast cultures obtained were stored by freeze-drying. The method described by Kirsop (1984) was used in this study. This involved the following steps: preparation of ampoules, preparation of yeast cultures, freeze drying and testing the viability of the freeze dried yeast cells.

Preparation of ampoules

The glass ampoules (Woods, Paisley, Glasgow, U.K.) were first washed and dried. A label, with the name of the Candida strain printed on it, was inserted into the ampoule. The ampoules were next plugged with non-absorbent cotton wool and autoclaved at 121^0C for 15 minutes.

Preparation of cultures

The yeast was first grown on SDA at 37^0C for 18 hours in an aerobic incubator (Gallenkamp, U.K.). A liquid medium was prepared, containing, 1 gram of inositol (BDH Chemicals, Limited., Poole, U.K.) in 20 ml of newborn calf serum (Gibco, Paisley, Scotland). This solution was filter sterilized using a 0.45 ʌm Minisart filter (Sartorius, Surrey, England). A loopful of yeast grown on SDA was inoculated into this liquid medium to make a heavy yeast suspension. Aliquots (0.5ml) of this suspension were dispensed into sterile freeze drying ampoules and plugged lightly with cotton wool.

Freeze-drying

This process was carried out in 2 steps: namely, primary and secondary freeze drying. The ampoules were arranged symmetrically on the centrifugal attachment of the freeze-dryer (Edwards freeze dryer, Crawley., England) and spun for at least 30 minutes during which time, primary freeze-drying was completed. After the completion of this stage, the centrifuge was switched off and the vials kept in the freeze dried state under vacuum for at least two and a half hours to remove as much moisture as possible. The ampoules were then removed from the freeze dryer, the cotton wool plugs pushed gently halfway down the tube and the vials constricted using an attachment supplied by the manufacturer for this purpose.

In the secondary phase of freeze drying the cultures were dried 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.

Viability of the freeze dried yeast isolate

A viability test was carried out with the newly prepared freeze dried ampoules. A file mark was made on the ampoule, near the middle of the cotton wool plug with a diamond cutter and a red hot wire loop was applied to crack the ampoule. The cotton wool plug was gently removed with forceps and using a pasteur pipette,0.5ml of anaerobic blood broth (Gibco; Limited., Paisley, Scotland) was added and carefully mixed to avoid frothing with the freeze dried culture. A loopful of this suspension was cultured on SDA and incubated in an aerobic incubator at 37^0C for 48 hours. The identity of the yeast strain was checked using the API 20C Auxanogram method as described earlier in Section 2.2.2.

2.2.4. Sugar media

The preparation of sugar media for experiments 2 and 3 (see Sections 2.2.10. and 2.2.11.) is described in Appendix A.

2.2.5. Lysozyme

The preparation of different concentrations of lysozyme (1000 µg/ml - 40 µg/ml) for Experiment 1 (see Section 2.2.9.) is described in Appendix A.

2.2.6. Haemocytometer cell counting

The haemocytometer chamber

The haemocytometer chamber is divided into nine large squares. The centre square is divided into 25 squares, each of which is sub-divided into 16 squares. The large squares each have an area of $1 mm^2$. When the coverslip is pressed down over the grid so that an interference pattern appear, the depth of the whole chamber is 0.1mm (Figure 2.1.). A volume of 10 µl of the yeast suspension was inserted into the haemocytometer chamber with a sterile pipette tip. One of the haemocytometer wells was filled by capillary action and care was taken to avoid flooding the chamber.

The cell Count

The cells in the centre square and the four large corner squares (i.e., total of 5 squares) were counted. The cells touching the upper and right hand perimeter lines are ignored, while those touching the lower and left hand perimeter lines were counted. If the total number of cells counted is n, then,

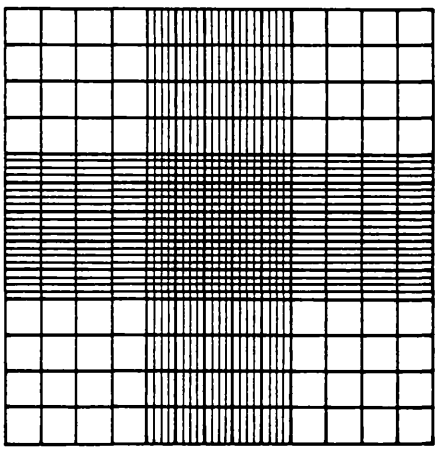

Figure 2.1. Diagrammatic representation of the counting grid used in the improved Naeubauer Haemocytometer Chamber.

The number of cells in 1 large square = n/5

The total volume of each large square = 1x1x0.1

= 0.1^3mm

= 0.0001^3cm

= 10^{-4} ml

Number of cells per ml yeast suspension= n/5 x 10^4.

2.2.7. Preparation of inoculum

The yeast strain under investigation was grown on Sabouraud's dextrose agar medium for 18 hours at 37^0C. Using this culture two different inocula (A) and (B) were prepared. Inoculum (A) was used in Experiment 1, when the yeasts were not pre-incubated in dietary carbohydrates, whereas inoculum (B) was used in Experiments 2 and 3 when the yeasts were grown in dietary carbohydrates before use in the lysozyme antifungal assays (Sections 2.2.10. and 2.2.11.).

Inoculum A

A loopful of the organism from the Sabouraud's dextrose plate was suspended in 10ml sterile distilled water to obtain a standard yeast inoculum of 6 to 8 x 10^6 cfu/ml. The total yeast cell count per ml was prepared using an improved Neubauer haemocytometer chamber (Hawksley, U.K.) as described in Section 2.2.6. The volume of

the inoculum used was adjusted with sterile distilled water if necessary.

Inoculum B

A loopful of the organism from the Saboraud's dextrose plate was suspended in 10ml sterile distilled water and 0.2ml of this suspension was added to 20ml of the appropriate sugar in Yeast Nitrogen Base (YNB) fluid medium. Cultures were grown in an aerobic orbital incubator (Gallenkamp Orbital Incubator) at 100 rpm for 24 hours at 37^0C. The cells were harvested and washed once in sterile distilled water by centrifugation at 1500g, for 10 minutes, (MSE Super Minor Centrifuge). A standard volume (10 µl) of the washed yeast pellet was suspended in 10ml sterile distilled water to obtain a standard yeast inoculum of 1 to 2 x 10^6 cfu/ml. The total cell count per ml of the yeast inoculum was estimated using an improved Neubauer haemocytometer chamber (Hawksley, U.K.) as described in Section 2.2.6. The volume of the inoculum used was adjusted with sterile distilled water if necessary.

2.2.8. Plan of study

Three different experiments were carried out in this Chapter. The first study was to determine the fungicidal effect of different concentrations of lysozyme on C. albicans GDH 1878. The second investigation was to assess the fungicidal effect of 20 µg/ml lysozyme on C. albicans GDH 1878 grown in YNB containing 500mM

concentrations of glucose, galactose, sucrose, lactose, maltose or xylitol. Finally the third experiment was conducted to determine the fungicidal effect of 20µg/ml lysozyme on twelve Candida isolates grown in different concentrations of sucrose (500mM; 125mM and 0.03mM) in YNB medium.

2.2.9. Experiment 1

The fungicidal effect of different concentrations of lysozyme on C. albicans GDH 1878

This was determined by the method of Tobgi et al. (1987) with minor modifications. Aliquots (0.5ml) of the lysozyme concentrations under study (1000, 500, 250, 62.5 and 40) µg/ml (see Appendix A) were dispensed into sterile plastic bijou bottles. Next, 0.5ml of the yeast inoculum prepared as described in Section 2.2.6 (Inoculum A), was added to each of the bottles containing lysozyme as described above giving the following final concentrations of lysozyme (500, 250, 125, 31.25 and 20) µg/ml. A negative control was carried out by placing 0.5ml of yeast inoculum (A) into 0.5ml of sterile distilled water.

At the start of the experiment (0 minutes), 0.1ml samples were removed from the test and control tubes and dispensed into sterile plastic bijoux bottles using a sterile pipette. The bijou bottles were incubated in an orbital incubator; 100 rpm; (Gallenkamp U.K) at 37^0C for 5 hours, and each hour 0.1ml was removed from all the bottles. The number of Candida in these samples was counted using a Spiral plater system (Spiral Diluter, Don Whitley, Yorkshire, U.K.).

(Figure 2.2.). First the spiral plater was set in the automatic mode and the stylus assembly was disinfected twice using a disinfectant solution (one presept tablet to 10 litres of water giving a solution containing 2500 ppm chlorine). The stylus and attached tubing was thoroughly rinsed with sterile distilled water to remove all trace of the disinfectant. Next the stylus was charged with the yeast suspension and a SDA plate placed on the turntable. The stylus was lowered on to the agar surface and the instrument started by depressing the starter switch. A spiral track containing a total volume of 50µl of the yeast suspension was dispensed over the surface of the SDA plate, starting near the centre and finishing at the periphery of the plate. The cultures were incubated aerobically at 37°C for 48 hours (Gallenkamp U.K.).

Counting was carried out by first sectoring the agar plate into specific sized sectors using a metal grid (Figure 2.3.). Each area marked on the grid corresponds to a known and constant volume of sample deposited on the agar plate. A sector containing a reasonable number of colonies was chosen and the number counted using a Gallenkamp colony counter (Gallenkamp, Germany). The yeast concentration of the samples was calculated as follows: The number of colony forming units in a sector was noted and divided by the volume of yeast suspension dispersed over the sector in order to calculate the number of colonies per unit volume. This volume is multiplied by 1000 to calculate the cfu/ml. However, if theyeast suspension has been diluted then the latter value needs to be multiplied by the dilution factor.

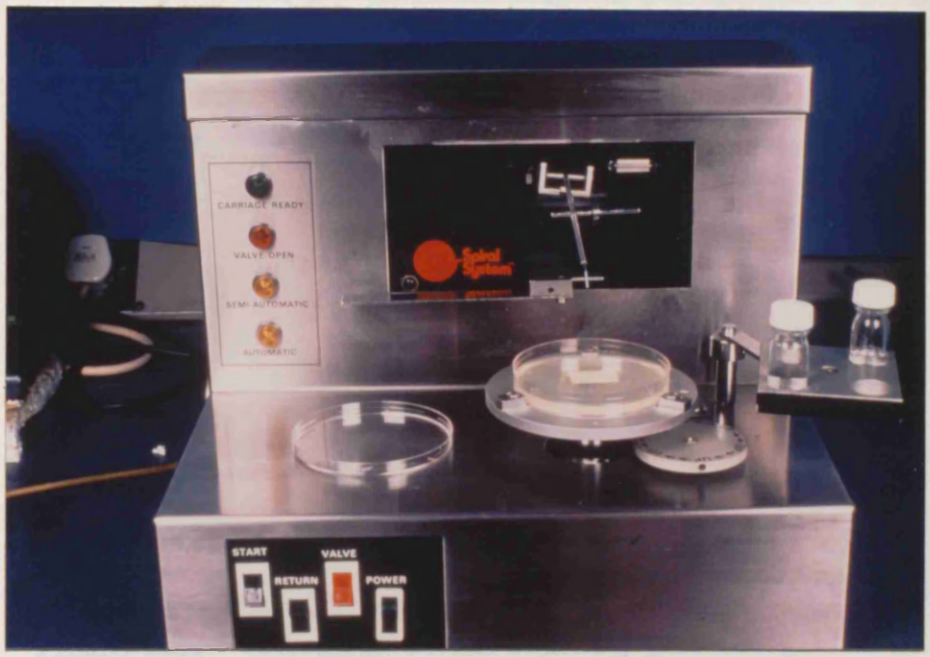

Figure 2.2. The Spiral Diluter in use with a Sabouraud's agar plate in position.

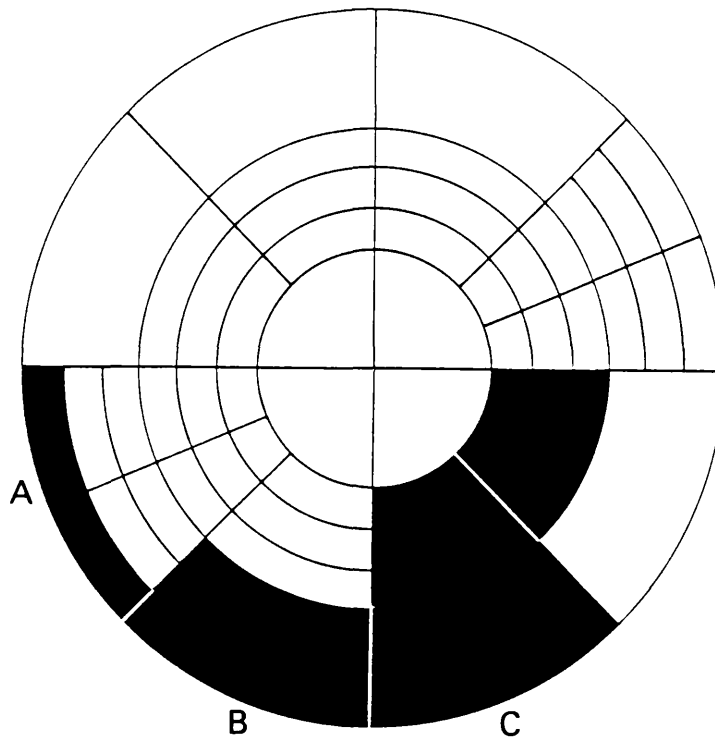

Figure 2.3. A diagram of the counting grid stamped on to a Sabouraud's dextrose agar plate. The volume of sample deposited in each of the sectors is pre-determined. For example sectors marked A, B and C correspond to 0.375 µl, 1.65 µl and 6.25 µl of sample volume deposited on the plate.

2.2.10. Experiment 2

The fungicidal effect of 20µg/ml on lysozyme on C. albicans GDH 1878 grown in 50mM glucose or in a number of sugar solutions

The experiment involved inoculating 0.2ml of the yeast inoculum prepared as described in Section 2.2.6. (Inoculum B) into 20ml of YNB supplemented with 50mM glucose, 500mM glucose, 500mM galactose, 500mM sucrose, 500mM maltose, 500mM xylitol or 500mM lactose. The cultures were grown aerobically at 37^0C for 24 hours (Gallenkamp Orbital Incubator U.K.). Cells were harvested after 24 hours, washed once with 20ml sterile distilled water and centrifuged at 1500 rpm for 10 minutes (MSE Super Minor Centrifuge). Ten microlitres of the yeast pellet so obtained were added to 10ml sterile distilled water and sonicated for two minutes at a setting of two (Sonicator, Model W-10, Plainview, New York) (Figure 2.4.). Preliminary studies were conducted to disaggregate the yeast suspensions at different settings and the sonicated yeast suspensions checked subsequently for viability. By this method the minimum time and sonication required (i.e., 2 minutes, at a setting of 2) to achieve even dispersal of the yeasts without loss of viability, was obtained.

The sonicated yeast suspension was added to sterile distilled water to give a final yeast suspension of 1 to 2 x 10^6cfu/ml. The counts were obtained by the use of an improved Neaubauer haemocytomer chamber (Hawksley, England). Aliquots (0.5ml) of this yeast

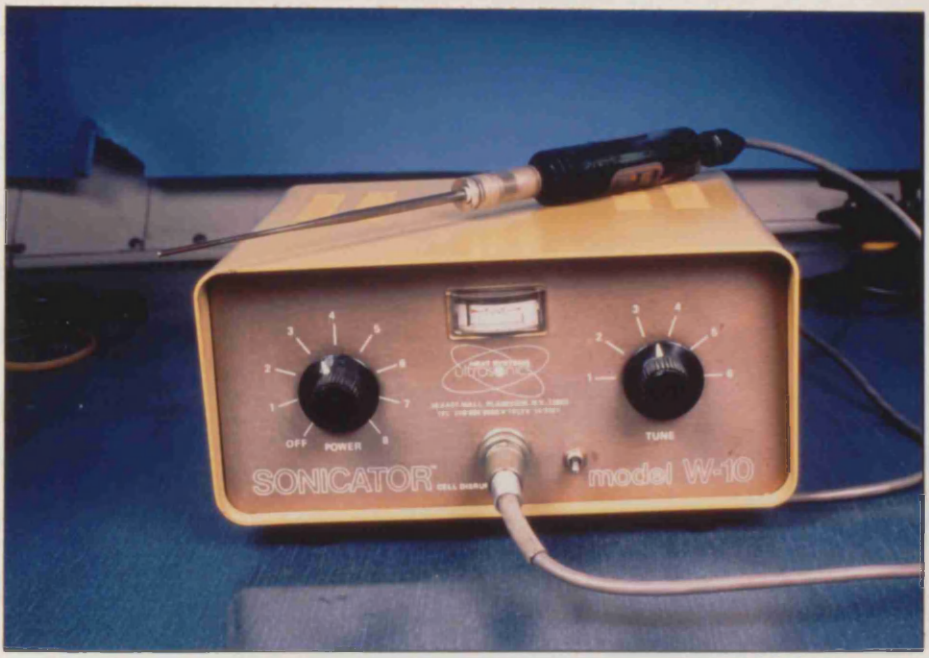

Figure 2.4. The Sonicator Cell Disruptor, Model W-10 (Heat Systems, Ultra Sonics, N.Y.) used in the dispersal of the yeast suspensions before inoculation onto Sabouraud's dextrose agar plates.

suspension were dispensed into sterile plastic bijoux bottles containing an equal volume of 40 µg/ml lysozyme giving a final lysozyme concentration of 20 µg/ml. At the beginning of the experiment (0 minute), 0.1ml samples were dispensed into sterile bijoux bottles using sterile pipettes, then 50 µl were spiral plated onto SDA plates in triplicate, as described in Section 2.2.9. Plates were subsequently incubated aerobically at 37^0C for 48 hours (Gallenkamp U.K.). The test bottles were incubated aerobically at 100 rpm at 37^0C for 1 hour (Gallenkamp Orbital Incubator U.K.). At the end of one hour, 50µl of each test sample was inoculated onto Sabouraud's dextrose agar plates in triplicate, using a spiral plater and incubated at 37^0C for 48 hours, as described in Section 2.2.9. The viable count of yeasts per ml of inoculum was estimated by sectoring each agar plate with a metal grid and counting the number of colonies in a chosen sector as described in Section 2.2.9.

The initial concentrations of yeasts in the different (500mM) sugar suspensions varied a little from that of the yeast concentration of the control sample (50mM glucose). Therefore the corrected mean value was calculated (50 µl) for all the test samples using the formulae:

$$\text{Corrected mean} = \frac{\text{Mean count of yeast, test sample (50µl)}}{\text{Initial yeastcon. of test sample (50µl)}} \times \text{Yeast con. of control sample (50µl)}$$

2.2.11. Experiment 3

The fungicidal effect of 20µg/ml lysozyme on twelve Candida isolates grown in different concentrations of sucrose (500mM; 125mM; and 0.03mM) in YNB medium

The 12 Candida isolates used in this study were as described in Table 2.1. and the experimental conditions were similiar to those described in Section 2.2.10. The concentration of lysozyme, tested was 20 µg/ml, and was the same as that used in the previous experiment. The yeasts however were grown in YNB containing three different concentrations of sucrose, namely 500mM: 125mM: and 0.03mM.

The initial yeast concentrations of the test sugar samples in different experiments varied between 70 to $90x10^4$ colony forming units per ml. Therefore these concentrations were standardised to $3.5x10^4$ colony forming units per 50 µl using the formulae:

$$\text{Standardised mean} = \frac{\text{Mean count of yeast, test sample (50µl)}}{\text{Initial yeast con. of test sample (50µl)}} \times \frac{70x10^4}{20}$$

2.2.12. Statistical analysis

Experiment 1 (Section 2.2.9.)

Statistical analysis was carried out to verify,

1) whether there was a significant relationship between lysozyme concentration and fungicidal activity of C. albicans GDH 1878 and

2) whether there was a significant relationship between time and concentration of lysozyme on the viability of C. albicans GDH 1878.

The mean results of experiment 1 were transformed to log values and these numbers were plotted against time. A two-way factorial analysis of variance of the results was carried out. A linear regression of log count on time was carried out for each concentration excluding the data obtained for 0 hours.

Experiment 3 (Section 2.2.11.)

A mathematical model was used to express the three sources of variability i.e., replicates, experiments and strains as nested random effects. An analysis of variance of the results was carried out using this model.

2.3. RESULTS

2.3.1. Relationship between lysozyme concentration, period of exposure to lysozyme and the fungicidal activity of C. albicans GDH 1878.

Experiments to study the reduction in the number of colony forming units per millilitre (cfu/ml) of C. albicans GDH 1878 after exposure to different concentrations of lysozyme (500, 250, 125, 31.25 and 20 µg/ml) for five hours were carried out on three separate occasions and the results are shown in Tables 2.2. to 2.4. The mean results for the three experiments are presented in Table 2.5. During the first hour of incubation there was a marked reduction in cfu/ml in all 5 tested concentrations of lysozyme (Table 2.5., Figure 2.5.). However, the reduction (i.e., the fungicidal effect) was greatest in the highest concentration and least in the lowest concentration of lysozyme. In the first hour of incubation 500 µg/ml lysozyme shows a reduction of cfu/ml from 3.37×10^6/ml to 3.60×10^3/ml and 20 µg/ml of lysozyme reduced the number of Candida from 3.37×10^6/ml to 1.39×10^5/ml. During the next 4 hours of incubation the cfu/ml falls to 1.73×10^2/ml and 4.48×10^3/ml in these two concentrations of lysozyme respectively.

Plots of log(count) against time, for each of the lysozyme concentrations on each of the three times tested are shown in Figures 2.6. (a), (b), (c), (d) and (e). Observing the patterns obtained for log(count) of the number of cfu/ml against time for each of the

Time (hours)	Concentration of lysozyme					
	500 µg/ml	250 µg/ml	125 µg/ml	31.25 µg/ml	20 µg/ml	Control Dis. water
0	$3.38x10^6$	$3.37x10^6$	$3.37x10^6$	$3.38x10^6$	$3.39x10^6$	$3.39x10^6$
1	$3.36x10^3$	$5.16x10^3$	$7.52x10^3$	$3.88x10^4$	$1.44x10^5$	$3.36x10^6$
2	$2.88x10^3$	$2.56x10^3$	$8.00x10^3$	$3.35x10^4$	$1.39x10^5$	$3.36x10^6$
3	$8.20x10^2$	$9.20x10^2$	$1.50x10^3$	$1.99x10^4$	$2.93x10^4$	$3.36x10^6$
4	$6.00x10^2$	$7.00x10^2$	$4.80x10^2$	$3.42x10^3$	$1.15x10^4$	$3.36x10^6$
5	$2.40x10^2$	$2.60x10^2$	$4.00x10^2$	$1.08x10^3$	$4.48x10^3$	$3.36x10^6$

Table 2.2. The number of colony forming units per ml of *C. albicans* GDH 1878 at different time intervals after exposure to different concentrations of lysozyme.

Time (hours)	Concentration of lysozyme					
	500 µg/ml	250 µg/ml	125 µg/ml	31.25 µg/ml	20 µg/ml	Control Dis. water
0	$3.36x10^6$	$3.36x10^6$	$3.35x10^6$	$3.35x10^6$	$3.36x10^6$	$3.37x10^6$
1	$3.64x10^3$	$4.80x10^3$	$9.60x10^3$	$4.97x10^4$	$1.33x10^5$	$3.37x10^6$
2	$2.88x10^3$	$2.20x10^3$	$6.56x10^3$	$3.69x10^4$	$1.33x10^5$	$3.37x10^6$
3	$7.80x10^2$	$9.40x10^2$	$1.14x10^3$	$1.45x10^4$	$4.53x10^4$	$3.37x10^6$
4	$5.00x10^2$	$7.60x10^2$	$7.40x10^2$	$2.60x10^3$	$1.34x10^4$	$3.37x10^6$
5	$1.60x10^2$	$2.20x10^2$	$2.80x10^2$	$8.40x10^2$	$5.76x10^3$	$3.37x10^6$

Table 2.3. The number of colony forming units per ml of *C. albicans* GDH 1878 at different time intervals after exposure to different concentrations of lysozyme.

Time (hours)	Concentration of lysozyme					
	500 µg/ml	250 µg/ml	125 µg/ml	31.25 µg/ml	20 µg/ml	Control dis. water
0	$3.39x10^6$	$3.35x10^6$	$3.39x10^6$	$3.37x10^6$	$3.38x10^6$	$3.38x10^6$
1	$3.80x10^3$	$4.12x10^3$	$7.84x10^3$	$5.94x10^4$	$1.41x10^5$	$3.38x10^6$
2	$2.68x10^3$	$2.20x10^3$	$8.00x10^3$	$2.97x10^4$	$1.09x10^5$	$3.38x10^6$
3	$9.00x10^2$	$8.80x10^2$	$1.38x10^3$	$2.78x10^4$	$3.73x10^4$	$3.38x10^6$
4	$5.60x10^2$	$6.20x10^2$	$5.20x10^2$	$2.72x10^3$	$1.24x10^4$	$3.38x10^6$
5	$1.20x10^2$	$2.40x10^2$	$4.60x10^2$	$8.00x10^2$	$3.20x10^3$	$3.38x10^6$

Table 2.4. The number of colony forming units per ml of C. albicans GDH 1878 at different time intervals after exposure to different concentrations of lysozyme.

Time (hours)	Concentration of lysozyme					
	500 µg/ml	250 µg/ml	125 µg/ml	31.25 µg/ml	20 µg/ml	Control Dis. water
0	$3.37x10^6$	$3.36x10^6$	$3.37x10^6$	$3.36x10^6$	$3.37x10^6$	$3.38x10^6$
1	$3.60x10^3$	$4.69x10^3$	$8.32x10^3$	$4.93x10^4$	$1.39x10^5$	$3.38x10^6$
2	$2.81x10^3$	$2.32x10^3$	$7.52x10^3$	$3.33x10^4$	$1.27x10^5$	$3.38x10^6$
3	$8.33x10^2$	$9.13x10^2$	$1.34x10^3$	$2.08x10^4$	$3.73x10^4$	$3.38x10^6$
4	$5.53x10^2$	$6.93x10^2$	$5.80x10^2$	$2.91x10^3$	$1.24x10^4$	$3.38x10^6$
5	$1.73x10^2$	$2.40x10^2$	$3.80x10^2$	$9.06x10^2$	$4.48x10^3$	$3.38x10^6$

Table 2.5. The mean number of colony forming units per ml of C. albicans GDH 1878 at different time intervals after exposure to different concentrations of lysozyme (Combined data from Tables, 2.2., 2.3. and 2.4.).

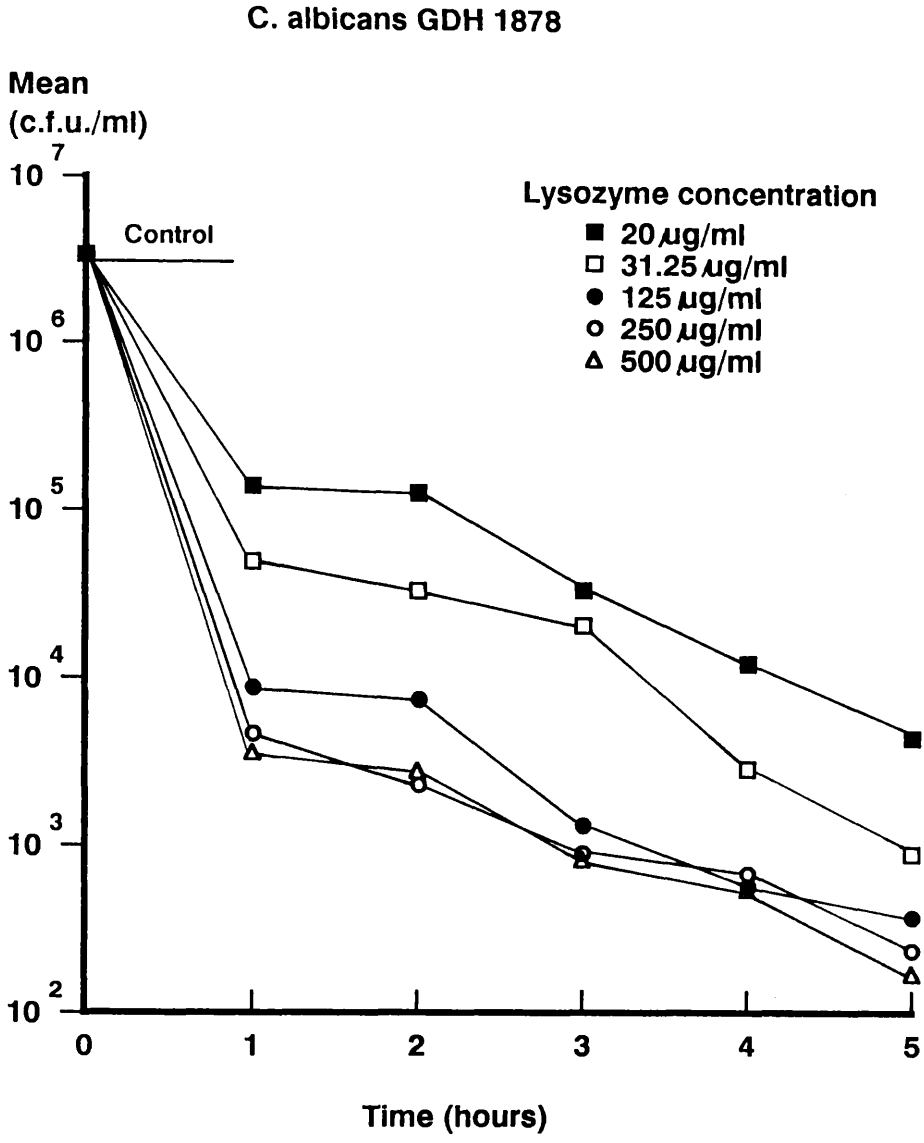

Figure 2.5 The number of colony forming units per ml of C. albicans GDH 1878 when incubated with different concentrations of lysozyme (500, 250, 125, 31.25 and 20) µg/ml for 5 hours.

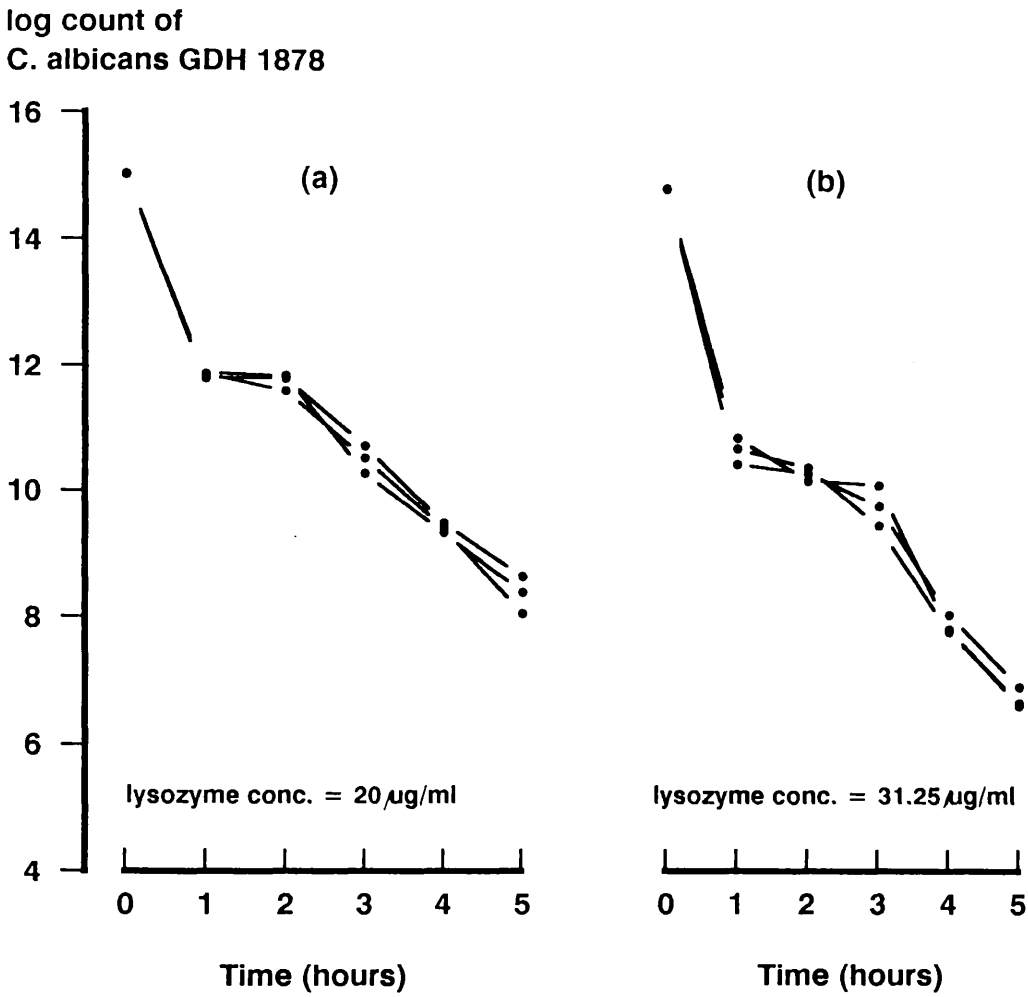

Figure 2.6a,b Log count of colony forming units per ml of C. albicans GDH 1878 after exposure to (a) 20 µg/ml, and (b) 31.25 µg/ml of lysozyme (Results of 3 experiments).

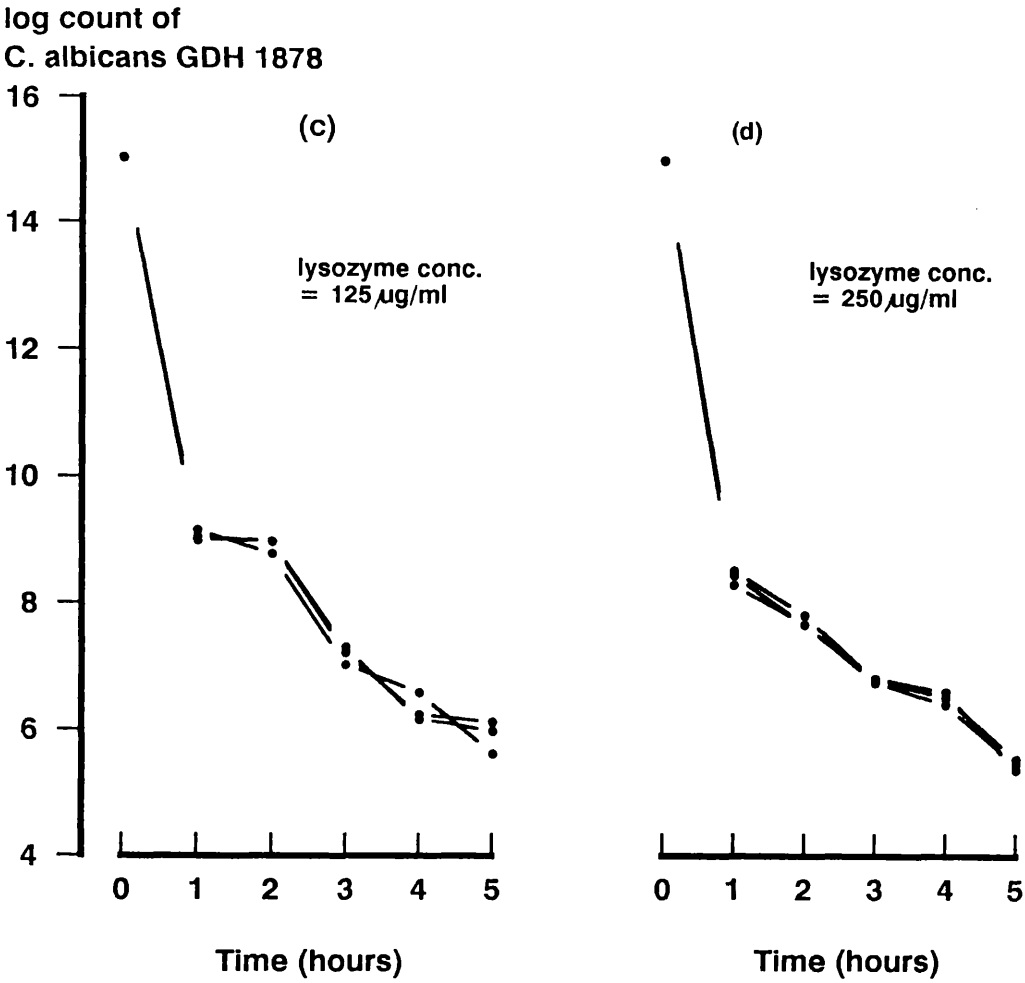

Figure 2.6c,d Log count of colony forming units per ml of C. albicans GDH 1878 after exposure to (a) 125 µg/ml, and (b) 250 µg/ml of lysozyme (Results of 3 experiments).

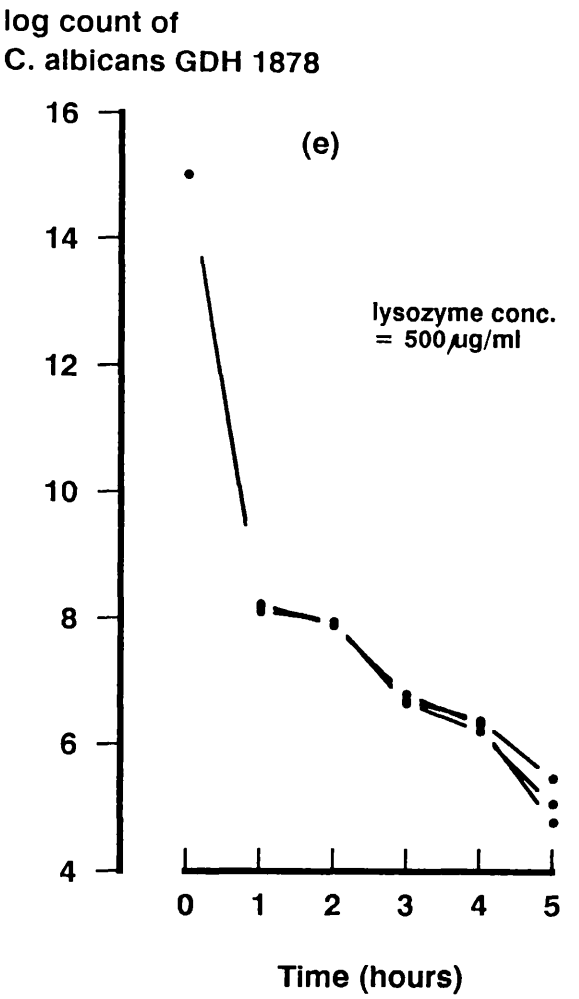

Figure 2.6e Log count of colony forming units per ml of C. albicans 1878 after exposure to (e) 500 µg/ml of lysozyme (Results of 3 experiments).

lysozyme concentrations, no overall model can be established to describe the viability of the yeast. This is because of the high killing effect of lysozyme during the first hour. This effect can also be seen in the plot of the average cfu/ml for each of the lysozyme concentrations throughout the experiments (Figure 2.7.). This dramatic fall in viability during the first hour is then followed by a reasonably stable log-linear decrease through 1 to 5 hours. The viability of the yeast decreases generally as the concentration of lysozyme increases (Figure 2.5.).

It is evident from the two-way factorial analysis of variance carried out on this data, (Table 2.6.), that both time and lysozyme concentration have a significant effect on the viability of the yeast ($p < 0.0001$). Although there is a time-concentration interaction it is difficult to describe the exact nature of these effects because of the dramatic reduction in cfu/ml of C. albicans GDH 1878 during the first hour of incubation at all 5 concentrations of lysozyme. This is further investigated below.

A linear regression analysis of the yeast viability (log count) with respect to time for each concentration was carried out excluding the data of the control. This is shown in Table 2.7. The intercepts appear to decrease exponentially as the lysoyzme concentration increases (Figure 2.8.), but the pattern for the slopes is not clear (Figure 2.9.).

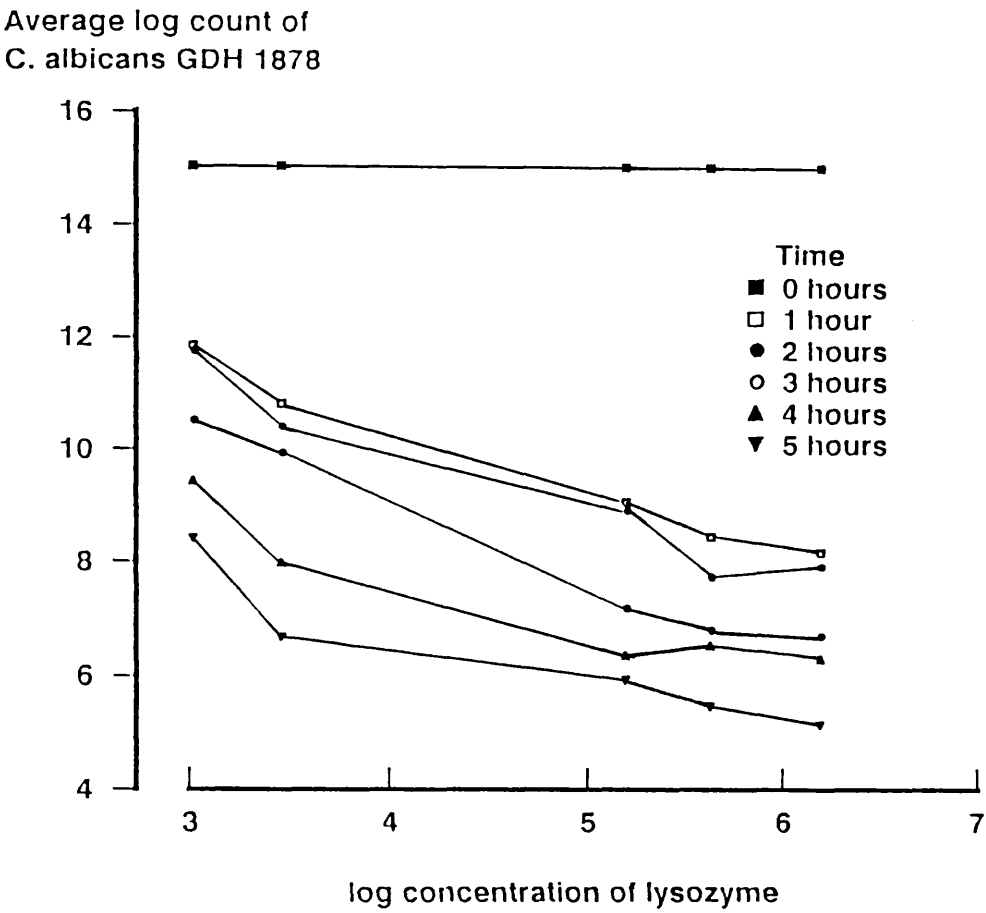

Figure 2.7 The log count of the average number of colony forming units per ml of C. albicans GDH 1878 over 5 hours, for each of the lysozyme concentrations over the 3 experiments (Combined data from Tables 2.2, 2.3, and 2.4).

SOURCE	D.F	MEAN SQUARE	F	PROBABILITY
CONC (C)	4	29.5147	1249.37	0.0000
TIME (T)	5	140.4511	5945.36	0.0000
CT INTERACTION	20	1.4776	62.55	0.0000
EXPERIMENTS	60	0.0236		

TABLE 2.6. **The results of the Two-way Factorial Analysis of Variance.**
DF = Degrees of freedom
F = Test statistic

Concentration (µg/ml)	Experiment	Intercept	Standard Error	Slope	Standard Error	R^2 (%)
	1	8.99	0.29	-0.68	0.09	95.3
500.00	2	9.22	0.30	-0.80	0.09	96.2
	3	9.35	0.37	-0.85	0.11	95.2
	1	9.25	0.20	-0.73	0.06	98.0
250.00	2	9.18	0.26	-0.72	0.08	96.7
	3	9.03	0.16	-0.70	0.05	98.6
	1	10.18	0.51	-0.89	0.15	91.8
125.00	2	10.22	0.37	-0.93	0.11	95.9
	3	10.07	0.53	-0.85	0.16	90.2
	1	12.03	0.59	-0.94	0.18	90.3
31.25	2	12.35	0.47	-1.08	0.14	95.0
	3	12.52	0.71	-1.10	0.21	89.9
	1	13.18	0.40	-0.94	0.12	95.3
20.00	2	13.07	0.41	-0.86	0.12	94.2
	3	13.22	0.38	-0.97	0.12	96.0

Table 2.7. **Results of Regression Analysis of log count/time for each concentration of lysozyme excluding time 0 hours.**

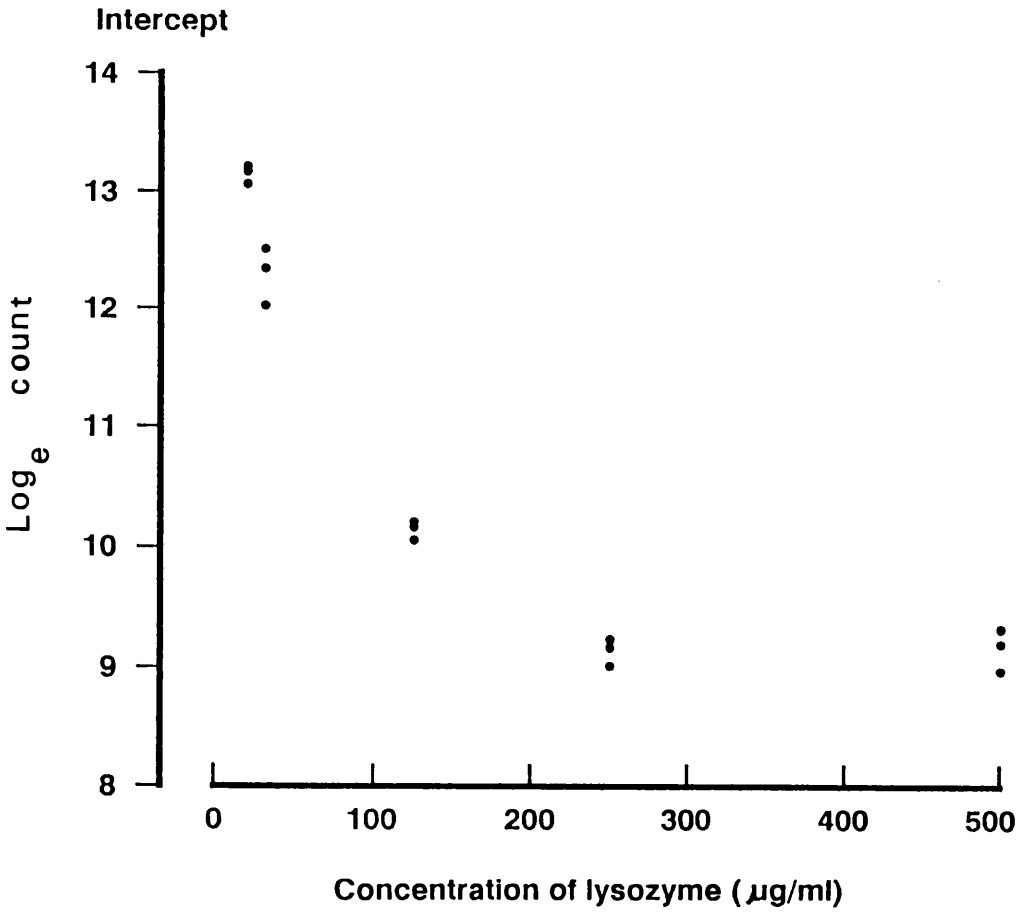

Figure 2.8 Graph showing the intercept for each of the different concentrations of lysozyme. Data from the linear regression analysis shown in Table 2.7.

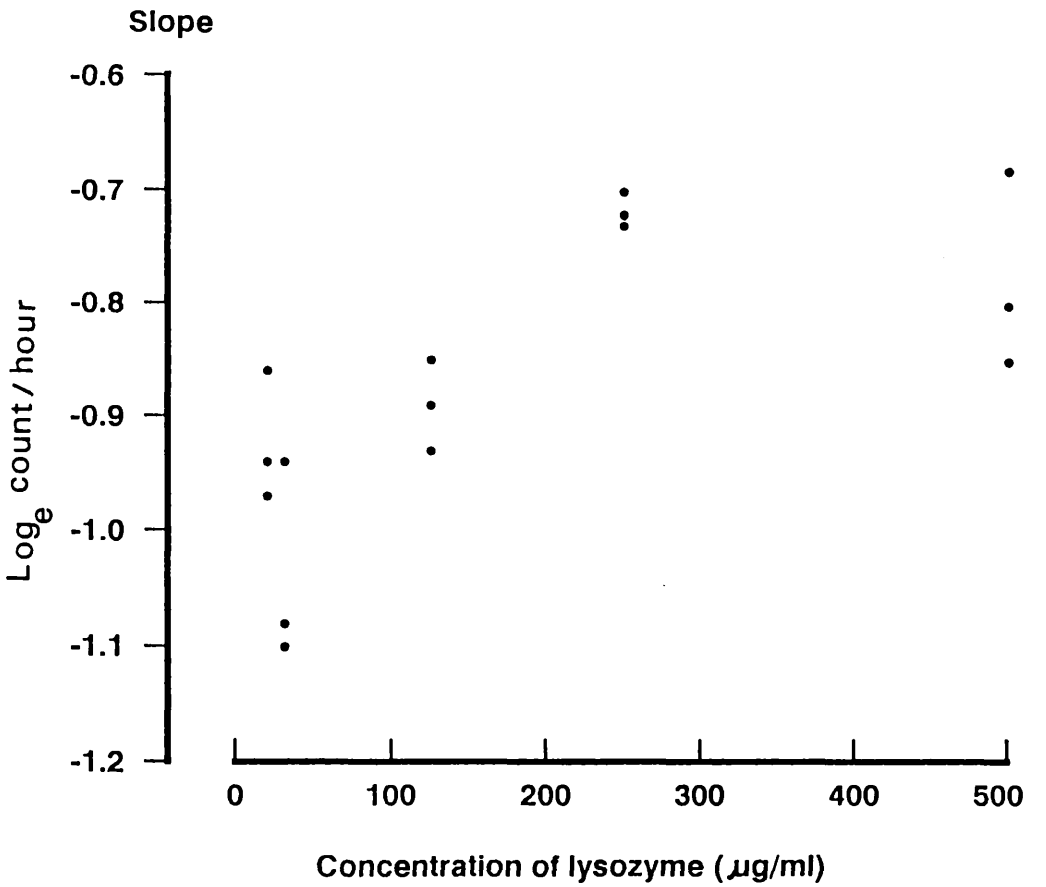

Figure 2.9 Graph showing the slope for each of the different concentrations of lysozyme. Data from the linear regression analysis shown in Table 2.7.

Based on this analysis it can be said that both time and lysozyme concentration affect the viability of C. albicans GDH 1878. There is also a time-concentration interaction, the exact nature of which cannot be established using these data.

2.3.2. The susceptibility of C. albicans GDH 1878 pre-incubated in YNB containing 500mM galactose, sucrose, glucose, maltose, xylitol and lactose, to 20 µg/ml lysozyme.

This experiment was carried out on 3 occasions using the sugars, glucose, galactose and sucrose in one set and maltose, xylitol and lactose in another set. The colony forming units per 50 microlitres (cfu/50µl) of C. albicans GDH 1878 pre-incubated in these sugars and then exposed to 20 µg/ml of lysozyme are shown in Tables 2.8 to 2.15. The corrected mean values (cfu/50µl) obtained for glucose over the 3 experiments show consistent results i.e., 231, 228 and 220 whereas the corrected mean values obtained for galactose and sucrose are more variable (Tables 2.8. to 2.10.). When the corrected mean values obtained for the three sugars relative to the control (50mM glucose) were compared it is evident that yeast cultured in sucrose and galactose before exposure to lysozyme are higher i.e., are more resistant to lysozyme than those cultured in 500mM glucose. The results are summarised in Table 2.11.

The results obtained for the 3 sugars maltose, xylitol and lactose are shown in Tables 2.12. to 2.15. The mean values obtained for maltose and xylitol are fairly similar except for lactose which

	Glucose (500mM)	Galactose (500mM)	Sucrose (500mM)	Control (50mM)
Plate 1	225	279	283	152
Plate 2	232	295	268	176
Plate 3	212	272	298	179
MEAN	223	282	283	169
C.M.*	231	305	302	169
Ratio of C.M./Control	1.37	1.80	1.78	1

Table 2.8. The number of cfu/50µl of C. albicans GDH 1878 (pre-incubated in glucose, galactose and sucrose) after exposure to 20µg/ml of lysozyme. Experiment 1.

*C.M. = Corrected mean (see Section 2.2.10.).

	Glucose (500mM)	Galactose (500mM)	Sucrose (500mM)	Control (50mM)
Plate 1	260	461	455	198
Plate 2	254	426	437	186
Plate 3	230	412	431	192
MEAN	248	433	441	192
C.M.*	228	410	412	192
Ratio of C.M./Control	1.19	2.13	2.14	1

Table 2.9. The number of cfu/50µl of C. albicans GDH 1878 (pre-incubated in glucose, galactose and sucrose) after exposure to 20µg/ml of lysozyme. Experiment 2.

*C.M. = Corrected mean (see Section 2.2.10.).

	Glucose (500mM)	Galactose (500mM)	Sucrose (500mM)	Control (50mM)
Plate 1	252	422	412	168
Plate 2	238	409	402	171
Plate 3	230	408	410	198
MEAN	240	413	408	179
C.M.*	220	390	375	179
Ratio of C.M./Control	1.23	2.17	2.09	1

Table 2.10. The number of cfu/50µl of *C. albicans* GDH 1878 (pre-incubated in glucose, galactose and sucrose) after exposure to 20 µg/ml of lysozyme. Experiment 3.

* C.M. = Corrected mean (see Section 2.2.10.).

	Glucose (500mM)	Galactose (500mM)	Sucrose (500mM)
Mean Ratio of C.M./Control	1.31	2.07	2.07

Table 2.11. Mean ratio of the number of cfu/50µl of *C. albicans* GDH 1878 (pre-incubated in glucose, galactose and sucrose) after exposure to 20µg/ml of lysozyme (data from Tables 2.8, 2.9 and 2.10).

	Maltose (500mM)	Xylitol (500mM)	Lactose (500mM)	Control (50mM)
Plate 1	218	208	100	121
Plate 2	230	225	96	141
Plate 3	239	218	119	146
MEAN	229	217	105	136
C.M.*	215	239	92	136
Ratio of C.M./Control	1.58	1.75	0.67	1

Table 2.12. The number of cfu/50µl of C. albicans GDH 1878 (pre-incubated in maltose, xylitol and lactose) after exposure to 20µg/ml of lysozyme. Experiment 1.

* C.M. = Corrected mean (see Section 2.2.10.).

	Maltose (500mM)	Xylitol (500mM)	Lactose (500mM)	Control (50mM)
Plate 1	314	238	67	216
Plate 2	342	262	78	238
Plate 3	346	268	82	221
MEAN	334	256	76	225
C.M.*	294	230	73	225
Ratio of C.M./Control	1.30	1.02	0.32	1

Table 2.13. The number of cfu/50µl of C. albicans GDH 1878 (pre-incubated in maltose, xylitol and lactose) after exposure to 20µg/ml of lysozyme. Experiment 2.

*C.M. = Corrected mean (see Section 2.2.10.).

	Maltose (500mM)	Xylitol (500mM)	Lactose (500mM)	Control (50mM)
Plate 1	238	224	66	127
Plate 2	222	247	82	140
Plate 3	227	231	74	132
MEAN	229	234	74	133
C.M.*	212	244	70	133
Ratio of C.M./Control	1.59	1.84	0.05	1

Table 2.14. The number of cfu/50µl of C. albicans GDH 1878 (pre-incubated in maltose, xylitol and lactose) after exposure to 20µg/ml of lysozyme. Experiment 3.

* C.M. = Corrected mean (see Section 2.2.10.).

	Maltose (500mM)	Xylitol (500mM)	Lactose (500mM)
Mean Ratio of C.M./Control	1.62	1.49	0.55

Table 2.15. Mean ratio of the number of cfu/50 µl of C. albicans GDH 1878 (pre-incubated in maltose, xylitol and lactose) after exposure to 20 µg/ml of lysozyme (data from Tables 2.12, 2.13 and 2.14).

shows very low mean values. This observation indicates that while maltose and xylitol exhibit a fairly similiar susceptibility towards lysozyme, lactose grown Candida is most susceptible to the killing effect of lysozyme.

Overall the pre-incubation of C. albicans GDH 1878 in 500mM galactose, glucose, sucrose, maltose, lactose and xylitol alters the subsequent susceptibility to 20 µg/ml as shown in Figure 2.10. Yeasts that were cultured in sucrose and galactose before exposure to lysozyme appear to be more resistant to the killing effect than those cultured in maltose, xylitol and glucose. Lactose is remarkably ineffective in protecting the yeast from this enzyme.

2.3.3. Fungicidal effect of 20 µg/ml lysozyme on twelve Candida isolates pre-incubated in three different concentrations of sucrose (500mM; 125mM; and 0.03mM) in YNB medium.

The results of the effect of 20 µg/ml of lysozyme on 12 Candida isolates belonging to 4 different species (C. albicans, C. tropicalis, C. glabrata and C. krusei) pre-incubated in three different concentrations of sucrose are shown in Tables 2.16. to 2.31.

The results for the 3 C. albicans strains GDH 1878; GDH 1786 and GDH 0019 are shown in Tables 2.16 to 2.18. The standardised mean cfu/50µl for all 3 C. albicans strains decreases as the concentration of sucrose decreases (Table 2.19.). However, C. albicans GDH 0019 demonstrated a marked resistance to the killing

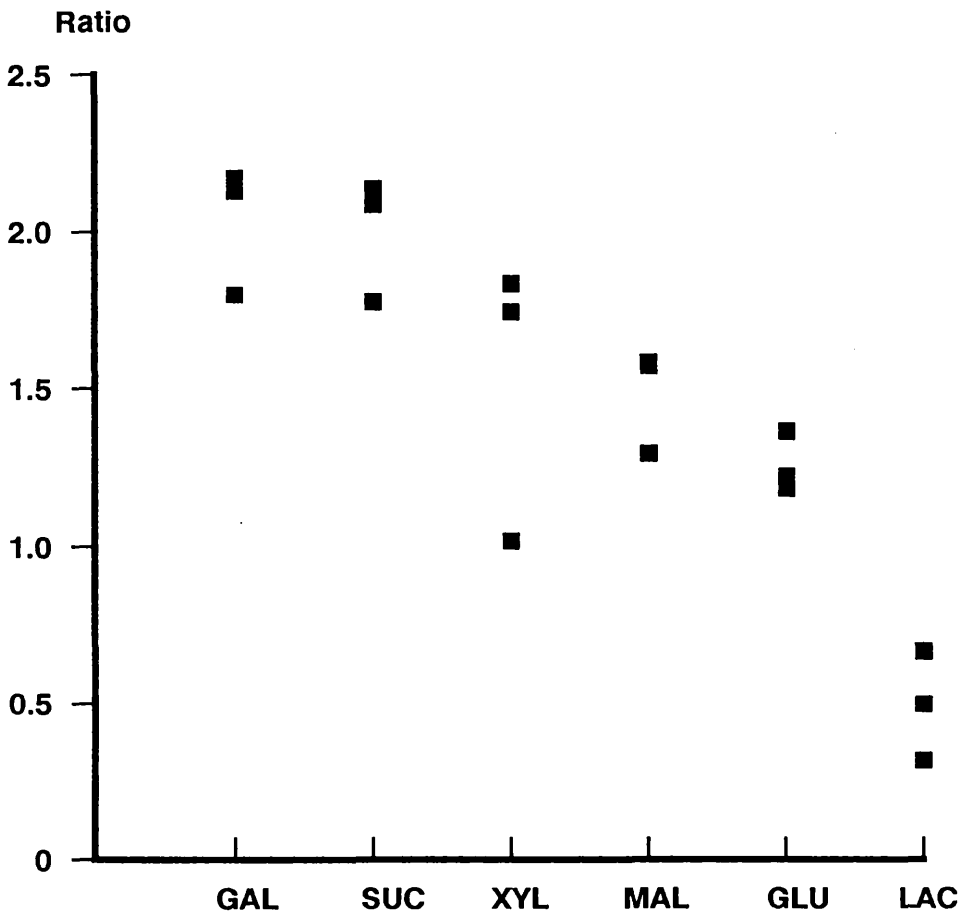

Figure 2.10 The ratio of the mean number of colony forming units per 50 µl of C. albicans GDH 1878 precultured in 500 mM galactose (GAL), sucrose (SUC), glucose (GLU), maltose (MAL), xylitol (XYL) and lactose (LAC) in YNB medium and exposed to 20 µg/ml lysozyme compared with controls containing 50 mM glucose (Results of 3 experiments).

C. albicans GDH 1878	Molarity of the sucrose solution 0.5M	0.125M	0.03M
		Experiment 1	
Number of yeasts			
Plate 1	364	210	68
Plate 2	380	280	50
Plate 3	336	280	59
Mean	360	256	59
S.M*	423	285	63
		Experiment 2	
Number of yeasts			
Plate 1	392	210	28
Plate 2	340	185	19
Plate 3	320	200	30
Mean	350	198	25
S.M	400	220	29
		Experiment 3	
Number of yeasts			
Plate 1	250	182	28
Plate 2	298	244	19
Plate 3	270	172	32
Mean	272	199	26
S.M	311	245	27

Table 2.16. The number of cfu/50µl, of C. albicans GDH 1878 pre-incubated in 0.5M, 0.125M and 0.03M sucrose/YNB for 24 hours and subsequently exposed to 20µg/ml lysozyme for 1 hour.

***S.M - Standardised mean (see Section 2.2.11.).**

C. albicans GDH 1786	Molarity of the sucrose solution 0.5M	0.125M	0.03M
		Experiment 1	
Number of yeasts			
Plate 1	188	42	33
Plate 2	168	24	30
Plate 3	168	48	30
Mean	174	38	31
S.M*	166	33	33
		Experiment 2	
Number of yeasts			
Plate 1	276	67	57
Plate 2	209	56	46
Plate 3	200	75	30
Mean	228	66	44
S.M	243	61	40
		Experiment 3	
Number of yeasts			
Plate 1	250	88	65
Plate 2	175	79	72
Plate 3	196	66	50
Mean	207	77	62
S.M	207	83	63

Table 2.17. The number of cfu/50µl, of C. albicans GDH 1786 pre-incubated in 0.5M, 0.125M and 0.03M sucrose/YNB for 24 hours and subsequently exposed to 20µg/ml lysozyme for 1 hour.

*S.M - Standardised mean (see Section 2.2.11.).

C. albicans GDH 0019	Molarity of the sucrose solution 0.5M	0.125M	0.03M
		Experiment 1	
Number of yeasts			
Plate 1	1770	1260	1170
Plate 2	1770	1560	960
Plate 3	1920	1740	1290
Mean	1820	1520	1140
S.M*	1693	1351	1013
		Experiment 2	
Number of yeasts			
Plate 1	2640	2070	1770
Plate 2	2400	2310	1860
Plate 3	2340	1950	1650
Mean	2460	2110	1760
S.M	2071	1985	1564
		Experiment 3	
Number of yeasts			
Plate 1	2340	1770	1560
Plate 2	2550	2160	1470
Plate 3	2400	1980	1140
Mean	2433	1970	1390
S.M	2162	1854	1293

Table 2.18. The number of cfu/50µl, of C. albicans GDH 0019 pre-incubated in 0.5M, 0.125M and 0.03M sucrose/YNB for 24 hours and subsequently exposed to 20µg/ml lysozyme for 1 hour.

*S.M - Standardised mean (see Section 2.2.11.).

	Molarity of the sucrose solution		
	0.5M	0.125M	0.03M
C. albicans GDH 1878	378*	250	40
C. albicans GDH 1786	205	59	43
C. albicans GDH 0019	1975	1729	1290
Mean of 3 strains	852	679	457

Table 2.19. Mean of standardised mean values of the 3 experiments for C. albicans

*Standardised mean (data from Tables 2.16. to 2.18.).

effect of lysozyme compared to the other 2 C. albicans strains.

The results for the 3 C. tropicalis strains GDH 1009; GDH 0998 and GDH 0057 are shown in Tables 2.20.to 2.22. and overall they tend to be consistent. The killing of all 3 strains by lysozyme increases as the concentration of sucrose falls although the difference between cells grown in 0.125M and 0.03M concentrations of sucrose are relatively small (Table 2.23.). However, of the three C. tropicalis strains tested, the mean cfu/50 μl obtained with C. tropicalis GDH 0057 was highest in all three concentrations of sucrose, suggesting greater resistance to the antifungal activity of lysozyme. An increase kill of about 50% occurred in cells grown in 0.03M compared with 0.5M sucrose (see Table 2.23).

The results for the three C. glabrata strains GDH 1337; GDH 1397 and GDH 0081 are shown in Tables 2.24. to 2.26. The results for the 3 strains of C. glabrata are clearly different from each other. C. glabrata GDH 1397 demonstrates a small decrease in yeast numbers with decreasing concentrations of sucrose, while C. glabrata GDH 0081, is uniformly resistant to lysozyme when grown in different concentrations of sucrose. On the contrary C. glabrata GDH 1337 strain shows an increase in viability with decreasing concentration of sucrose (the opposite result from that expected) Table 2.27.

The results for the three C. krusei strains NCPF 3165; GDH 1742 and GDH 1331 are shown in Tables 2.28. to 2.30. Examining the results of C. krusei NCPF 3165 (Table 2.28.) it is evident that this

C. tropicalis GDH 1009	Molarity of the sucrose solution 0.5M	0.125M	0.03M
		Experiment 1	
Number of yeasts			
Plate 1	44	25	18
Plate 2	46	31	13
Plate 3	35	22	15
Mean	41	26	15
S.M*	47	27	17
		Experiment 2	
Number of yeasts			
Plate 1	62	48	25
Plate 2	65	40	35
Plate 3	50	37	25
Mean	59	41	28
S.M	62	41	29
		Experiment 3	
Number of yeasts			
Plate 1	75	60	30
Plate 2	80	42	22
Plate 3	78	40	19
Mean	77	47	23
S.M	77	44	24

Table 2.20. The number of cfu/50µl, of C. tropicalis GDH 1009 pre-incubated in 0.5M, 0.125M and 0.03M sucrose/YNB for 24 hours and subsequently exposed to 20µg/ml lysozyme for 1 hour.

*S.M - Standardised mean (see Section 2.2.11.).

C. tropicalis GDH 0998	Molarity of the sucrose solution 0.5M	0.125M	0.03M
		Experiment 1	
Number of yeasts			
Plate 1	39	29	30
Plate 2	42	27	21
Plate 3	35	24	15
Mean	38	26	22
S.M*	38	28	23
		Experiment 2	
Number of yeasts			
Plate 1	40	32	20
Plate 2	45	28	25
Plate 3	50	31	18
Mean	45	30	21
S.M	51	31	22
		Experiment 3	
Number of yeasts			
Plate 1	38	21	14
Plate 2	43	18	25
Plate 3	45	28	21
Mean	42	22	20
S.M	42	22	22

Table 2.21. The number of cfu/50µl, of C. tropicalis GDH 0998 pre-incubated in 0.5M, 0.125M and 0.03M sucrose/YNB for 24 hours and subsequently exposed to 20 µg/ml lysozyme for 1 hour.

*S.M - Standardised mean (see Section 2.2.11.).

C. tropicalis GDH 0057	Molarity of the sucrose solution 0.5M	0.125M	0.03M
		Experiment 1	
Number of yeasts			
Plate 1	140	116	80
Plate 2	176	120	60
Plate 3	140	100	84
Mean	152	112	74
S.M.*	152	112	75
		Experiment 2	
Number of yeasts			
Plate 1	120	88	68
Plate 2	140	76	72
Plate 3	148	80	48
Mean	136	81	62
S.M	120	76	61
		Experiment 3	
Number of yeasts			
Plate 1	180	112	76
Plate 2	156	120	100
Plate 3	200	140	80
Mean	178	124	85
S.M	168	124	87

Table 2.22. The number of cfu/50µl, of *C. tropicalis* GDH 0057 pre-incubated in 0.5M, 0.125M and 0.03M sucrose/YNB for 24 hours and subsequently exposed to 20µg/ml lysozyme for 1 hour.

*S.M - Standardised mean (see Section 2.2.11.).

	Molarity of the sucrose solution		
	0.5M	0.125M	0.03M
C. tropicalis GDH 1009	62*	37	23
C. tropicalis GDH 0998	44	27	22
C. tropicalis GDH 0057	147	104	74
Mean of 3 strains	84	56	39

Table 2.23. Mean of Standardised mean values of the 3 experiments for C. tropicalis.

* Standardised mean (data from Tables 2.19. to 2.21.).

C. glabrata GDH 1337	Molarity of the sucrose solution 0.5M	0.125M	0.03M
		Experiment 1	
Number of yeasts			
Plate 1	40	939	1181
Plate 2	53	1060	1242
Plate 3	60	1121	1424
Mean	51	1040	1282
S.M*	52	1094	1314
		Experiment 2	
Number of yeasts			
Plate 1	20	808	1040
Plate 2	18	784	896
Plate 3	25	768	960
Mean	21	786	965
S.M	21	786	965
		Experiment 3	
Number of yeasts			
Plate 1	19	464	768
Plate 2	20	416	808
Plate 3	15	360	896
Mean	18	413	824
S.M	18	389	803

Table 2.24. The number of cfu/50µl, of C. glabrata GDH 1337 pre-incubated in 0.5M, 0.125M and 0.03M sucrose/YNB for 24 hours and subsequently exposed to 20µg/ml lysozyme for 1 hour.

*S.M - Standardised mean (see Section 2.2.11.).

C. glabrata GDH 1397	Molartiy of the sucrose solution 0.5M	0.125M	0.03M
		Experiment 1	
Number of yeasts			
Plate 1	4933	7066	6800
Plate 2	6666	5066	5333
Plate 3	5200	5333	3733
Mean	5600	5813	4960
S.M*	5600	5407	5087
		Experiment 2	
Number of yeasts			
Plate 1	6533	4133	6800
Plate 2	5200	5066	4933
Plate 3	6000	6133	5333
Mean	5911	5106	5680
S.M	5254	4750	5163
		Experiment 3	
Number of yeasts			
Plate 1	7733	6933	5600
Plate 2	8666	6533	7200
Plate 3	6800	5333	6000
Mean	7733	6266	6266
S.M	7278	6113	5696

Table 2.25. The number of cfu/50µl, of *C. glabrata* GDH 1397 pre-incubated in 0.5M, 0.125M and 0.03M sucrose/YNB for 24 hours and subsequently exposed to 20µg/ml lysozyme for 1 hour.

*S.M - Standardised mean (see Section 2.2.11.).

C. glabrata GDH 0081	Molarity of the sucrose solution 0.5M	0.125M	0.03M
		Experiment 1	
Number of yeasts			
Plate 1	5066	5333	4266
Plate 2	4800	4266	5466
Plate 3	4933	4666	4533
Mean	4933	4754	4754
S.M*	4933	5004	5282
		Experiment 2	
Number of yeasts			
Plate 1	5600	5066	5733
Plate 2	4933	5466	4266
Plate 3	4000	5066	4800
Mean	4844	5200	4933
S.M	5382	5200	5192
		Experiment 3	
Number of yeasts			
Plate 1	4266	4800	5333
Plate 2	5333	5466	4266
Plate 3	5466	5066	4800
Mean	5021	5110	4800
S.M	5150	4810	5052

Table 2.26. The number of cfu/50µl, of C. glabrata GDH 0081 pre-incubated in 0.5M, 0.125M and 0.03M sucrose/YNB for 24 hours and subsequently exposed to 20µg/ml lysozyme for 1 hour.

*S.M - Standardised mean (see Section 2.2.11.).

	Molarity of the sucrose solution		
	0.5M	0.125M	0.03M
C. glabrata GDH 1397	6044*	5423	5315
C. glabrata GDH 1337	30	756	1028
C. glabrata GDH 0081	5155	5004	5176
Mean of 3 strains	3743	3727	3839

Table 2.27. Mean of Standardised mean values of the 3 experiments for C. glabrata.

* Standardisd mean (data from Tables 2.22. to 2.24.).

C. krusei NCPF 3165	Molarity of the sucrose solution 0.5M	0.125M	0.03
		Experiment 1	
Number of yeasts			
Plate 1	0	0	8
Plate 2	0	1	17
Plate 3	1	1	21
Mean	0.3	0.6	15
		Experiment 2	
Number of yeasts			
Plate 1	1	4	24
Plate 2	1	6	26
Plate 3	0	8	27
Mean	0.6	6	25
S.V	0.5	6	25
		Experiment 3	
Number of yeasts			
Plate 1	2	3	15
Plate 2	1	2	16
Plate 3	1	2	21
Mean	1	2	17
S.M	1	2	16

Table 2.28. The number of cfu/50µl, of C krusei NCPF 1365 pre-incubated in 0.5M, 0.125M and 0.03M sucrose/YNB for 24 hours and subsequently exposed to 20µg/ml lysozyme for 1 hour.

*S.M -Standardised mean (see Section 2.2.11.).

C. krusei GDH 1742	Molarity of the sucrose solution 0.5M	0.125M	0.03M
		Experiment 1	
Number of yeasts			
Plate 1	14	50	576
Plate 2	10	45	536
Plate 3	12	41	600
Mean	12	45	570
S.M*	12	45	507
		Experiment 2	
Number of yeasts			
Plate 1	20	30	328
Plate 2	20	37	344
Plate 3	18	29	392
Mean	19	32	354
S.M	19	28	308
		Experiment 3	
Number of yeasts			
Plate 1	15	30	380
Plate 2	14	28	444
Plate 3	20	32	460
Mean	19	30	428
S.M	18	27	398

Table 2.29. The number of cfu/50µl, of *C. krusei* GDH 1742 pre-incubated in 0.5M, 0.125M and 0.03M sucrose/YNB for 24 hours and subsequently exposed to 20µg/ml lysozyme for 1 hour.

*S.M - Standardised mean (see section 2.2.11.).

C. krusei GDH 1331	Molarity of the sucrose solution 0.5M	0.125M	0.03M
		Experiment 1	
Number of yeasts			
Plate 1	60	64	272
Plate 2	68	72	288
Plate 3	64	64	272
Mean	64	66	273
S.M*	56	59	316
		Experiment 2	
Number of yeasts			
Plate 1	10	56	400
Plate 2	35	76	440
Plate 3	24	64	432
Mean	23	65	424
S.M	23	62	376
		Experiment 3	
Number of yeasts			
Plate 1	72	172	328
Plate 2	84	132	360
Plate 3	44	144	304
Mean	66	149	330
S.M	62	149	306

Table 2.30. The number of cfu/50µl, of *C. krusei* GDH 1331 pre-incubated in 0.5M, 0.125M and 0.03M sucrose/YNB for 24 hours and subsequently exposed to 20µg/ml lysozyme for 1 hour.

*S.M - Standardised mean (see Section 2.2.11.).

strain is susceptible to the killing effect of lysozyme when cultured in media containing 0.5M and 0.125M sucrose, while yeasts survive in greater numbers when grown in 0.03M sucrose. Overall the killing of this strain increases with increasing concentration of sucrose.

The results of the three experiments with C. krusei GDH 1742 show fairly similar results (Table 2.31.). These strains show little difference in viability when cultured in 0.5M or 0.125M sucrose but a marked increase in yeast numbers when cells grown in 0.03M sucrose. Overall the killing effect of these strains increase with increasing concentrations of sucrose, (Table 2.31.).

The mathematical model used to compare the 4 Candida species and the analysis of variance table based on the model are shown in Table 2.32. and Table 2.33. respectively. The results of the analysis of variance based on the mathematical model (Table 2.32.) demonstrate that in terms of fixed effects, sugar concentration has no significant effect ($P<0.31$) on the killing effect of lysozyme. There is also evidence of a significant ($P<0.03$) but not large differences among species and a more considerable sugar/species interaction ($P<0.002$). To elaborate on this result a graph of average log count of cfu/50µl across strains, experiments and replicates against log sucrose concentrations is given in Figure 2.11. It is clear from this that C. albicans and C. tropicalis were less sensitive to lysozyme as the concentration of sucrose in the preincubated YNB medium increases whereas the strains of C. glabrata and C. krusei were more sensitive to lysozyme as the concentration of the sugar increased.

	Molarity of the sucrose solution		
	0.5M	0.125M	0.03M
C. krusei NCPF 3165	1*	3	18
C. krusei GDH 1742	16	20	404
C. krusei NCPF 1331	47	90	333
Mean of 3 strains	21	37	251

Table 2.31. Mean of Standardised mean values of the 3 experiments for C. krusei.

* Standardised mean (data from Tables 2.25. to 2.27.).

The mathematical form of the model:

$$X_{ijhlm} = u + B_i + (BC)_{ij} + C_j + S_{k(i)} + (SC)_{jh(i)} + E_{e(ih)} + (EC)_{je(ih)} + R_{ijhlm}$$

for the usual restrictions on B_i etc.,

ie., $B_i = 0$ etc.,

and the variance terms being independent Normal components

ie., $S_{h(i)}$ N (0, $_{strains}$) etc.

Here X_{ijhlm} is the Log(count + 1/2) of the i^{th} Yeast (species) on the j^{th} Concentration of Sugar for the h^{th} Strain on the l^{th} Experiment and the m^{th} Replicate.

The logarithm of the count (plus a half) was taken as the appropriate measurement for the model basically to stabilise the variance.

Table 2.32. Details of mathematical model used to compare 4 Candida species.

Source	Error Term	Degrees of Freedom	Mean Square	F	p-value
Sugar/(S)	S/ST(SP)	2	5.28	1.26	0.31
Species/(SP)	ST(SP)	3	295.70	4.84	0.03
Strains within species(ST(SP))	E(ST(SP))	8	61.09	107.00	<0.0001
Sugar/Species Interaction (S/SP)	S/ST(SP)	6	24.84	5.95	0.002
Exeriments within strains within species E(ST(SP))	R	24	0.57	13.49	<0.0001
Sugar/Strain Interaction within species S/ST(SP)	S/E(ST(SP))	16	4.18	22.15	<0.0001
Sugar/Experiment Interaction within strains within species S/E(ST(SP))	R	48	0.189	4.45	<0.0001
Replicates within everything (R)		216	0.0423		

Table 2.33. Analysis of Variance Table (on log scale) based on the mathematical model given in Table 2.32.

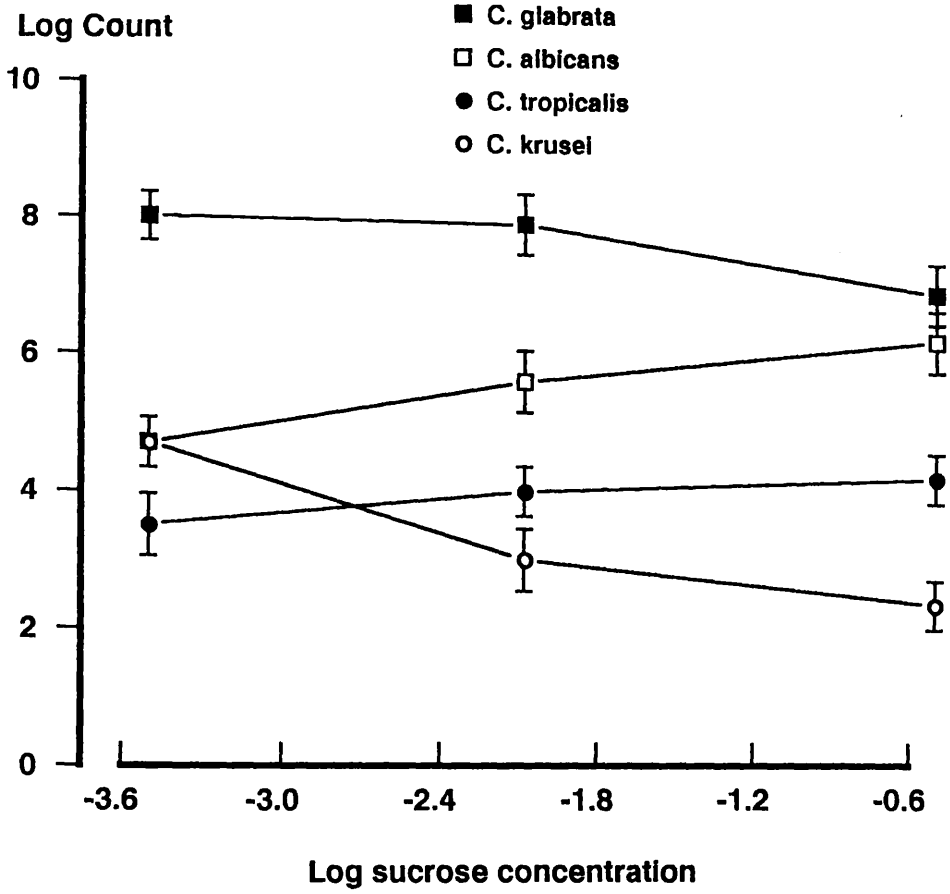

Figure 2.11. The average log counts of the colony forming units per ml of Candida species across strains, experiments and replicates cultured in 0.5M, 0.125M and 0.03M sucrose and exposed to 20 μg/ml lysozyme. Errors indicate range of values.

Table 2.34. shows the estimates of the components of variability and it is clear that the major source of variability is that among strains. The other two components (i.e., experiments within strains and replicates within experiments within strains) are of a similar magnitude to each other but substantially less than that due to variability among strians.

Component	SD
Strains within species	1.64
Experiments within strains within species	0.33
Replicates within experiments within strains within species	0.21

Table 2.34. Components of Variance (on log scale). derived from Table 2.33.

SD = Standard deviation

2.4. DISCUSSION

2.4.1. Experiment 1

The first series of experiments reported in this Chapter, with a single isolate of C. albicans GDH 1878 isolated from the saliva indicated that sensitivity to lysozyme increases in a concentration and time-dependent manner. These results are similar to those obtained by Tobgi et al. (1987) who conducted similar experiments with a single isolate of C. albicans (GDH 1957) obtained from the oral cavity of a patient with chronic atrophic candidosis. The range of the concentration of lysozyme used in the present study was 20 to 500 µg/ml. However, in the experiment carried out by Tobgi et al. (1987) a wider range of lysozyme concentrations (0.5 to 1000 µg/ml) were used. In both these experiments the yeast suspensions were prepared in distilled water, and the susceptibility to the enzyme studied after a 5 hour exposure to the enzyme. The results of the above studies contradict those of Kamaya (1970) who found that it was necessary to supplement the assay medium (water) with 0.1% (5.5 mmol) glucose to demonstrate the candidacidal activity of 5 clinical isolates of C. albicans. Furthermore, Collins and Papagianis (1974) in a later study failed to demonstrate the growth inhibition of three isolates of C. albicans by lysozyme without the addition of 0.03 µg Amphotericin B per ml. Both Kamaya (1970) and Collins and Papagianis (1974) used distilled water suspensions of yeast and hen egg white lysozyme at concentrations > 2000 µg/ml. There are a number of reasons which may explain the differences between the results of the

present study and those of other workers. For example, the strains of C. albicans used in the different studies were not the same. Another variable is the quality of the lysozyme used, as the source of lysozyme varied in different experiments. Thus while Kamaya (1970) obtained the enzyme from Nutritional Biochemicals in U.S.A., and Collins and Papagianis (1974) obtained it from Sigma, U.S.A. In the current study and that of Tobgi et al. (1987), the source was also Sigma Chemical Co., St. Loius, MO. In addition to these differences the concentrations of the enzyme used by different workers also varied.

Tobgi (1990) also studied the lytic activity of 2 µg/ml of lysozyme on 22 different C. albicans isolates which comprised 13 oral, 8 vaginal and 1 skin isolate. The susceptibility of C. albicans strains was calculated by using the following formula: Fly = - log(CFU test suspension/CFU control suspension (where Fly = Fungicidal activity of lysozyme, and CFU = Colony forming units). The higher the Fly value the more susceptible the isolate is to lysozyme. The results indicated a wide variation in the susceptibility of C. albicans strains to lysozyme with Fly values ranging from 1.1 to 5.2 among the 22 C. albicans strains tested (Tobgi, 1990). C. albicans GDH 1878 was tested by Tobgi, and had a Fly value of 2.4 when tested with lysozyme at a concentration of 2 µg/ml and a Fly value of 3.10 when tested with 20 µg/ml lysozyme. In this study the same strain (GDH 1878) had a Fly value of 2.88 when tested against a 20 µg/ml of lysozyme. This slight difference in the results of the two studies could be due to the reasons given earlier in this section. The

majority of the strains tested by Tobgi (1990) demonstrated values between 2 to 3 and this wide range of the Fly values strongly suggest that differences in susceptibility to lysozyme exist among the different strains within a given species. Hence the results of experiments conducted with a single isolate of Candida should be interpreted with caution.

It is interesting that Tobgi (1990) reported a dramatic reduction in the anti-candidal activity of lysozyme, when experiments were performed in phosphate-buffered saline compared with distilled water. This may be due to the presence of inorganic salts in the buffer which adsorb to the highly cationic lysozyme and thus renders them less active (Collins and Pappagianis, 1974). Since saliva too contains a number of inorganic ions e.g., Na^+, K^+, PO_4^{--}, H^+, and HCO_3^{--}, these might interact with lysozyme in vivo. However, there is no data in the literature on the effect of saliva on the anticandidal activity of lysozyme and further work is required to ascertain the effect of the ionic constituents of saliva on this enzyme.

Although there is relatively little information in the literature in relation to lysozyme and Candida interactions, studies of strain variations in the susceptibility of bacteria such as S. mutans to lysozyme have been demonstrated (Iacono et al., 1980). It is thought that extracellular polysaccharide synthesised by the bacteria may play a role in this variation. The strain variation in susceptibility of C. albicans isolates may also be explained in similar terms as C. albicans is known to elaborate an outer surface

layer depending on the identity and concentration of carbohydrate in the growth media (Douglas et al., 1981; Poulain et al., 1978), the age of the culture (Pugh and Cawson, 1978) and the source of isolation, for example symptomatic and asymptomatic carriers of Candida (McCourtie and Douglas, 1984). The next part of the current study was therefore devoted to investigating the effect of the growth medium on the subsequent susceptibility of C. albicans GDH 1878 to lysozyme.

2.4.2. Experiment 2

This series of experiments were carried out by culturing the yeasts for 24 hours in YNB with one of 6 different sugars, harvesting them and subsequently exposing the Candida to the effect of a standard concentration of lysozyme (20 µg/ml). A concentration of 20 µg/ml of lysozyme was chosen to represent the physiological level of lysozyme in the oral cavity since it is known that the concentration of this enzyme varies from 1 to 57 µg/ml saliva in vivo (Stuchel and Mandel, 1983)

The results of this series of experiments showed that C. albicans GDH 1878 pre-cultured in lactose is most susceptible when subsequently exposed to lysozyme, while growth in YNB plus glucose, maltose, xylitol, sucrose or galactose resulted in an increasing resistance to the antifungal effect of lysozyme in that particular order.

A possible explanation for this increased resistance to lysozyme may be related to the inability of lysozyme to gain access to the peptidoglycan of the cell wall or to other cell structures (such as the cytoplasmic membrane and components of an autolytic enzyme system) due to the presence of extracellular manno-protein, known to be produced by the yeast when grown in the presence of high concentrations of sugars such as galactose and sucrose (McCourtie & Douglas 1981; 1984).

There are studies in the literature which indicate that the amount of extracellular polymeric material formed by C. albicans may reduce susceptibility to antifungal agents. For example McCourtie and Douglas, (1984) found that C. albicans cells pre-incubated in galactose were significantly more resistant to spheroplast formation than cells incubated in glucose when subsequently exposed to the cytolytic enzyme Zymolyase-5000. They postulated that the extracellular polymeric material acts as a physical barrier and prevents the accessibility of Zymolyase-5000. This polymeric barrier might block the cell wall target site for the enzyme or it may prevent it acting on the cell membrane, as suggested by Marquis et al. (1982). One or more of these mechanisms may be operating in the galactose and sucrose mediated resistance to lysozyme activity observed in the current study. McCourtie and Douglas (1985) have characterised the extracellular polymeric material which is produced when C. albicans is grown in media containing 50mM glucose, 500mM galactose and 500mM sucrose as a mannoprotein. The main constituents of the latter are as follows; carbohydrates (65-82%),

proteins (7%), phosphorous (0.5%) and glucosamine (1.5%). C. albicans strains cultured in 500mM galactose produce maximal extracellular material followed by sucrose, maltose, glucose, fructose and 50mM glucose (McCourtie & Douglas, 1981). The results obtained in the present study seem to confirm these observations as galactose and sucrose pre-incubated C. albicans were the most resistant to lysozyme followed by maltose, xylitol, glucose and lactose; the latter being least resistant.

The current results also imply that in vivo in the presence of excess dietary sugars such as galactose (found primarily as a component of lactose, a disaccharide present in the milk of mammals, (Cole and Eastoe, 1988) C. albicans may elaborate extracellular mannoproteins which may protect the yeasts from the fungicidal activity of salivary lysozyme. It is known that this extracellular material enhances the adhesion of Candida to epithelial and acrylic denture surfaces (Samaranayake and MacFarlane, 1982a; 1980; Douglas 1987) In addition, dietary carbohydrates are a ready source of nutrients for candidal proliferation in saliva (Knight and Fletcher, 1971). Indeed there are a number of studies which appear to relate the initiation and aggravation of oral candidosis to a high carbohydrate diet (Neil, 1965; Gentles and La Touche, 1969; Ritchie et al. 1969; Knight and Fletcher, 1971 and Zgraggen and Graf, 1975). The possible effect of dietary sugars on oral candidosis is shown in Figure 2.12, and it is clear that carbohydrates in general may promote oral candidosis by a number of mechanisms. Not only does the current studies tend to support this view but suggests another mechanism by which this process may occur.

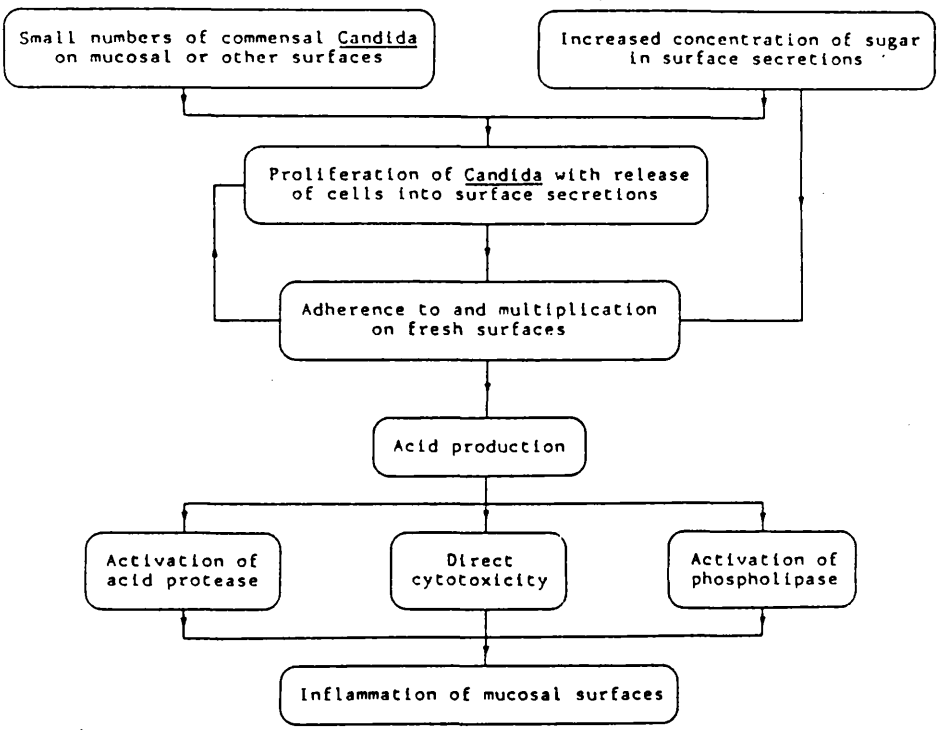

Figure 2.12. The possible effects of dietary sugars on oral candidosis (Adapted from Samaranayake and MacFarlane, 1985).

2.4.3. Experiment 3

While the above studies demonstrate the effect of dietary sugars on a single species of Candida it is not known whether other Candida species behave similarly on exposure to dietary sugars. Hence a third series of experiments were carried out to verify the effect of varying concentrations of a common dietary carbohydrate (sucrose) on 4 different Candida species, namely C. albicans, C. tropicalis, C. glabrata and C. krusei. These organisms were pre-cultured in (0.5M; 0.125M and 0.03M) sucrose before exposure to (20 µg/ml) lysozyme.

From these studies it would appear that C. albicans and C. tropicalis were more resistant to the killing effect of lysozyme when pre-incubated with sucrose when compared to C. glabrata and C. krusei; C. albicans and C. krusei being the least and the most sensitive, respectively.

Using a growth inhibition assay, similar to that used in Experiment 1 of the present study Tobgi et al. (1987) studied the in vitro sensitivity of 6 Candida species precultured in sabouraud's dextrose agar to 2 µg/ml lysozyme and found that there were differences in susceptibility to lysozyme among the species. They found that C. glabrata was most resistant to lysozyme followed by C. albicans, C. guilliermondii, C. tropicalis, C. parapsilosis and C. krusei (most sensitive). The latter rank order of the susceptibility of Candida species to lysozyme is dissimilar to the present results although in both studies C. krusei was found to be the most sensitive

species to lysozyme. These differences could be due to a number of factors e.g., the growth medium used (SDA supplemented with 4% glucose compared with YNB with 3.6% glucose); concentration of lysozyme (2 µg/ml compared with 20 µg/ml); and also differences in strains used by Tobgi et al. (1987) and the present study.

From the current studies and those of Tobgi et al. (1987) it is clear that there are distinct variations in the susceptibility of different Candida species and different strains within a species to lysozyme. This may reflect 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 general, when the yeasts were preincubated in a range of concentrations of sucrose, C. albicans and C. tropicalis demonstrated increased resistance to lysozyme while C. glabrata and C. krusei showed decreased resistance. This indicates that the mechanism of lysozyme resistance of C. tropicalis may be similar to that of C. albicans as discussed in Section 2.4.2. Thus it is possible that they too produce extracellular polysaccharides on exposure to sucrose. However, further work is required to clarify this postulate.

On the other hand C. glabrata and C. krusei overall show increased susceptibility to lysozyme when preincubated in increasing

concentrations of sucrose and it is difficult to offer a reason for this phenomenon. As there are no similar studies in the literature, the present results need to be substantiated by further experiments, ideally using a number of isolates from each of the above Candida species.

The above interactions might have an effect on Candida populations in the oral cavity. However, caution should be used in extrapolating these results to the in vivo situation as the diluent in the current experiments was distilled water. Saliva which contains a high concentration of inorganic ions (see Section 1.4.4.) may reduce the overall inhibitory potency of the enzyme (Collins and Papagianis, 1974) and this may be one method by which Candida evades the action of salivary lysozyme which can be present in concentrations ranging from 1 to 57 µg/ml., in mixed saliva (Stuchel and Mandel 1983).

The concentrations of salivary glucose is less than 1 mg/100ml, but this can increase slightly by some of the sugars released from salivary glycoproteins by the enzymatic action of bacteria (Nikiforuk, 1985b). However, higher salivary glucose concentrations up to 0.33mg/100ml can be found in diseased states such as diabetics compared with 0.21mg/100ml in healthy individuals (Thorstensson et al., 1989). Many workers have also reported an increased predisposition for candidal infections among diabetics (Knight and Fletcher, 1971; Harrison and Bowen, 1987; Lamey et al. 1988), although others have not been able to confirm this relationship

(Phelan and Stephan, 1986; Peters et al., 1966). It is possible that an increase in salivary glucose concentration may help Candida species to evade the action of lysozyme (see Section 1.5.6.). However, in the current study in which a number of sugars were compared for their inhibitory effect on the subsequent exposure of C. albicans to lysozyme, it was found that galactose, sucrose, and maltose were more effective in preventing the killing effect of lysozyme than glucose. As sucrose consumption in the daily food is greater than glucose it is possible that Candida may be protected by this sugar in the oral cavity.

In clinical terms, the differential susceptibility of Candida species to lysozyme in vitro may explain why some species such as C. albicans and C. tropicalis are more frequently isolated from the oral cavities of patients with normal salivary flow compared with others such as C krusei. On the other hand in patients with severe xerostomia due to Sjogren's syndrome, a variety of Candida species including C. krusei are not infrequently isolated (MacFarlane, 1990). It is possible that the higher prevalence of C. krusei in such patients is in part related to the virtual absence of the protective effect of lysozyme.

2.4.4. Conclusions

1. The viability of C. albicans GDH 1878 is inhibited in the presence of lysozyme. It is very clear that time and lysozyme concentration plays a dominant role in the killing effect of

this enzyme. Although there is a strong linear relationship between the Candida count and time, the relationship with lysozyme concentration is not as clear (see Figures 2.8 and 2.9).

2. C. albicans GDH 1878 when cultured in the presence of 500mM concentrations of various dietary carbohydrates such as sucrose, galactose, glucose, maltose, xylitol and lactose show varying degrees of susceptibility to the killing effect of 20 µg/ml of lysozyme. Galactose and sucrose produced the most protective effect while lactose produced the least protective effect.

3. The two Candida species C. albicans and C. tropicalis demonstrated increasing susceptibility to 20 µg/ml lysozyme when grown in the presence of decreasing concentrations of sucrose (0.5M, 0.125M and 0.03M) in YNB medium. However, C. glabrata and C. krusei were different to the other 2 Candida species and showed decreasing killing effect with increasing concentrations of sucrose in YNB medium.

CHAPTER 3

THE PROTEOLYTIC AND SACCHAROLYTIC ACTIVITY OF CANDIDA SPECIES IN SALIVA

3.1. INTRODUCTION

Although acidic proteinases of Candida species are believed to be involved in the pathogenesis of candidosis (see Section 1.5.4.) there is little information about the ability of Candida to break down salivary proteins. Germaine, Tellefson and Johnson (1978), first reported the growth and degradation of salivary proteins by C. albicans at a pH of 4. In their experiments, very slight proteolysis was seen at pH 5 and no degradation occurred at pH 6 or 7. Polyacrylamide gel electrophoresis profiles indicated no selectivity of the proteins utilized by Candida. However, subsequent in vitro experiments of salivary proteolysis carried out by Germaine and Tellefson, (1981) indicated that saliva was a potent inhibitorof candidal proteinase synthesis and therefore they concluded that Candida proteinase do not contribute to the virulence of the organism in vivo.

The proteolytic and glycolytic activity of C. albicans in glucose supplemented and glucose-free pooled human saliva were examined by Samaranayake et al.(1983a). These experiments demonstrated that glucose-free saliva did not support the growth of C. albicans. However, there was significant candidal growth in saliva samples supplemented with glucose and a sharp drop in the pH from 7.5

to 3.5 within 48 hours. Associated with the pH drop, was a significant quantitative reduction in salivary proteins which was confirmed by isoelectric focusing. While this appeared to be due to degradation by proteolytic enzymes, the possibility that some protein loss was due to adsorption to the surface of yeast cells could not be definitively excluded. The present study was performed to confirm the findings of Samaranayake et al. (1983a), clarify the reason for the quantitative loss of salivary proteins and to study the proteolytic and saccharolytic activity of species not previously studied, i.e., C. tropicalis, C. glabrata and C. krusei.

3.2. MATERIALS AND METHODS

3.2.1. Microorganisms

The following four Candida isolates were used in the experiments described in this chapter; C. albicans GDH 1878, C. tropicalis GDH 1009, C. glabrata GDH 1397 and C. krusei NCPF 3165. (see Table 2.1. for further details of these strains).

3.2.2. Identification and storage of Candida

The yeasts were identified and stored as described in Sections 2.2.2. and 2.2.3.

3.2.3. Preparation of Bovine Serum Albumin Agar, Napthalene black solution and Decolourizing solution for the plate test for proteinase production

see Appendix A.

3.2.4. Plate test for proteinase production using bovine serum albumin

The experimental procedure described by Staib (1965) was used for testing proteinase production by Candida species. All 12 strainsof Candida mentioned in Table 2.1. were grown on Sabouraud's dextrose agar plates at 37^0C for 24 hours in an aerobic incubator (Gallenkamp U.K.). The yeast cells were washed and harvested in

sterile distilled water by centrifuging at 1500 rpm for 10 minutes (MSE Super Minor Centrifuge). From the yeast pellet thus obtained a yeast suspension of $1x10^6$ cells/ml was prepared by haemocytometer counting (Improved Neaubauer Haemocytometer Chamber) as described in Section 2.2.6. Using a sterile Eppendorf pipette 10 µl of this yeast suspension was inoculated onto a bovine serum albumin agar plate (see Appendix A). The plates were incubated aerobically in a humidified container for five days at 37°C (Gallenkamp U.K.), then flooded with 1.25% naphthalene black solution in 90% methanol/water (v/v), for 15 minutes. Decolorization was performed for one and a half days using several changes of the decolorizing solution, and examined for the formation of a translucent zone around the colony, (Figure 3.1.).

All strains of C. albicans (4 strains) and C. tropicalis (4 strains) tested, produced translucent haloes around the colonies after incubation for 5 days at 37°C. In comparison all strains of C. glabrata (4 strains) and C. krusei (4 strains) tested did not produce any translucency around the colonies. Although the latter Candida species did not degrade BSA it was decided to screen all 4 species for salivary protein degradation. Therefore one strain each of C. albicans GDH 1878; C. tropicalis GDH 1009; C. glabrata GDH 1397 and C. krusei NCPF 3165 was chosen randomly for these studies (see Table 2.1).

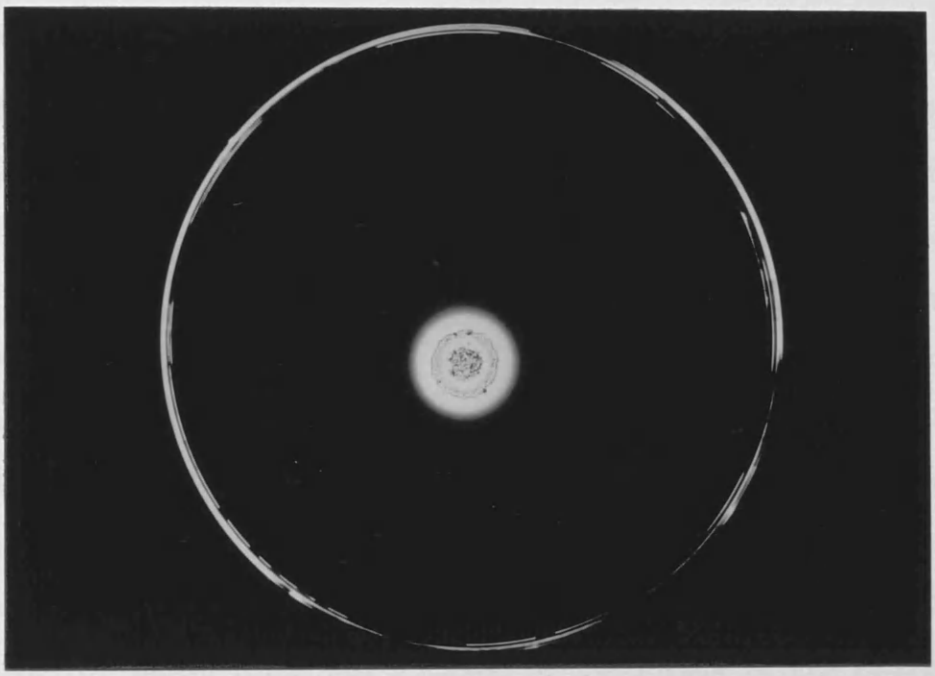

Figure 3.1. Bovine serum albumin plate inoculated with C. albicans GDH 1878 for 5 days then decolorized with 1.25% Naphthalene black showing the translucent halo around the colony indicating proteolysis.

3.2.5. Parotid saliva

Parotid saliva was collected by placing a modified Carlson-Crittenden Cup (Carlson and Crittenden, 1915, modified by Mason et al. (1966) over the orifice of Stenson's duct (Figure 3.2.). This cup was obtained from Mr. N. L. Harvey, Lochgilphead, Scotland). The Carlson-Crittenden cup is made from teflon and stainless steel and is sterilized by autoclaving. Three healthy young individuals, (1 female, 2 males) volunteered for saliva collection. Saliva was always collected between 10 a.m. and 12 noon. The Carlson-Crittenden cup was first placed over the parotid orifice, then the small rubber bulb which is connected to the outer circle of the Carlson-Crittenden Cup was squeezed to exhaust air from the outer ring, which draws and holds the collector in place. Using a sterile plastic disposable pipette 4ml of lemon juice was applied to the dorsum of the tongue. The saliva collected during the first 2 minutes of stimulation was discarded, since it is known that variation in the composition of saliva occurs during the early period of stimulation (Dawes, 1967). A 10ml volume of stimulated parotid saliva was collected over ice from each individual in a sterile plastic universal container. The saliva samples from the three volunteers were then pooled and the mixture filtered through a 0.2μm Minisart filter (Satorius, Surrey, England). The sterility of the pooled saliva was checked by plating a loopful of the saliva onto Sabouraud's dextrose agar and Blood agar plates (Gibco Limited., Paisley, Scotland), (see Appendix A for the preparation of Sabouraud's dextrose agar and Blood agar plates). These plates were incubated aerobically at 37°C for 24 hours. The saliva samples were

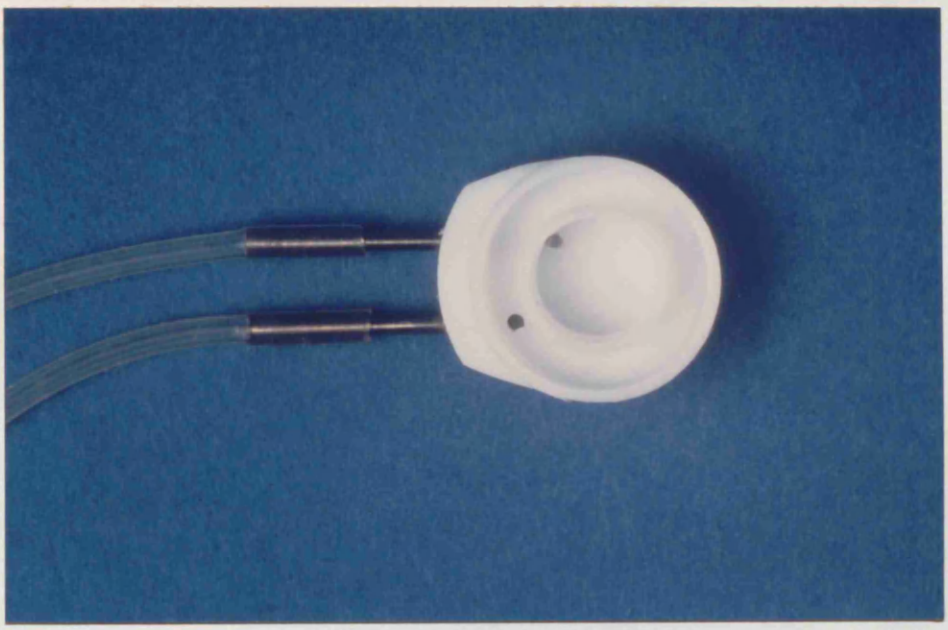

Figure 3.2. Modified Carlson-Crittenden cup (Mason et al., 1966).

stored at -20°C as soon as possible to minimize post-collection modification of salivary proteins and, used within 24 hours.

3.2.6. Whole saliva

Unstimulated whole saliva was collected in plastic universal containers over ice, by expectoration. Ten millilitres of whole saliva from each of four young donors (2 females, 2 males) were collected for use in the experiments. These 4 donors were different from those who donated parotid saliva. Whole saliva was collected between 10.00 a.m and 12 noon, and samples were pooled and centrifuged at 15,000 rpm (MSE High Speed 18; Fisons, Crawley, England) for 15 minutes at 4°C. The supernatant was then passed through a 0.2μm Minisart filter (Satorius, Surrey, England). A volume of 1ml of sterile 1M glucose (see Appendix A) was added to 4ml of sterile saliva to give a final concentration of 0.2M glucose in the saliva sample. Using a sterile 1ml pipette 0.05ml of streptomycin/ penicillin solution (10,000 μg/ml and 10,000 units/ml, respectively), (Gibco; Scotland) were added to inhibit growth of any bacteria which remained in the mixed salivary samples. The sterility of saliva was checked by streaking a loopful of the sample onto SDA and BA (Gibco Limited., Paisley, Scotland), and the plates were incubated at 37°C for 48 hours (Gallenkamp U.K.). The saliva samples were stored at -20°C for up to 24 hours. After thawing the saliva samples to room temperature, the experiments described in Sections 3.2.12., 3.2.18 and 3.2.19. were carried out.

3.2.7. Preparation of yeast inoculum for saliva

The yeast strains under investigation were grown on Sabouraud's dextrose agar (SDA; Gibco Limited, Paisley, Scotland), in an aerobic incubator (Gallenkamp U.K.) at 37°C for 24 hours. A loopful of the growth was added to 10ml of sterile distilled water and 0.2ml of this suspension was added to 20ml of 200mM glucose in YNB liquid medium (see Appendix A). The culture was grown aerobically in an orbital incubator (100 rpm) (Gallenkamp Orbital Incubator) at 37°C for 24 hours. The cells were harvested and washed once in sterile distilled water by centrifuging at 1500g, for 10 minutes, (MSE Super Minor Centrifuge). The washed yeast pellet was then used as the inoculum.

3.2.8. Preparation of killed yeasts

In some experiments dead yeast cells were used as controls. The yeast under test was cultured aerobically on Sabouraud's dextrose agar for 18 hours at 37°C (Gallenkamp U.K.), and a loopful of the resultant growth suspended in 5ml of sterile distilled water. The bottles were immersed in boiling water for twenty minutes, then left to cool. After the cells naturally settled in the bottom of the bottles, the supernatant was discarded and the dead cells used in the experiments. The viability and sterility of the dead cells were tested by streaking a loopful of the cell deposit on to SDA and BA and incubating the plates aerobically at 37°C for 48 hours (Gallenkamp

U.K.). A dead yeast inoculum of 2.0 to 8.0 x 10^6 was prepared for use in assays by haemocytometer counting.

3.2.9. Cultivation of Candida species in saliva

The yeast pellet obtained as described in Section 3.2.7. was touched by a sterile wire loop and inoculated into sterile parotid saliva or whole saliva (see Sections 3.2.5. and 3.2.6.) supplemented with 0.2M glucose to give a final concentration of (2.0 to 8.0) x 10^6 cfu/ml. The concentration of Candida was determined by using an improved Neubauer haemocytometer chamber as described in Section (2.2.6.).

3.2.10. Assessment of acid production

Acid production in the test and the control samples was determined by two methods.

A By measurement of hydrogen ion concentration (pH)

B By the quantification and identification of different acid end products in the test and control saliva samples using isotachophoresis.

A pH change

The pH of each sample was measured by using an EIL 2320 pH meter (Electronic Instruments Ltd., England) fitted with a Beckmann

narrow diameter (5mm.) glass electrode (Beckman-Ltd., Buckinghamshire, England). The electrodes were standardised before use with standard buffers pH 7 and pH 4 respectively. At the appropriate times, 100 µl of the saliva sample were removed using a sterile micro-pipette and placed in a sterile 0.5 ml plastic micro-tube (Sarstedt, Leicester, U.K.). The micro-electrode was then placed in the tube and the pH value recorded.

B Identifying and quantifying acids by isotachophoresis

The acid anions were analysed by isotachophoresis using a LKB 2127 Tachophor (LKB Instruments, Bromma, Sweden, Figure 3.3.) fitted with conductivity and ultraviolet detection systems (Geddes and Weetman, 1981). The separation took place in a 610 mm Teflon capillary tube of 0.5 mm diameter maintained at a constant temperature of 12°C.

The leading electrolyte was 5 mM hydrochloric acid adjusted to pH 4.2 by the addition of 6-amino-n-hexanoic acid. Hydroxypropylmethylcellulose (0.2 % w/v) was added to sharpen the boundaries between acids. The terminating electrolyte was 4 mM n-octanoic acid adjusted to pH 5.5 by the addition of 2-amino-2 (hyroxymethyl)propane-1, 3-diol (Tris). These chemicals were supplied by BDH Chemicals Ltd., Poole, England, with the exception of the hydroxypropylmethylcellulose which was supplied by the Sigma Chemical Co. Ltd., Poole, England.

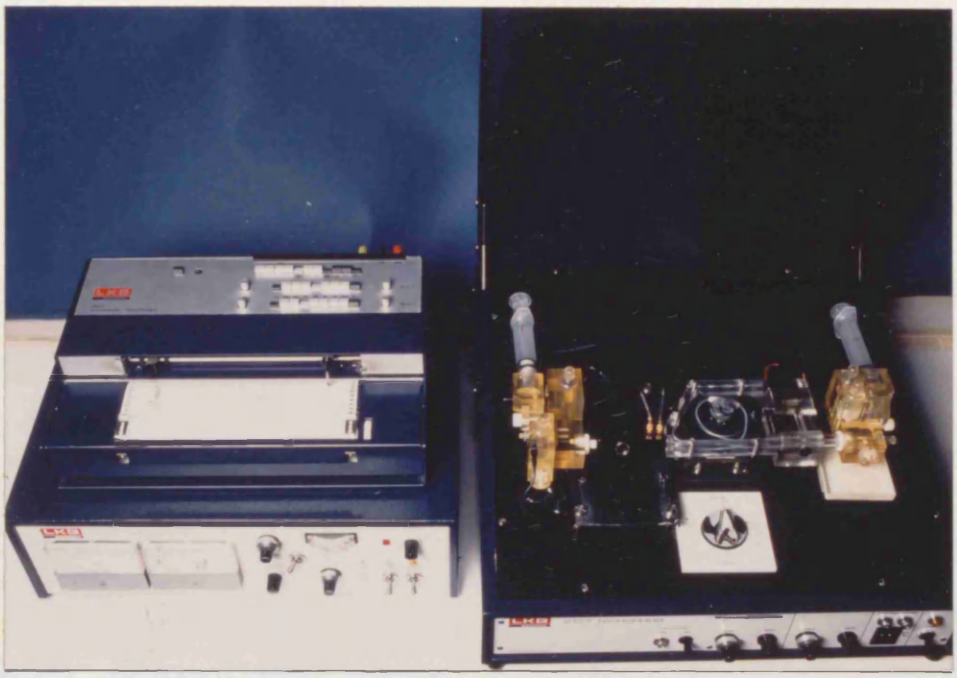

Figure 3.3. LKB 2127 Tachophor & Chart Recorder used for analysis of acid anions

Standard solutions, of 5 mM formic, pyruvic, phosphoric, lactic, succinic, acetic and propionic acids, supplied by BDH Chemicals Ltd., Poole, England, were run through the system and calibration curves obtained from which the zone lengths of the tracings could be converted into nanomolar concentrations (Figure 3.4).

Samples of saliva were then run through the tachophor at 50 uA. The traces obtained were then analysed, and the amount of each acid expressed in millimoles/litre.

3.2.11. Measurement of growth

Growth of Candida isolates was estimated using an improved Neaubauer haemocytometer chamber (Hawksley, England) as described in Section 2.2.6.).

3.2.12. Measurement of protein in the test saliva samples

BCA Protein Assay

The BCA protein assay (Smith et al., 1985) was carried out to determine the protein concentration of the saliva samples. All the reagents for this assay were supplied by Pierce and Warriner, 44 Upper Nathgate street, Chester, UK., Limited.

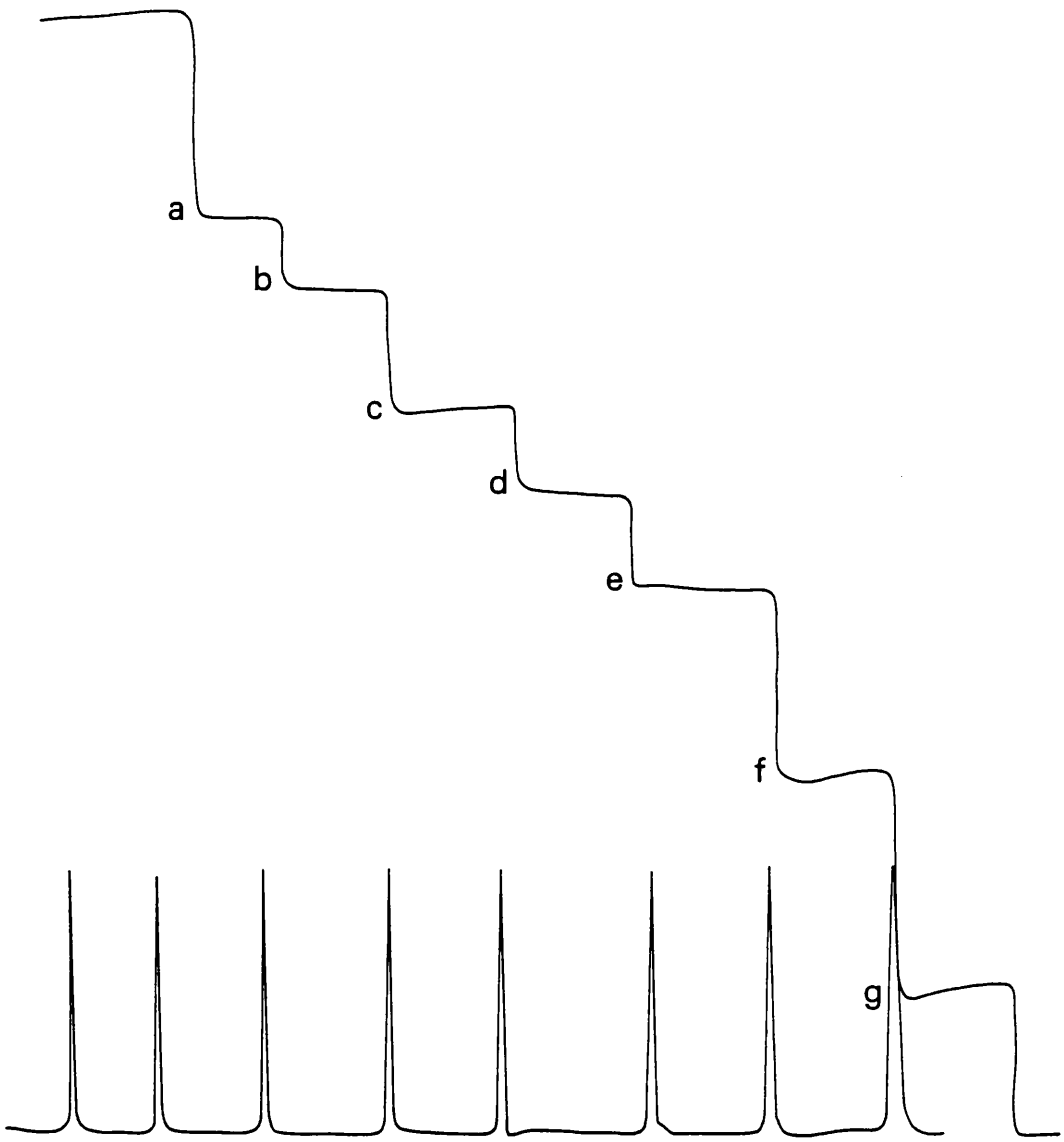

Figure 3.4. Example of tracing, used in calibration obtained from standard acid solution, a) formate, b) pyruvate, c) phosphate, d) lactate, e) succinate, f) acetate, g) propionate.

The working reagent consists of the following two components; Reagent A contains sodium carbonate, sodium bicarbonate, bicinchoninic acid, sodium tatarate and sodium hydroxide, while Reagent B contains 4% $CuSO_4.5H_2O$. The working reagent is prepared by adding 50 parts of A to 1 part of B. The mixed reagent is light green in colour and is stable for one week at room temperature.

Preparation of protein standard solution

A 5ml solution of "Pentex" bovine albumin (1mg/ml) (Miles, Scientific, Stoke Pages, Bucks.) was prepared in sterile distilled water. A one in ten dilution of this standard solution was prepared in distilled water and the absorbance at 280nm was measured in a SP8-100UV/visible Spectrophotometer (PYE UNICAM) using disposable plastic cuvettes. The actual concentration of protein in the standard solution was calculated from its extinction coefficient (A_{280} of a 1% w/v solution, l = 1 cm., was determined as 6.55).

$$\text{i.e., Protein concentration of standard} = \frac{A_{280} \times 100}{6.55} \text{ mg/ml}$$

Standard calibration curve

A standard curve was prepared for each batch of assays using a range of dilutions of the standard protein solutions. Two millilitres of BCA reagent was added to 0.1 ml of the protein

containing solutions and the absorbance determined at 562 nm. The protein concentration in mg/ml for each dilution was then calculated and a graph of absorbance at 562nm as ordinates, versus protein concentration in mg/ml as abscissae, was plotted. The standard protein assay calibration curve thus obtained is shown in Figure 3.5.

Assay for saliva

The following protocol has been found suitable for the assay of protein in saliva where sample volumes are small. The saliva samples were diluted with distilled water as follows; 20 µl of parotid saliva was added to 80 µl of distilled water; while 10 µl of whole saliva was added to 90 µl of distilled water. Aliquots (2.0ml) of BCA working reagent were added to each salivary sample and mixed well. The tubes were then placed in a water bath (Grant Instruments Limited., Cambridge, Glasgow), at 37^0C and after 30 minutes the tubes were immersed in cold water at 4^oC to stop the reaction rapidly. The tubes were read at 562 nm against the blank using the disposable cuvettes in the SPS-100UV/visible Spectrophotometer (PYE UNICAM). The protein concentration for each saliva sample was then calculated using the standard protein assay calibration curve as shown in Figure 3.5.

3.2.13. Preparation of salivary proteins sample for sodium dodecyl sulphate polyacrylamide gelelectrophoresis

In preparing the salivary protein samples for zone electrophoresis a buffer system is required to dissociate the

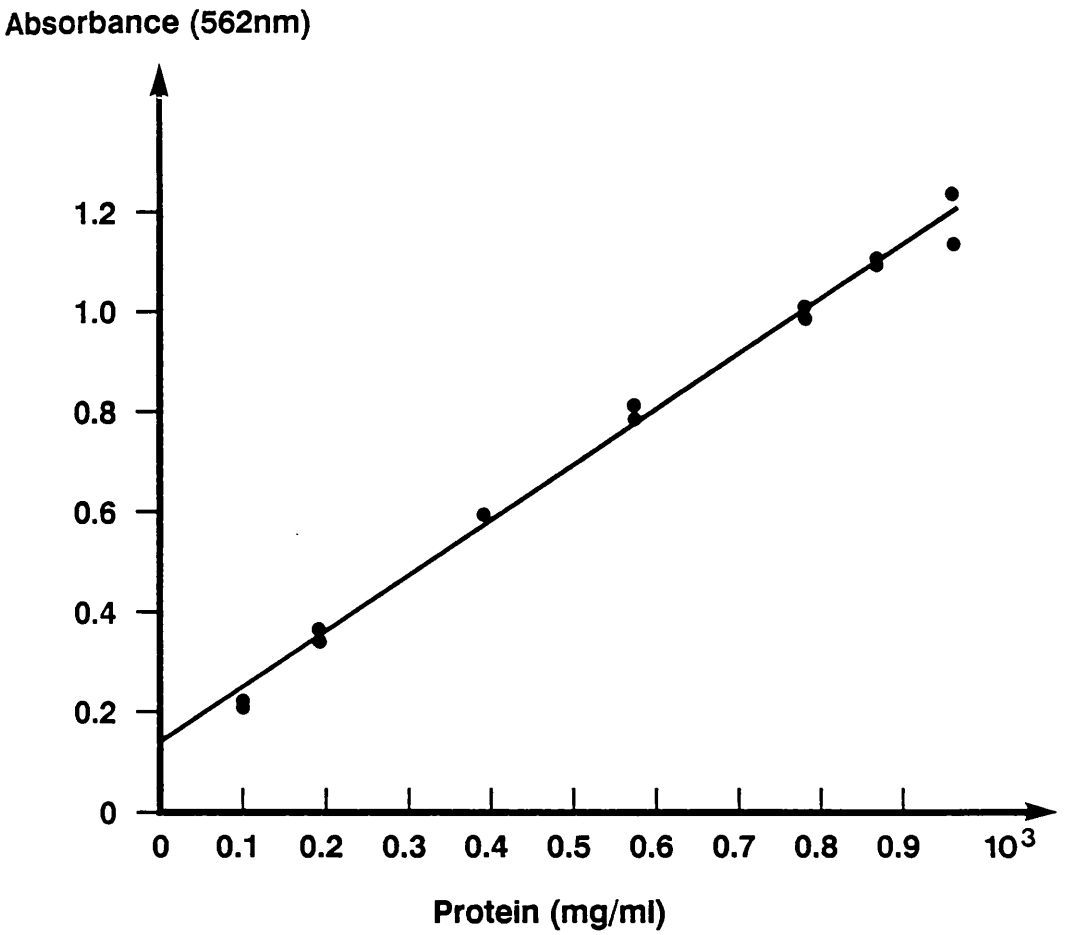

Figure 3.5. Protein assay calibration curve using bovine serum albumin as a standard.

proteins into their individual polypeptide units. The method used in this study was as follows.

Sample preparation

Aliquots (100µl) of either mixed or parotid saliva were added to 10 µl of a sample dissolving solution, (see Appendix B) and 10 µl glycerol then heated in a boiling bath for 2 minutes to ensure the denaturation of proteins. To this heated mixture 5 µl of bromophenol was added and these samples stored at -20°C. Before loading onto gels the samples were warmed by immersing the tubes in a water bath at 37° for 5 minutes. (For the preparation of gels see Appendix B and Tables 3.1. and 3.2).

The dissociating agent used in this study was sodium dodecyl sulphate (SDS). The salivary proteins are denatured by heating the protein mixture at 100°C in the presence of excess SDS and a reducing agent, dithiothreit, to cleave disulphide bonds. This results in the binding of polypeptides to SDS in a constant weight ratio, 1.4 grams of SDS binds to 1 gram of polypeptide. These SDS-polypeptide complexes have identical charge densities and migrate in polyacrlamide gels according to polypeptide size.

3.2.14. Molecular weight standards

Whenever a gel was run, low molecular weight markers (MW 14,400-96,000, Pharmacia) were also run in a parallel track to the

Stock acrylamide	12.5ml
Running buffer	15.0ml
Water	2.25ml

Mix and degas then add the following

TEMED*	5 µl
Ammonium persulphate	100 µl
20% SDS+	0.15ml

Table 3.1. Constituents of the Running gel
*** Trimethyl ethylene diamine**
+ Sodium dodecyl sulphate

Stock acrylamide	2ml
Stacking buffer	8.8ml
Water	1.16ml

Mix and degas then add the following:

TEMED*	3 µl
Ammonium persulphate	40 µl
20% SDS+	60 µl

Table 3.2. Constituents of the Stacking gel.
*** Trimethyl ethylene diamine**
+ Sodium dodecyl sulphate

sample tracks. By running a sample of molecular weight markers parallel to the salivary protein sample, the molecular weight of the sample polypeptides can be determined (Hames, 1981).

3.2.15. 1-Dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis

The method of Laemli (1970) was used for 1-dimensional SDS-PAGE of saliva.

SDS-polyacrylamide gels consist of a lower portion, the running gel and an upper portion, the stacking gel. All chemicals and solutions required for SDS-PAGE gels are given in the Appendix B.

A 12.5% acrylamide gel solution (Table 3.1.) wasprepared and cast between 2 sheets of flat glass separated by a 1.5 mm thick neoprene spacer. This gel solution was overlayed with iso-propyl alcohol to a height of about 0.5 cm. both to exclude oxygen which inhibits polymerisation and to ensure a flat gel miniscus. The gel is left undisturbed, to polymerise at room temperature for 2 to 3 hours.

Once the resolving gel has polymerised the overlay is poured off and washed with 10 ml of the running buffer (see Appendix B). The stacking gel (Table 3.2.) was then prepared and poured on top of the running gel. Immediately, a perspex comb (10 wells) was inserted between the glass plates and into the gel mixture. The gel is left undisturbed for about 2 hours to set. Next the comb and the bottom spacer were removed and the resulting gel was placed either in

the electrophoresis tank and used immediately, or alternatively, the gel was stored for a period of up to 24 hours at 4^0C wrapped in cling film (A & G Beverage, Edinburgh). The assembled slab gel holder is shown in Figure 3.6.

3.2.16. Sample loading

A volume of 50 µl of the prepared saliva samples were added to each well. The concentration of protein of each sample was calculated using the BCA protein assay method (Smith et al., 1985).

Calculation of the protein load

Assuming the concentration of PPS sample is = Y µg/ml.

1ml sample = Y µg.

125 µl of treated saliva sample = 1/10 x original concentration

= Y/10 µg

50 µl = (Y/10) X (100/125)

= (Y/10) X (4/5)

concentration of test sample = 4Y/50 µg.

3.2.17. The electrophoresis tank

The electrophoresis tank (BRL, Model V 16, Bethesda, Research Laboratories, Cambridge, U.K.) comprises an upper and lower reservoir both of which contain the reservoir buffer (Figure 3.7.).

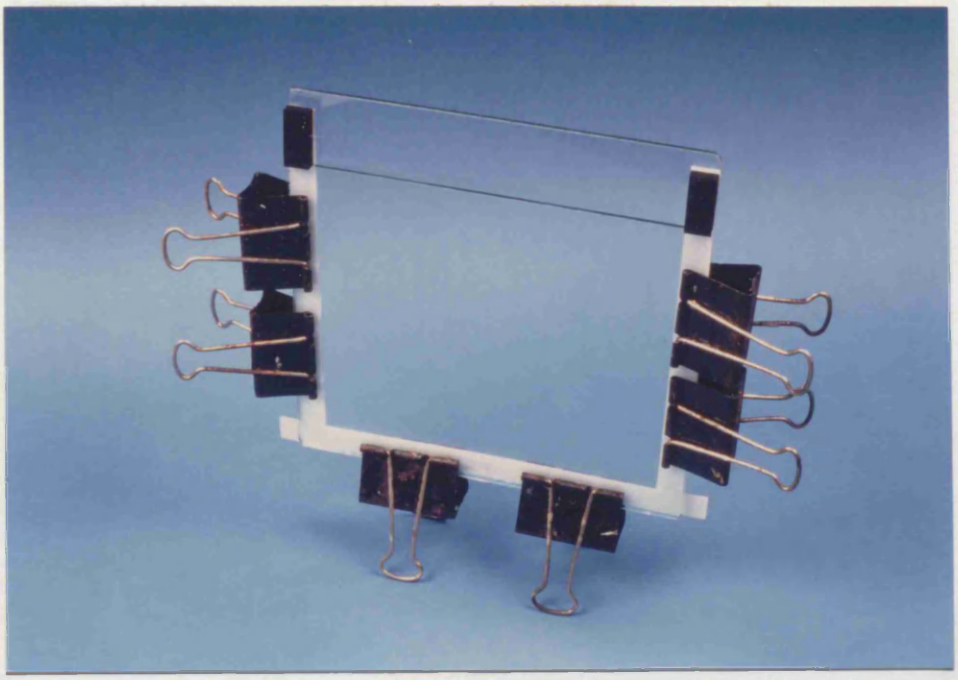

Figure 3.6. The slab gel holder assembly, prior to addition of gel solution

Figure 3.7. The Electrophoresis tank used for running SDS-PAGE gels.

The tank is connected to a control power supply unit VoKam power pack (Shandon Southern, SAE 2761) which is capable of supplying up to 400V and 80mA and which can be adjusted to give a constant voltage across the gel with a variable current. The initial voltage (as used for a slow run) was set at 60V and the current was set at 20 mA.

The gel is attached to the tank by the use of clamps. The top of the polyacrylamide gel is in communication with the upper reservoir into which the negative electrode dips, while the bottom of the gel is in communication with the lower reservoir into which the positive electrode dips. When a difference of voltage is applied to the gel, the proteins being negatively charged, run into the stacking gel from the well. The proteins separate out according to their molecular weight as the complex mixture migrates down the gel. The gels were run overnight for 16 hours at 60 volts increasing to 160 volts the next day until the bromophenol tracker dye front reached 5 cm from the bottom edge of the gel. The gel was then removed from the electrophoresis tank, still clamped on to the glass plates. Next the gel was removed from between the glass plates and placed carefully in the staining solution, 1% coomasie brilliant blue R-250 (St. Lewis, MO.) in an acetic and ethanol mixture, for one day. Gels were then destained for 2-3 days with several changes of 10% (v/v) acetic acid. Finally gels were sealed in polythene and stored at $4^{o}C$.

3.2.18. Determination of the proteolytic and saccharolytic activity of Candida in pooled parotid and pooled whole saliva

Assay method

Five millilitres of sterile pooled parotid (described in Section 3.2.5.) or sterile pooled whole saliva (as described in Section 3.2.6) were inoculated with (2.0 to 8.0)x10^6cells/ml of the yeast under test (as described in Sections 3.2.7. and 3.2.9.). Two controls were used in this assay; the first was an uninoculated saliva sample which was incubated with the test sample as a control for non-specific changes in the salivary constituents during the experimental period (C1). The second control was a saliva sample inoculated with a dead yeast suspension which was co-incubated with the test samples to see if any change in the protein concentration occurred due to adsorption of salivary protein on to the dead yeast cell surface (C2). The third control is described in Section 3.2.19.

The pH of the yeast suspension (see Section 3.2.10), growth (cfu/ml), (see Section 3.2.11.) and protein concentration (see Section 3.2.12.) were measured immediately at 0 hours, 8 hours, 24 hours, 32 hours, 48 hours, 56 hours and 72 hours. Salivary protein samples at the end of 0, 24, and 48 hours of incubation were prepared for SDS-PAGE electrophoresis as described in Section 3.2.13. and stored at -20^{o}C until PAGE analysis was performed. Samples of 50 µl of the test and controls (C1, C2), at 0 hours, 8 hours, 24 hours, 32

hours, 48 hors, 56 hours and 72 hours were removed into sterile micro ampoules and stored at -20^{o} for isotachophoresis (see Section 3.2.10b).

3.2.19. The third control saliva sample

The third control (C3) was carried out by incubating the saliva sample with live yeasts at 4^{0}C. This control was removed from the fridge and incubated at room temperature for half an hour each day for the three consecutive days on which the assay was carried out. The purpose of this control was to investigate if salivary proteins were adsorbed onto the surface of viable yeasts at a combination of 4^{o}C and room temperature. Salivary protein samples at the end 0, 24, 48 and 72 hours of incubation were prepared for SDS-PAGE electrophoresis as described in Section 3.2.13. and stored at -20^{o}C untill PAGE analysis was performed.

3.2.20. Statistical analysis

The results obtained for yeast growth and protein degradation were transformed into their log values, and graphs were constructed using the results of the 4 parameters studied i.e. growth, pH, acid anion production and protein degradation, against time.

To investigate if there were any differences amongst the 4 Candida species for each variable, a Repeated Measures Analysis

of Variance was carried out. If there were any differences amongst the Candida species using this test a One-way analysis of Variance was carried out at each distinct time point for each variable to determine at which time points these occurred.

3.3. RESULTS

3.3.1. Growth of C. albicans GDH 1878 when cultured in pooled parotid saliva

The results of the growth of C. albicans GDH 1878 in pooled parotid saliva supplemented with 200mM glucose are shown in Tables 3.3 to 3.5. The mean growth increased slowly but steadily during the first 24 hours, from 2.89×10^6 to 3.00×10^7 and subsequently appeared to stabilise at about 60 hours (6.2×10^7) (Figure 3.8). On microscopic examination, the yeasts were in the blastospore phase throughout these experiments.

No growth occurred in the control saliva samples (Tables 3.3 to 3.4). Subculture of the test and control suspensions, on blood agar failed to reveal any bacterial growth, confirming the efficacy of the antibiotic supplements.

3.3.2. The pH changes of pooled parotid saliva during the culture of C. albicans GDH 1878

The pH changes which occurred in cultures of C. albicans GDH 1878 in pooled parotid saliva supplemented with 200mM glucose are shown in Tables 3.6 to 3.8. The mean pH of the cultures changed from 8.9 to 7.8, during the first 48 hours of growth after which a slight change occurred up to 72 hours. The pH of the control saliva samples remained stable throughout the 72 hour period in which the experiments were carried out (8.7 to 8.9). Thus, although a fall in

Time hours	Colony forming units per ml		
	Experiment 1	Control 1A	Control 1B
0	2.86×10^6	0	3.56×10^6
8	0.95×10^7	0	2.50×10^6
24	2.69×10^7	0	2.48×10^6
32	3.50×10^7	0	2.30×10^6
48	3.96×10^7	0	2.20×10^6
56	4.64×10^7	0	1.90×10^6
72	6.96×10^7	0	1.80×10^6

Table 3.3. The growth of C. albicans GDH 1878 cultured in parotid saliva supplemented with 200mM glucose over a 3 day period (Experiment 1 and the two Control experiments 1A and 1B).
Control 1A - Whole saliva supplemented with 200mM glucose, only.
Control 1B - Heat killed C. albicans GDH 1878 incubated in whole saliva supplemented with 200mM glucose.

Time hours	Colony forming units per ml Experiment 2	Control 2A	Control 2B
0	2.92×10^6	0	3.24×10^6
8	1.10×10^7	0	3.20×10^6
24	3.31×10^7	0	2.80×10^6
32	3.84×10^7	0	2.74×10^6
48	4.32×10^7	0	2.60×10^6
56	5.64×10^7	0	2.10×10^6
72	6.84×10^7	0	1.98×10^6

Table 3.4. The growth of C. albicans GDH 1878 cultured in parotid saliva supplemented with 200mM glucose over a 3 day period (Experiment 2 and the two Control experiments 2A and 2B).
Control 2A - Parotid saliva supplemented with 200mM glucose, only.
Control 2B - Heat killed C. albicans GDH 1878 incubated in parotid saliva supplemented with 200mM glucose.

Time hours	Mean colony forming units per ml Experiment 1+2	Control 1A+1B	Control 2A+2B
0	2.89×10^6	0	3.40×10^6
8	1.02×10^7	0	2.85×10^7
24	3.00×10^7	0	2.64×10^6
32	3.67×10^7	0	2.52×10^6
48	4.14×10^7	0	2.00×10^6
56	5.14×10^7	0	2.00×10^6
72	6.90×10^7	0	1.89×10^6

Table 3.5. The mean growth of C. albicans GDH 1878 cultured in parotid saliva supplemented with 200mM glucose over a 3 day period.

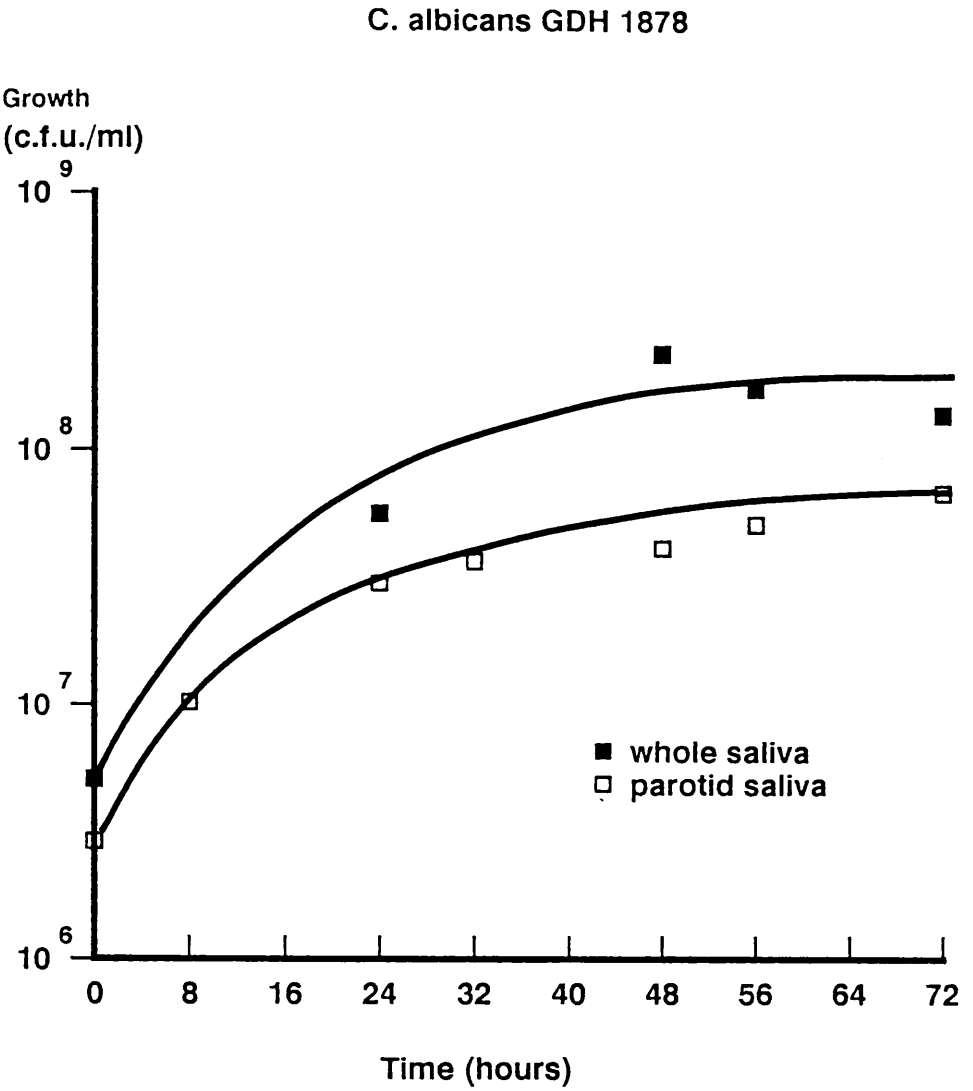

Figure 3.8 The mean growth of C. albicans GDH 1878 cultered in pooled parotid saliva and pooled whole saliva supplemented with 200mM glucose during 72 hours incubation at 37°C under aerobic conditions.

Time hours	pH value Experiment 1	Control 1A	Control 1B
0	8.90	8.80	8.90
8	8.80	8.90	8.85
24	8.40	8.75	8.90
32	8.20	8.80	8.90
48	7.80	8.80	8.80
56	7.65	8.80	8.80
72	7.60	8.70	8.70

Table 3.6. The variation in pH of C. albicans GDH 1878 cultured in parotid saliva supplemented with 200mM glucose over a 3 day period (Experiment 1 and the two Control experiments 1A and 1B).
Control 1A - Parotid saliva supplemented with 200mM glucose only.
Control 1B - Heat killed C. albicans GDH 1878 incubated in parotid saliva supplemented with 200mM glucose.

	pH value		
Time hours	Experiment 2	Control 2A	Control 2B
0	8.90	8.90	8.90
8	8.40	8.80	8.80
24	7.80	8.90	8.90
32	7.90	8.90	8.80
48	7.80	8.80	8.80
56	7.80	8.90	8.80
72	7.70	8.80	8.80

Table 3.7. The variation in pH of C.albicans GDH 1878 cultured in parotid saliva supplemented with 200mM glucose over a 3 day period (Experiment 2 and the two Control experiments 2A and 2B).
Control 2A - Parotid saliva supplemented with 200mM glucose only.
Control 2B - Heat killed C. albicans GDH 1878 incubated in parotid saliva supplemented with 200mM glucose.

Time hours	Mean pH value Experiment 1+2	Control 1A+2A	Control 1B+2B
0	8.90	8.85	8.90
8	8.60	8.85	8.82
24	8.19	8.82	8.90
32	8.05	8.85	8.85
48	7.80	8.80	8.80
56	7.72	8.85	8.80
72	7.76	8.75	8.75

Table 3.8. The mean pH of C. albicans GDH 1878 cultured in parotid saliva supplemented with 200mM glucose over a 3 day period.

pH occurred, the parotid saliva samples remained at an alkaline pH throughout the 3 day period of the experiments (Figure 3.9).

3.3.3. Production of acids by C. albicans GDH 1878 when cultured in pooled parotid saliva

The acid anions produced by C. albicans GDH 1878 in pooled parotid saliva supplemented with 200mM glucose are shown in Tables 3.9 to 3.11. The concentration of the pyruvate and the acetate anions tended to increase gradually and consistently over the 72 hour period although yeast proliferation slowed down after 48 hours of incubation. The mean pyruvate concentration increased from 0.00 to 7.63 millimoles/litre and acetate increased from 0.46 to 8.61 millimoles/litre during the 3 day period of the experiments. In comparison the concentration of the other acid anions investigated i.e., formate, lactate, succinate and propionate, were relatively static during the experimental period (formate: 0.15 - 0.27; lactate: 0.87 - 1.20; succinate: 0.16 - 1.38; and propionate: 0.30 - 1.29) (Figure 3.10.).

The concentrations of the acid anions in the 2 control saliva samples showed very little change during the course of the experiment. The total acid anions during the 3 day period of the experiments changed from 1.67 to 2.04 millimoles/litre in the parotid saliva sample supplemented with glucose (C1A), and from 1.86 millimoles/litre to 1.98 millimoles/litre in the parotid sample inoculated with the dead yeasts (C1B). This variation of 0.1 to 0.3

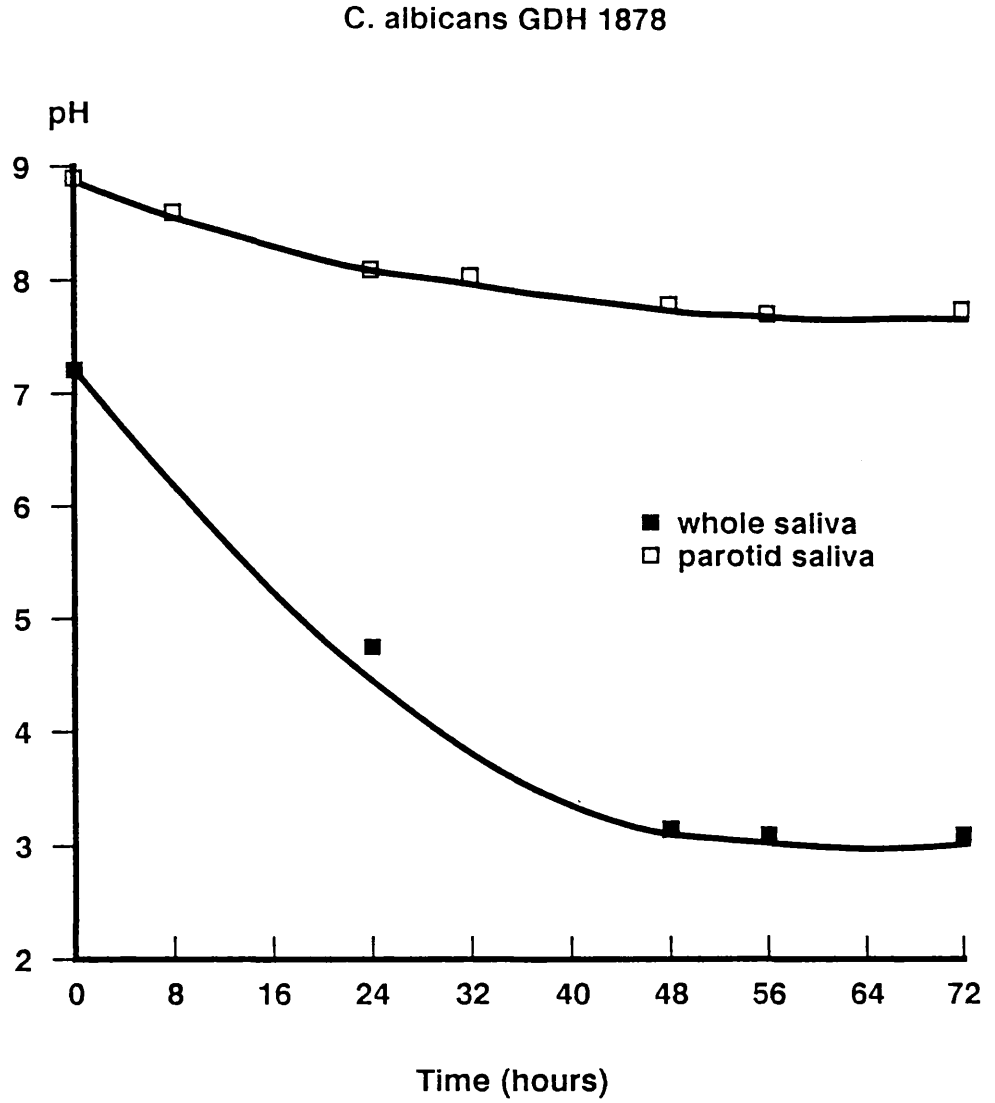

Figure 3.9 The mean pH of C. albicans GDH 1878 cultured in pooled parotid saliva and pooled whole saliva supplemented with 200mM glucose during 72 hours incubation at 37°C under aerobic conditions.

Time	Sample	Formate	Pyruvate	Lactate	Succinate	Acetate	Propionate	Total acids
	T1	0.18*	ND	0.85	0.26	0.49	0.40	2.18
0+	C1A	0.24	ND	0.91	0.32	0.11	0.19	1.77
	C1B	0.22	ND	0.94	0.36	0.13	0.26	1.91
	T1	0.20	ND	0.91	0.33	0.72	0.52	2.68
8	C1A	0.15	ND	0.87	0.23	0.48	0.48	2.21
	C1B	0.17	ND	0.94	0.37	0.34	0.47	2.29
	T1	0.18	3.10	1.20	0.43	4.02	0.53	9.46
24	C1A	0.11	ND	1.07	0.18	0.34	0.38	2.08
	C1B	0.18	ND	0.93	0.29	0.32	0.39	2.10
	T1	0.29	4.38	0.98	0.76	5.05	0.67	12.13
32	C1A	0.08	ND	0.96	0.28	0.53	0.70	2.55
	C1B	0.08	ND	1.01	0.34	0.66	0.67	2.76
	T1	0.40	6.94	0.91	0.99	4.84	0.81	14.89
48	C1A	0.09	0.05	0.86	0.26	0.85	0.59	5.18
	C1B	0.13	0.05	0.94	0.29	0.68	0.65	2.74
	T1	0.33	7.13	0.96	0.87	5.42	0.41	15.12
56	C1A	0.13	0.08	0.92	0.07	0.15	0.22	1.57
	C1B	0.17	0.08	0.90	0.08	0.22	0.29	1.61
	T1	0.19	7.38	1.12	1.46	8.05	0.90	19.10
72	C1A	0.17	0.15	0.90	0.07	0.30	0.23	1.82
	C1B	0.17	0.12	0.86	0.10	0.22	0.37	1.82

Table 3.9. The acid anions produced by C. albicans GDH 1878 cultured in pooled parotid saliva supplemented with 200mM glucose. T1 = Test sample 1; C1A = Parotid saliva supplemented with 200mM glucose only; C1B = Heat killed C. albicans GDH 1878 incubated in parotid saliva supplemented with 200mM glucose.
+ = hours; * = millimoles/litre; ND = Not detected.

Time	Sample	Formate	Pyruvate	Lactate	Succinate	Acetate	Propionate	Total acids
	T2	0.12*	ND	0.89	0.07	0.44	0.20	1.72
0+	C2A	0.10	ND	0.86	0.03	0.41	0.18	1.58
	C2B	0.12	ND	0.94	0.09	0.47	0.20	1.82
	T2	0.13	0.05	0.86	0.05	0.63	0.22	1.94
8	C2A	0.10	ND	0.83	0.05	0.38	0.13	1.49
	C2B	0.13	ND	0.84	0.07	0.40	0.18	1.62
	T2	0.27	5.43	1.21	0.71	3.45	0.99	12.05
24	C2A	0.13	0.05	0.91	0.14	0.22	0.32	1.77
	C2B	0.12	0.01	0.81	0.27	0.68	0.20	2.09
	T2	0.18	3.83	0.97	0.49	4.05	0.91	10.43
32	C2A	0.12	0.05	0.92	0.30	0.49	0.25	2.13
	C2B	0.15	ND	1.02	0.34	0.57	0.22	2.30
	T2	0.24	6.53	1.05	0.87	5.41	0.51	14.61
48	C2A	0.14	0.05	1.08	0.23	0.67	0.22	2.39
	C2B	0.13	0.05	0.89	0.15	0.69	0.18	2.09
	T2	0.28	7.15	1.09	1.30	5.59	1.50	16.91
56	C2A	0.15	ND	0.88	0.16	0.55	0.22	1.96
	C2B	0.06	0.08	0.83	0.27	0.59	0.40	2.23
	T2	0.36	7.88	1.29	1.30	9.18	1.69	21.70
72	C2A	0.17	0.05	0.82	0.31	0.55	0.36	2.26
	C2B	0.14	0.05	0.83	0.30	0.51	0.32	2.15

Table 3.10. The acid anions produced by C. albicans GDH 1878 cultured in pooled parotid saliva supplemented with 200mM glucose. T2 = Test sample 2; C2A = Parotid saliva supplemented with 200mM glucose only; C2B = Heat killed C. albicans GDH 1878 incubated in parotid saliva supplemented with 200mM glucose; + = hours; * = millimoles/litre; ND = not detected.

Time	Sample	Formate	Pyruvate	Lactate	Succinate	Acetate	Propionate	Total acids
	T	0.15*	ND	0.87	0.16	0.46	0.30	1.95
0+	CA	0.17	ND	0.88	0.17	0.26	0.18	1.67
	CB	0.17	ND	0.94	0.22	0.30	0.23	1.86
	T	0.16	0.02	0.88	0.19	0.67	0.37	2.31
8	CA	0.12	ND	0.85	0.14	0.43	0.30	1.85
	CB	0.15	ND	0.89	0.22	0.37	0.32	1.95
	T	0.22	4.26	1.20	0.57	3.73	0.76	10.76
24	CA	0.12	0.02	0.99	0.16	0.28	0.35	1.92
	CB	0.15	0.01	0.87	0.28	0.50	0.29	2.09
	T	0.23	4.10	0.97	0.62	4.55	0.79	11.28
32	CA	0.10	0.02	0.94	0.29	0.51	0.47	2.34
	CB	0.11	ND	1.01	0.34	0.61	0.44	2.53
	T	0.32	6.73	0.98	0.93	5.12	0.66	14.75
48	CA	0.11	0.05	0.97	0.24	0.76	0.40	3.78
	CB	0.13	0.05	0.91	0.22	0.68	0.49	2.41
	T	0.30	7.14	1.02	1.08	5.50	0.95	16.01
56	CA	0.14	0.04	0.90	0.11	0.35	0.22	1.76
	CB	0.11	0.08	0.86	0.17	0.40	0.34	1.92
	T	0.27	7.63	1.20	1.38	8.61	1.29	20.40
72	CA	0.17	0.10	0.86	0.19	0.42	0.29	2.04
	CB	0.15	0.08	0.84	0.20	0.36	0.34	1.98

Table 3.11. The mean value of acid anions produced by C. albicans GDH 1878 cultured in pooled parotid saliva, supplemented with 200mM glucose over a 3 day period.

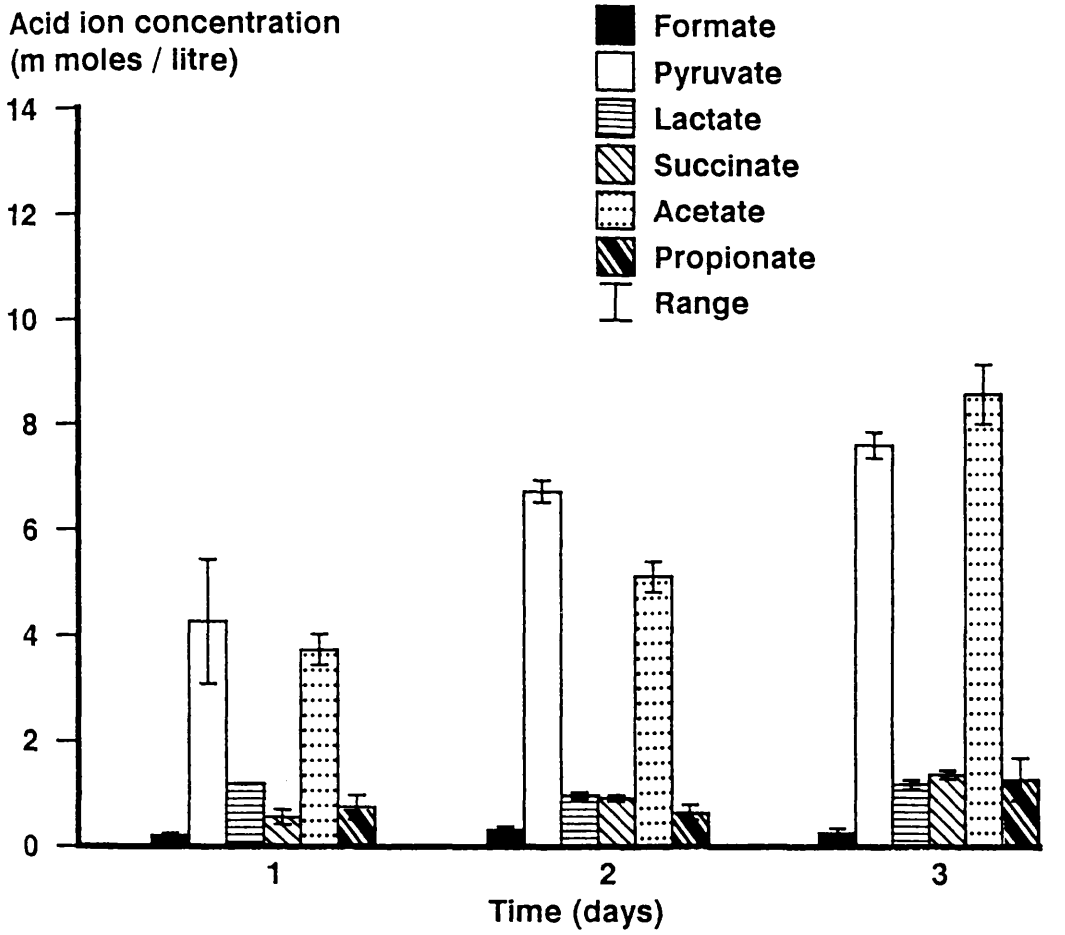

Figure 3.10 The mean acid anion concentration produced by C. albicans GDH 1878 when cultured in pooled parotid saliva supplemented with 200 mM glucose during 3 days aerobic incubation at 37°C.

millimoles/litre in the acid concentration of the control saliva samples could be due to the highly complex nature of saliva. As a variety of enzymes are present in saliva (see Section 1.4.) enzymic digestion of salivary components could occur resulting in the production of small amounts of acids.

3.3.4. Change in parotid salivary protein concentration during the growth of C. albicans GDH 1878

The changes which occurred in parotid salivary protein concentration during the growth of C. albicans GDH 1878 in pooled parotid saliva supplemented with 200mM glucose are shown in Tables 3.12 to 3.14. The mean percentage reduction of protein was gradual and generally proportional to the period of incubation. The mean % reduction of protein during 0-8h = 4.5; 8-24h = 11.5; 24-32h = 18.0; 32-48h = 27.6; 48-56h = 32.0; 56-72h = 43.7. Thus, after incubating the culture for 72 hours there was a 43 percent decrease in the parotid salivary proteins. These results are summarised in Figure 3.11.

The protein concentration of the 2 control cultures changed very little over the experimental period compared to the test saliva samples. A slight increase in protein concentrations occurred in both of the control cultures from 7. 2 to 7.5 mg/ml. Culture of samples from both test and control suspensions in each of the the above experiments on blood agar failed to reveal any bacterial growth, confirming the sterility of the parotid saliva samples.

Time hours	Concentration of protein mg/ml Experiment 1	Control 1A	Control 1B
0	7.2	7.2	7.2
8	6.2	7.5	7.6
24	6.4	7.7	7.7
32	5.9	7.2	7.3
48	5.2	7.6	7.3
56	4.9	7.5	7.2
72	4.1	7.6	7.6

Table 3.12. The protein concentration at different time intervals when C. albicans GDH 1878 was cultured in parotid saliva supplemented with 200mM glucose over a 3 day period (Experiment 1 and the two Control experiments 1A and 1B).
Control 1A - Parotid saliva supplemented with 200mM glucose only.
Control 1B - Heat killed C. albicans GDH 1878 incubated in parotid saliva supplemented with 200mM glucose.

Concentration of protein mg/ml			
Time hours	Experiment 2	Control 2A	Control 2B
0	7.1	7.1	7.1
8	6.8	7.1	7.6
24	6.3	7.4	7.5
32	5.9	7.3	7.4
48	5.2	7.5	7.1
56	4.8	7.5	7.4
72	3.9	7.4	7.3

Table 3.13. The concentration of protein at different time intervals when C. albicans GDH 1878 was cultured in parotid saliva supplemented with 200mM glucose over a 3 day period (Experiment 2 and the two Control experiments 2A and 2B).
Control 2A - Parotid saliva supplemented with 200mM glucose only.
Control 2B - Heat killed C. albicans GDH 1878 incubated in parotid saliva supplemented with 200mM glucose.

Time hours	Mean concentration of protein mg/ml Experiment 1+2	Control 1A+2A	Control 1B+2B
0	7.2	7.2	7.2
8	6.8	7.3	7.6
24	6.3	7.5	7.6
32	5.9	7.2	7.3
48	5.2	7.5	7.2
56	4.8	7.5	7.3
72	4.0	7.5	7.5

Table 3.14. The mean concentration of protein at different time intervals when C. albicans GDH 1878 was cultured in parotid saliva supplemented with 200mM glucose over a 3 day period.

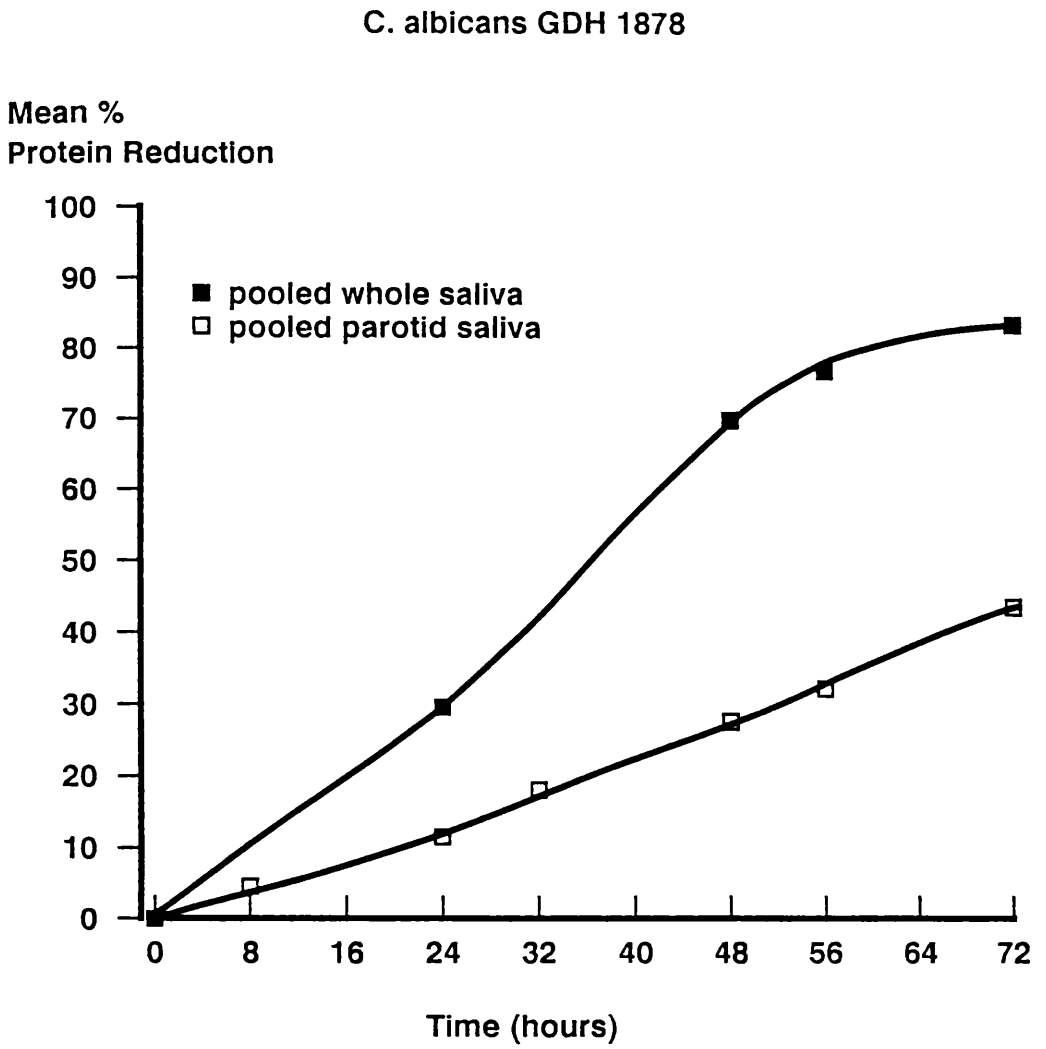

Figure 3.11 The mean percentage reduction of protein during growth of C. albicans GDH 1878 in pooled parotid saliva and pooled whole saliva supplemented with 200 mM glucose during 3 days aerobic incubation at 37°C.

3.3.5. SDS-PAGE of parotid saliva during culture with C. albicans GDH 1878

The culture supernatants of parotid saliva after incubation with C. albicans GDH 1878 were examined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) for alterations in their salivary protein profiles, at 0, 24, and 48 hours as described in Sections 3.2.15. to 3.2.17. The electrophoretic analysis patterns of the supernatant of the test and control samples obtained at 0 and 48 hours are shown in Figures 3.12 (a) & (b). It is clear that the protein patterns alter very little throughout the duration of the experiments, when assessed by visual examination, although as described earlier, biochemical assay of salivary protein revealed a mean reduction of 11% and 32% at 24 and 48 hours respectively. It is possible that densitometric analysis of the gels would have demonstrated differences at 24 and 48 hours, but this was not performed.

The results of the above experiments indicate that C. albicans GDH 1878 grows relatively poorly in pooled parotid saliva i.e., from 2.89×10^6 to 6.90×10^7 cfu/ml in 3 days. Yeast growth reached a maximum at 48 hours 4.1×10^7 cfu/ml, changing very little during the final 24 hours of incubation. The pH of the saliva fell by 1 unit (8.9 to 7.8), and while the acid anion concentration of pyruvate and acetate was 7.63mmol/l and 8.61mmol/l respectively the values for formate, lactate, succinate and propionate were very much less i.e., less than 2 mmol/l. The protein concentration fell from

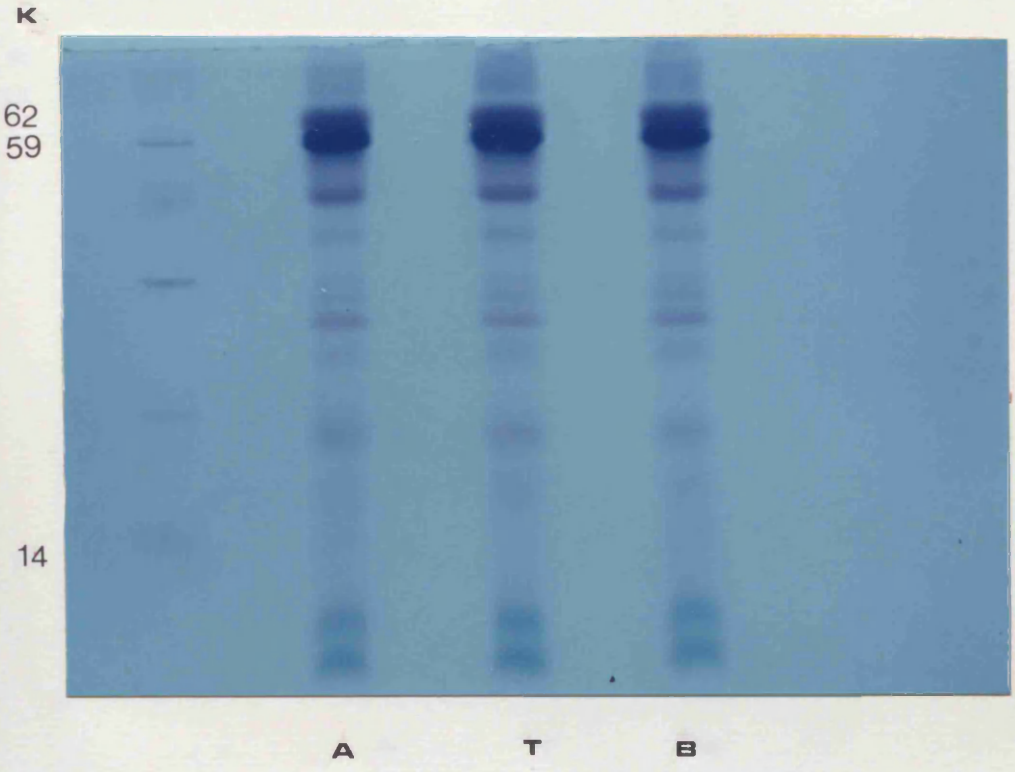

Figure 3.12 (a) The SDS-PAGE analysis patterns obtained at 0 hours of the Test and 2 Control parotid saliva samples cultured with C. albicans GDH 1878. T1 = Test Parotid saliva sample; C1A = Parotid saliva supplemented with 200mM glucose only; C1B = Heat killed C. albicans GDH 1878 incubated in parotid saliva supplemented with 200mM glucose.

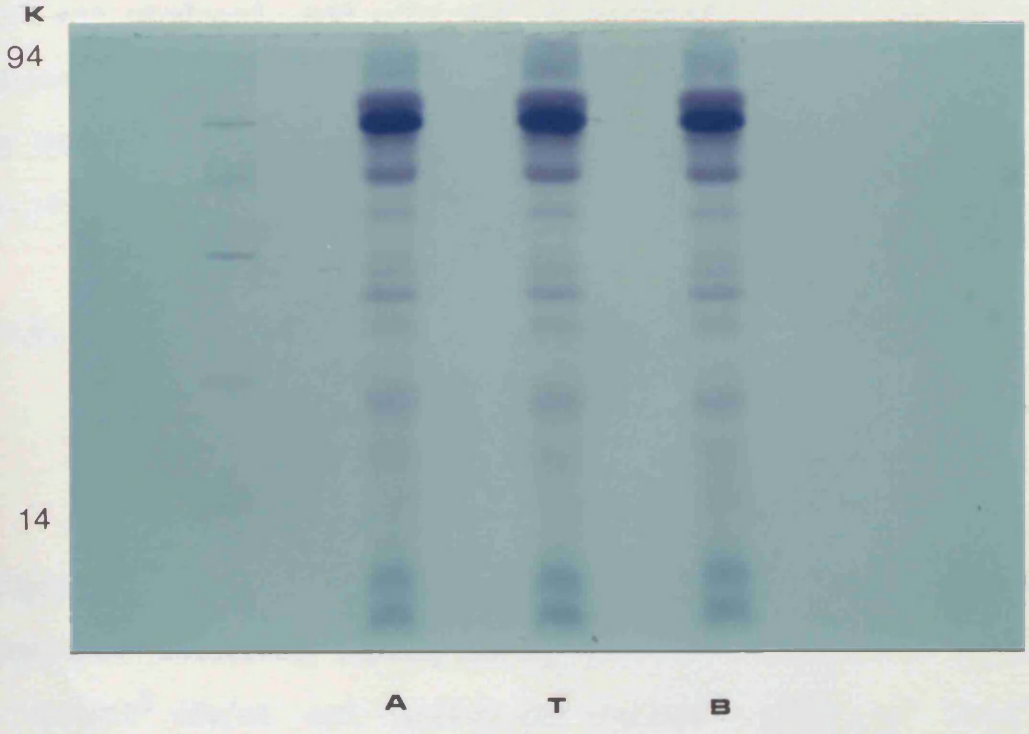

Figure 3.12 (b) The SDS-PAGE analysis patterns obtained at 48 hours of the Test and 2 Control parotid saliva samples cultured with C. albicans GDH 1878. T1 = Test Parotid saliva sample; C1A = Parotid saliva supplemented with 200mM glucose only; C1B = Heat killed C. albicans GDH 1878 incubated in parotid saliva supplemented with 200mM glucose.

7.2 to 4.0 mg/ml in 3 days indicating a 43% reduction in the parotid salivary proteins. Overall there was modest growth of C. albicans GDH 1878 in parotid saliva, the cultures remained alkaline, very little acid was produced, and only 43% of salivary protein degradation occurred. Therefore it was decided to find out if pooled whole saliva was any better for use in growth experiments (see Sections 3.3.6. to 3.3.10.).

3.3.6. Growth of C. albicans GDH 1878 when cultured in pooled whole saliva

The growth of C. albicans GDH 1878 in pooled whole saliva supplemented with 200mM glucose is shown in Tables 3.15 to 3.17. The yeast grew relatively rapidly during the first 48 hours from 5.06×10^6 to 2.36×10^8 cfu/ml and reached the stationery phase at about 48 hours (Figure 3.8. presented earlier). On microscopical examination, the yeasts were in the blastospore phase throughout the experiments and no yeast growth occurred in either of the control saliva samples. Subculture of the test and control suspensions, on blood agar failed to reveal any bacterial growth, confirming the efficacy of the antibiotic supplements.

3.3.7. The pH changes in pooled whole saliva during the culture of C. albicans GDH 1878

The pH change of C. albicans GDH 1878 in pooled whole saliva supplemented with 200mM glucose is shown in Tables 3.18 to 3.20. The

Time hours	Colony forming units per ml Experiment 1	Control 1A	Control 1B
0	3.91×10^6	0	3.7×10^6
8	ND	ND	ND
24	2.00×10^7	0	2.0×10^6
32	ND	ND	ND
48	1.92×10^8	0	1.0×10^5
56	1.81×10^8	0	1.2×10^4
72	1.42×10^8	0	1.0×10^4

Table 3.15. The growth of C. albicans GDH 1878 cultured in whole saliva supplemented with 200mM glucose over a 3 day period (Experiment 1 and the two Control experiments 1A and 1B).
Control 1A - Whole saliva supplemented with 200mM glucose, only.
Control 1B - Heat killed C. albicans GDH 1878 incubated in whole saliva supplemented with 200mM glucose.

ND - Not determined

Time hours	Colony forming units per ml Experiment 2	Control 2A	Control 2B
0	6.21×10^6	0	3.68×10^6
8	4.57×10^7	0	3.50×10^6
24	9.26×10^7	0	2.40×10^6
32	9.85×10^7	0	2.00×10^6
48	2.80×10^8	0	3.00×10^5
56	1.68×10^8	0	2.60×10^4
72	1.36×10^8	0	1.20×10^4

Table 3.16. The growth of C. albicans GDH 1878 cultured in whole saliva supplemented with 200mM glucose over a 3 day period (Experiment 2 and the two Control experiments 2A and 2B).
Control 2A - Whole saliva supplemented with 200mM glucose, only.
Control 2B - Heat killed C. albicans GDH 1878 incubated in whole saliva supplemented with 200mM glucose.

Time hours	Mean colony forming units per ml Experiment 1+2	Control 1A+2A	Control 1B+2B
0	5.06×10^6	0	3.69×10^6
8*	4.57×10^7	0	3.50×10^6
24	5.63×10^7	0	2.2×10^6
32*	9.85×10^7	0	2.0×10^6
48	2.36×10^8	0	2.0×10^5
56	1.74×10^8	0	1.9×10^4
72	1.39×10^8	0	1.1×10^4

Table 3.17. The mean growth of C. albicans GDH 1878 cultured in whole saliva supplemented with 200mM glucose over a 3 day period.
* Results from Experiment 2 only.

	pH value		
Time hours	Experiment 1	Control 1A	Control 1B
0	7.28	7.20	7.30
8	ND	ND	ND
24	4.90	7.10	7.10
32	ND	ND	ND
48	3.10	7.00	7.10
56	3.00	7.00	7.00
72	3.00	7.00	7.00

Table 3.18. The variation in pH of C. albicans GDH 1878 cultured in whole saliva supplemented with 200mM glucose over a 3 day period (Experiment 1 and the two Control experiments 1A and 1B).
Control 1A - Whole saliva supplemented with 200mM glucose only.
Control 1B - Heat killed C. albicans GDH 1878 incubated in whole saliva supplemented with 200mM glucose.

ND - Not determined

Time hours	pH value Experiment 2	Control 2A	Control 2B
0	7.15	7.10	7.10
8	5.95	7.10	7.10
24	4.60	7.10	7.15
32	3.20	7.10	7.10
48	3.20	7.20	7.10
56	3.20	7.10	7.20
72	3.20	7.10	7.20

Table 3.19. The variation in pH of C. albicans GDH 1878 cultured in whole saliva supplemented with 200mM glucose over a 3 day period (Experiment 2 and the two Control experiments 2A and 2B).
Control 2A - Whole saliva supplemented with 200mM glucose only.
Control 2B - Heat killed C. albicans GDH 1878 incubated in whole saliva supplemented with 200mM glucose.

	Mean pH value		
Time hours	Experiment 1+2	Control 1A+2A	Control 1B+2B
0	7.21	7.15	7.20
8*	5.95	7.10	7.10
24	4.75	7.10	7.12
32*	3.20	7.10	7.10
48	3.15	7.10	7.10
56	3.10	7.05	7.10
72	3.10	7.05	7.10

Table 3.20. The mean pH of C. albicans GDH 1878 cultured in whole saliva supplemented with 200mM glucose over a 3 day period.

* Results of 1 experiment only.

mean pH of the cultures changed from 7.21 to 3.10 over the 72 hour period with a sharp drop occurring during the first 24 hours of incubation (7.21 to 4.75). This rapid decline in pH is clearly seen in Figure 3.9. which was presented earlier.

There was no change in the pH of the two control samples, during the experimental period.

3.3.8. Production of acids by C. albicans GDH 1878 when cultured in pooled whole saliva

The acid anions produced by C. albicans GDH 1878 in whole saliva supplemented with 200mM glucose and the two controls are shown in Tables 3.21 to 3.23. The qualitative and quantitative analysis of short-chain carboxylic acids as analysed by isotachophoresis indicate the production of the following acids, in descending order of concentration: pyruvate, acetate, succinate, propionate, lactate and formate. Figure 3.13., shows the range of the acid anions produced and it is clear that the production of pyruvate is the predominant factor in these assays. The maximum concentration of pyruvate 15.50 mmol/litre was found at 32 hours. A lower concentration of pyruvate (11.9 mmol/l) was detected after 48 hours of incubation. The range of concentrations of the other acid anions produced after 48 hours of incubation are as follows: acetate (1.83 to 1.33) mmol/l; succinate (0.12 to 1.35) mmol/l; propionate (0.71 to 0.75) mmol/l; lactate (0.45 to 0.72)mmol/l and formate (0.22 to 0.45) mmol/l. There was no further increase in the concentration

Time	Sample	Formate	Pyruvate	Lactate	Succinate	Acetate	Propionate	Total acids
	T1	0.29*	ND	0.70	0.09	2.26	1.08	4.42
0+	C1A	0.31	ND	0.72	0.10	1.92	ND	3.05
	C1B	0.29	ND	0.55	0.08	2.41	1.04	4.37
	T1	-	-	-	-	-	-	-
8	C1A	-	-	-	-	-	-	-
	C1B	-	-	-	-	-	-	-
	T1	0.34	2.20	0.66	0.51	1.52	0.88	6.11
24	C1A	0.28	ND	0.71	0.12	2.09	ND	3.20
	C1B	0.29	ND	0.62	0.10	2.31	1.12	4.44
	T1	-	-	-	-	-	-	-
32	C1A	-	-	-	-	-	-	-
	C1B	-	-	-	-	-	-	-
	T1	0.48	11.58	0.79	1.14	1.54	1.25	16.78
48	C1A	0.31	ND	0.55	0.15	2.37	ND	3.38
	C1B	0.28	ND	0.54	0.13	2.20	1.06	4.21
	T1	0.73	10.11	0.99	1.57	1.21	1.21	15.82
72	C1A	0.31	0.11	0.54	0.24	2.47	ND	3.67
	C1B	0.29	0.08	0.54	0.23	2.49	0.93	4.56

Table 3.21. The acid anions produced by C. albicans GDH 1878 cultured in pooled whole saliva with 200mM glucose over a 3 day period. T1 = Test sample 1; C1A = Whole saliva supplemented with 200mM glucose only; C1B = Heat killed C. albicans GDH 1878 incubated in whole saliva supplemented with 200mM glucose.
+ = hours; * = millimoles/litre; ND = Not detected; - = Not done.

Time	Sample	Formate	Pyruvate	Lactate	Succinate	Acetate	Propionate	Total acids
	T2	0.15*	ND	0.20	0.15	1.40	0.35	2.25
0+	C2A	0.17	ND	0.21	0.14	1.45	0.31	2.28
	C2B	0.18	ND	0.22	0.15	1.52	0.32	2.39
	T2	0.18	0.56	0.30	0.27	1.30	0.39	3.00
8	C2A	0.17	ND	0.22	0.17	1.38	0.33	2.27
	C2B	0.15	ND	0.21	0.11	1.50	0.31	2.28
	T2	0.29	7.47	0.42	0.91	1.56	0.32	10.97
24	C2A	0.17	ND	0.23	0.24	1.69	0.33	2.66
	C2B	0.14	ND	0.24	0.17	2.03	0.36	2.94
	T2	0.41	15.50	0.60	1.17	1.51	0.24	19.43
32	C2A	0.13	ND	0.30	0.36	1.71	0.22	2.72
	C2B	0.10	ND	0.26	0.88	1.38	0.18	2.80
	T2	0.42	12.34	0.66	1.56	1.12	0.26	16.35
48	C2A	0.13	ND	0.25	0.44	1.72	0.33	2.87
	C2B	0.14	ND	0.25	0.45	1.93	0.39	3.16
	T2	0.35	10.97	0.66	1.92	2.11	0.39	16.40
56	C2A	0.13	ND	0.26	0.45	1.83	0.28	2.95
	C2B	0.11	ND	0.27	0.49	1.91	0.15	2.93
	T2	0.37	9.78	0.68	1.08	3.92	0.88	16.71
72	C2A	0.11	ND	0.25	0.54	2.04	0.18	3.12
	C2B	0.15	ND	0.23	0.50	2.06	0.17	3.11

Table 3.22. The acid anions produced by C. albicans GDH 1878 cultured in pooled whole saliva supplemented with 200mM glucose over a 3 day period. T2 = Test sample 2; C2A = Whole saliva supplemented with 200mM glucose only; C2B = Heat killed C. albicans GDH 1878 incubated in whole saliva supplemented with 200mM glucose.
+ = hours; * = millimoles/litre; ND = Not detected.

Time	Sample	Formate	Pyruvate	Lactate	Succinate	Acetate	Propionate	Total acids
	T	0.22*	ND	0.45	0.12	1.83	0.71	3.33
0+	CA	0.24	ND	0.46	0.12	1.68	0.15	2.66
	CB	0.23	ND	0.38	0.11	1.96	0.68	3.38
	T	0.18	0.56	0.30	0.27	1.30	0.39	3.00
8**	CA	0.17	ND	0.22	0.17	1.38	0.33	2.27
	CB	0.15	ND	0.21	0.11	1.50	0.31	2.28
	T	0.31	4.83	0.54	0.71	1.54	0.60	8.54
24	CA	0.22	ND	0.47	0.18	1.89	0.16	2.93
	CB	0.21	ND	0.43	0.13	2.17	0.74	3.69
	T	0.41	15.50	0.60	1.17	1.51	0.24	19.43
32**	CA	0.13	ND	0.30	0.36	1.71	0.22	2.72
	CB	0.10	ND	0.26	0.88	1.38	0.18	2.80
	T	0.45	11.96	0.72	1.35	1.33	0.75	16.57
48	CA	0.22	ND	0.40	0.29	2.04	0.16	3.12
	CB	0.21	ND	0.39	0.29	2.06	0.72	3.68
	T	0.55	9.94	0.83	1.32	2.56	1.04	16.26
72	CA	0.21	0.05	0.39	0.39	2.25	0.09	3.39
	CB	0.22	0.04	0.38	0.36	2.27	0.55	3.83

Table 3.23. The mean value of acid anions produced by C. albicans GDH 1878 cultured in pooled whole saliva supplemented with 200mM glucose over a 3 day period.

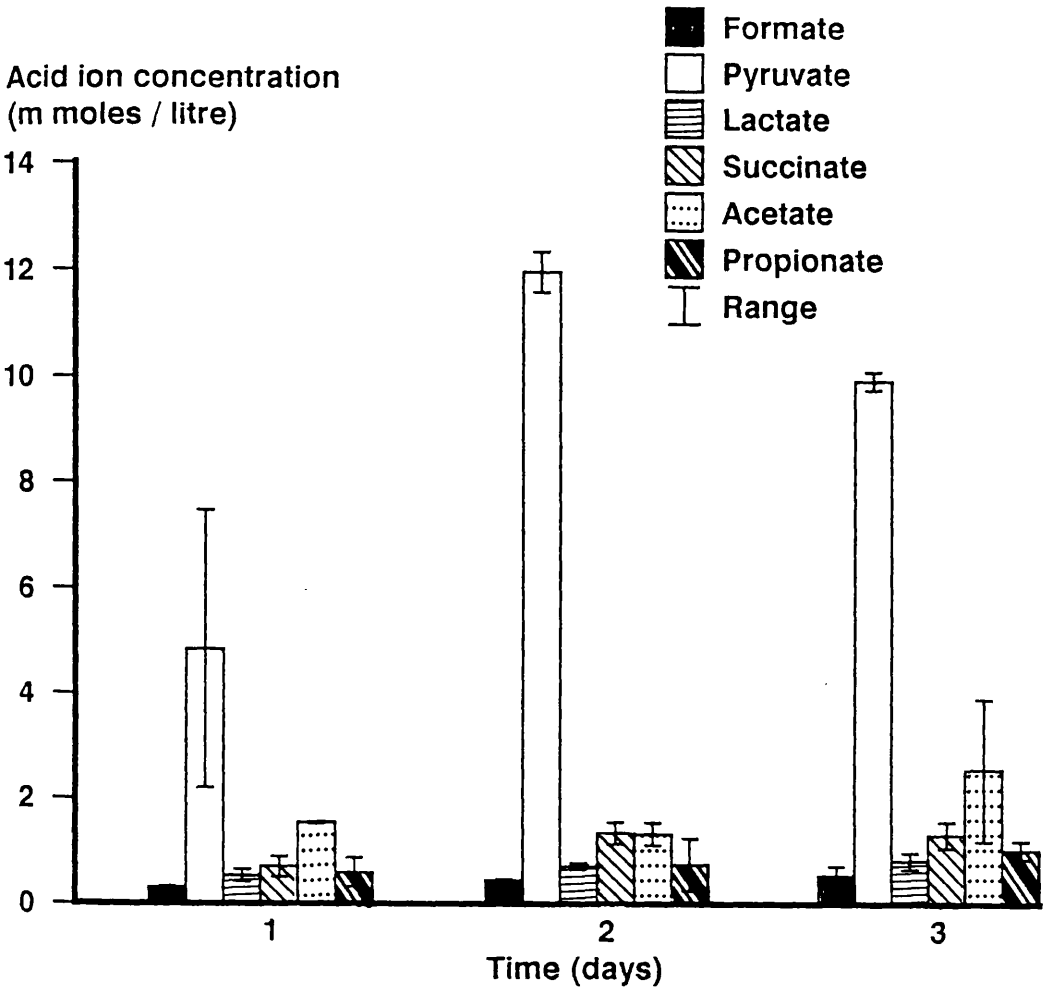

Figure 3.13 The mean total acid anions produced by C. albicans GDH 1878 cultured in pooled whole saliva supplemented with 200 mM glucose and incubated aerobically at 37°C for 3 days.

of pyruvate or succinate during the next 24 hours of incubation, while the other acid anions showed only small changes in concentration.

3.3.9. Change in protein concentration during the growth of C. albicans GDH 1878 in pooled whole saliva

The changes in pooled salivary protein concentrations during the growth of C. albicans GDH 1878 in pooled whole saliva supplemented with 200mM glucose are shown in Tables 3.24. to 3.26. and Figure 3.11. (presented earlier). There was a sharp reduction of mean protein concentration during the 72 hour period of incubation from 5.6 to 0.9 mg/ml with the maximum loss from 3.9 to 1.7 mg/ml, taking place during the second day (Table 3.26.). However, there was very little or no change in the protein concentrations of the two control experiments (Tables 3.24. and 3.25.).

3.3.10. SDS-PAGE of pooled whole saliva after culture with C. albicans GDH 1878

The culture supernatants of the pooled whole saliva samples were examined by SDS-PAGE for alterations in their salivary protein profiles as described in Sections 3.2.15. to 3.2.17. The salivary protein profiles of pooled whole saliva samples at 0 hours is shown in Figure 3.14 (a). After 48 hours incubation with C. albicans most of the protein bands in the test saliva sample became lighter (3.14 (b)) suggesting proteolysis. After 72 hours of incubation, all protein bands of the test samples disappeared. A slight reduction in

Time hours	Concentration of protein mg/ml Experiment 1	Control 1A	Control 1B
0	5.8	5.7	6.0
8	ND	ND	ND
24	4.5	5.6	5.8
32	ND	ND	ND
48	2.0	5.8	6.0
56	1.5	5.1	6.0
72	1.0	5.1	5.6

Table 3.24. The protein concentration at different time intervals when C. albicans GDH 1878 was cultured in whole saliva supplemented with 200mM glucose over a 3 day period (Experiment 1 and the two Control exeriments 1A and 1B).
Control 1A - Whole saliva supplemented with 200mM glucose only.
Control 1B - Heat killed C. albicans GDH 1878 incubated in whole saliva supplemented with 200mM glucose)
ND - Not determined

Concentration of protein mg/ml			
Time hours	Experiment 2	Control 2A	Control 2B
0	5.4	5.5	5.5
8	5.2	5.5	5.8
24	3.4	5.7	5.7
32	2.1	5.9	6.0
48	1.3	5.8	6.0
56	1.0	5.6	5.8
72	0.8	5.6	5.8

Table 3.25. The protein concentration at different time intervals when C. albicans GDH 1878 was cultured in whole saliva supplemented with 200mM glucose over a 3 day period (Experiment 2 and the two Control experiments 2A and 2B).
Control 2A - Whole saliva supplemented with 200mM glucose only.
Control 2B - Heat killed C. albicans GDH 1878 incubated in whole saliva supplemented with 200mM glucose.

Time hours	Mean concentration of protein mg/ml Experiment 1+2	Control 1A+2A	Control 2A+2B
0	5.6	5.6	5.7
8*	5.2	5.5	5.8
24	3.9	5.6	5.7
32*	2.1	5.9	6.0
48	1.7	5.8	6.0
56	1.3	5.3	5.9
72	0.9	5.3	5.7

Table 3.26. The mean concentration of protein at different time intervals when C. albicans GDH 1878 was cultured in whole saliva supplemented with 200mM glucose over a 3 day period.

* Results of experiment 2 only.

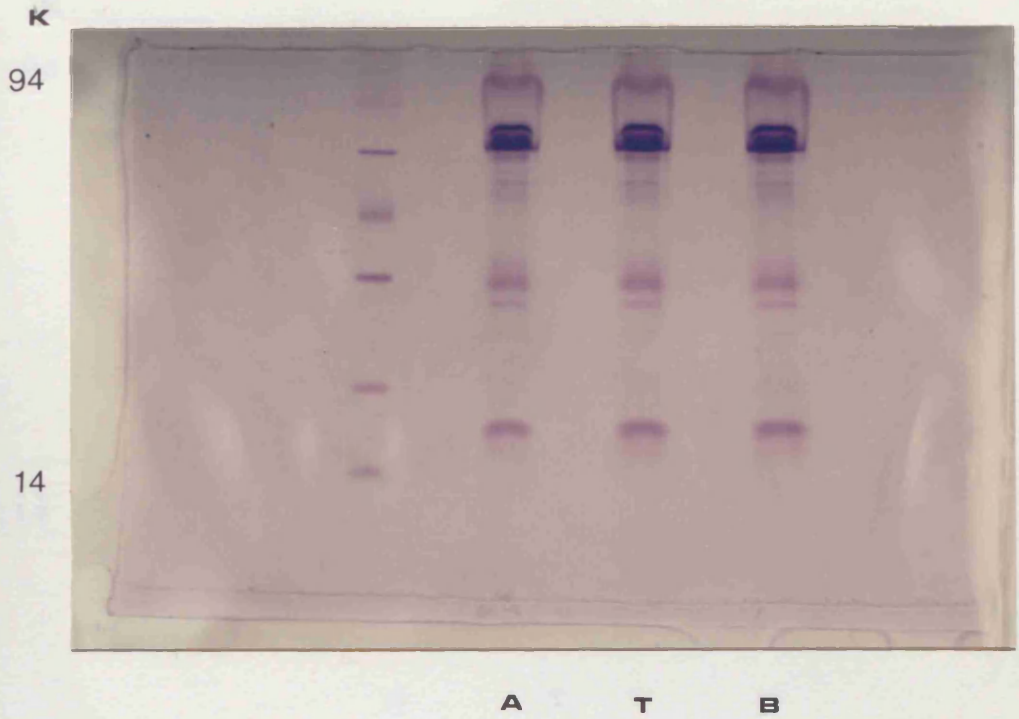

Figure 3.14 (a) The SDS-PAGE analysis patterns obtained at 0 hours of the Test and 2 Control whole saliva samples (C1A & C1B) cultured with C. albicans GDH 1878. T1 = Whole saliva test sample; C1A = Whole saliva supplemented with 200mM glucose only; C1B = Heat killed C. albicans GDH 1878 incubated in whole saliva supplemented with 200mM glucose.

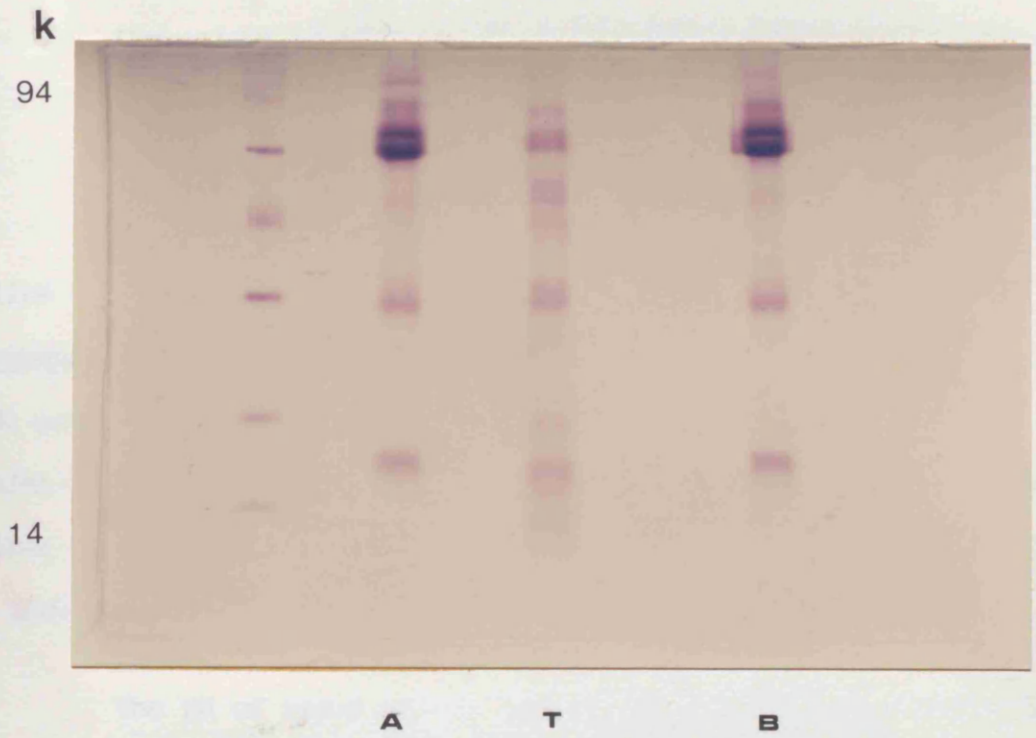

Figure 3.14(b). The SDS-PAGE analysis patterns obtained at 48 hours of the Test and 2 Control whole saliva samples (C1A & C1B) cultured with C. albicans GDH 1878. T1 = Whole saliva test sample; C1A = Whole saliva supplemented with 200mM glucose only; C1B = Heat killed C. albicans GDH 1878 incubated in whole saliva supplemented with 200mM glucose.

the intensity of staining of protein bands were also seen in the control saliva samples.

Comparison of the growth of C. albicans GDH 1878 in pooled parotid and whole saliva supplemented with 200mM glucose

When the growth of C. albicans GDH 1878 in pooled parotid saliva and in pooled whole saliva is compared it is clear that C. albicans GDH 1878 proliferates much better in the latter (Figure 3.8. presented earlier). Thus, in parotid saliva the number of cfu/ml increased from 2.89×10^6 to 6.90×10^7, while in pooled whole saliva an increase of 5.06×10^6 to 1.39×10^8 occurred over the 72 hours of incubation.

The pH of whole saliva samples showed a sharper drop from 7.2 to 3.1, when compared with parotid saliva 8.9 to 7.8, the latter remaining alkaline throughout the experimental period (Figure 3.9. presented earlier). The qualitative analysis of short chain carboxylic acids by isotachophoresis indicated that C. albicans GDH 1878 produce the same acid anions in both pooled parotid and pooled whole saliva. However, there were quantitative differences in the concentration of acid anions produced using the two forms of saliva. For example, C. albicans GDH 1878 produce a high concentration of pyruvate in whole saliva (15.50 mmol/l) but in parotid saliva acetate (8.61 mmol/l) was a major acid end product. The other acids, namely: lactate, succinate and propionate were produced in slightly higher concentration in pooled whole saliva than in parotid saliva.

When the percentage reduction of protein in whole and parotid salivary samples were compared it was evident that there was a higher percentage reduction of protein in pooled whole saliva compared with parotid saliva (Figure 3.11. presented earlier). The fact that the percentage reduction of protein in the control salivary samples was negligible, strongly suggests that the reduction of protein was due to the proteolytic activity of yeast. The SDS-PAGE protein patterns of the pooled parotid and pooled whole saliva protein samples (Figures 3.12. and 3.14. presented earlier) clearly indicate that there is a large difference between the proteolytic activity of C. albicans GDH 1878 in the two different kinds of saliva: A 83% reduction of protein was observed in pooled whole saliva while only 43% was obtained in pooled parotid sample.

From these results it was concluded that pooled whole saliva is preferable to parotid saliva for studying the growth of C. albicans GDH 1878 in saliva. As a result whole saliva was used in the experiments to study growth of other Candida species, i.e., C. tropicalis GDH 1009, C. glabrata GDH 1397 and C. krusei NCPF 3165.

3.3.11. Growth of C. tropicalis GDH 1009 when cultured in pooled whole saliva

The growth of C. tropicalis GDH 1009 in pooled whole saliva supplemented with 200mM glucose is shown in Tables 3.27 to 3.29. Yeast growth increases steadily from zero (3.96×10^6) to 32 hours (1.01×10^8) at which time stationery phase of growth was obtained

Time hours	Colony forming units per ml Experiment 1	Control 1A	Control 1B
0	4.03×10^6	0	3.76×10^6
8	2.30×10^7	0	3.40×10^6
24	6.94×10^7	0	2.10×10^6
32	1.01×10^8	0	1.68×10^6
48	1.30×10^8	0	8.90×10^5
56	1.13×10^8	0	6.80×10^5
72	1.12×10^8	0	3.80×10^5

Table 3.27. The growth of C. tropicalis GDH 1009 cultured in whole saliva supplemented with 200mM glucose over a 3 day period (Experiment 1 and the two Control experiments 1A and 1B).
Control 1A - Whole saliva supplemented with 200mM glucose, only.
Control 1B - Heat killed C. tropicalis GDH 1009 incubated in whole saliva supplemented with 200mM glucose.

Time hours	Colony forming units per ml		
	Experiment 2	Control 2A	Control 2B
0	3.90×10^6	0	5.60×10^6
8	2.56×10^7	0	3.52×10^6
24	7.55×10^7	0	2.56×10^6
32	1.02×10^8	0	1.78×10^6
48	1.18×10^8	0	9.80×10^5
56	1.21×10^8	0	7.80×10^5
72	1.20×10^8	0	4.00×10^5

Table 3.28. The growth of C. tropicalis GDH 1009 cultured in whole saliva supplemented with 200mM glucose over a 3 day period (Experiment 2 and the two Control experiments 2A and 2B).
Control 2A - Whole saliva supplemented with 200mM glucose, only.
Control 2B - Heat killed C. tropicalis GDH 1009 incubated in whole saliva supplemented with 200mM glucose.

Time	Mean colony forming units per ml		
	Experiment 1+2	Control 1A+1B	Control 2A+2B
0	$3.96x10^6$	0	$4.68x10^6$
8	$2.43x10^7$	0	$3.46x10^6$
24	$7.24x10^7$	0	$2.33x10^7$
32	$1.01x10^8$	0	$1.73x10^6$
48	$1.24x10^8$	0	$9.35x10^5$
56	$1.17x10^8$	0	$7.3x10^5$
72	$1.16x10^8$	0	$3.9x10^5$

Table 3.29. The mean growth of C. tropicalis GDH 1009 cultured in whole saliva supplemented with 200mM glucose over a 3 day period.

(Figure 3.15.). On microscopical examination, the yeasts were in the blastospore phase throughout the experiments, and no growth occurred in the control saliva samples.

Subculture of the test and control suspensions, on blood agar failed to reveal any bacterial growth, confirming the efficacy of the antibiotic supplements.

3.3.12. The pH changes in pooled whole saliva during the culture of C.tropicalis GDH 1009.

The pH change of C. tropicalis GDH 1009 in pooled whole saliva supplemented with 200mM glucose is shown in Tables 3.30 to 3.32. The mean pH of cultures changed from 7.4 to 4.05, over the 72 hour period with a sharp drop occurring during the first 24 hours of the experiment (7.4 to 4.3). This is shown in Figure 3.16.

There was little change in the pH of the 2 control samples during the experimental period.

3.3.13. Production of acids by C. tropicalis GDH 1009 when cultured in pooled whole saliva

The acid anions produced by C. tropicalis GDH 1009 in whole saliva supplemented with 200mM glucose and the two controls are shown in Tables 3.33 to 3.35. The qualitative and quantitative analysis of short-chain carboxylic acids as analysed by isotachophoresis indicate the production of the following acids, after 72 hours incubation in

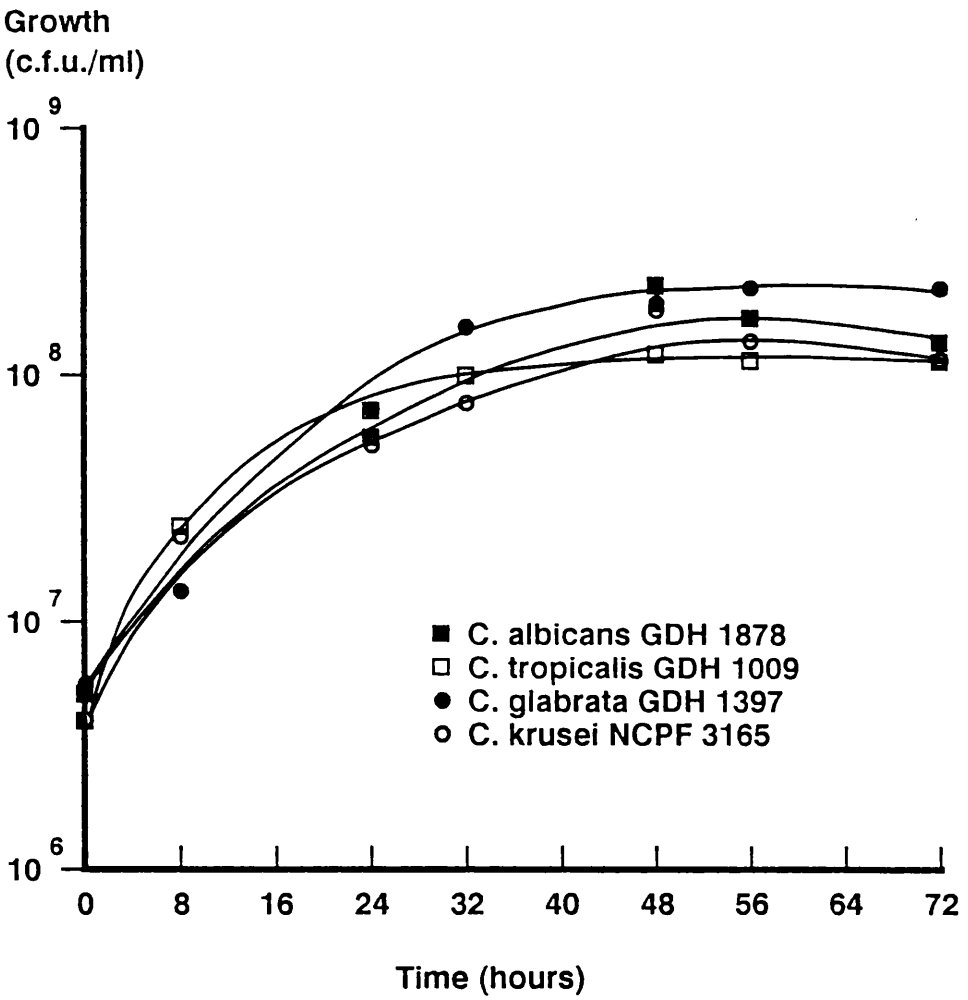

Figure 3.15 The mean growth curves of C. albicans GDH 1878; C. tropicalis GDH 1009; C. glabrata GDH 1397 and C. krusei NCPF 3165, cultured in pooled whole saliva supplemented with 200 mM glucose at 37°C and incubated aerobically for 72 hours.

Time hours	pH value Experiment 1	Control 1A	Control 1B
0	7.40	7.30	7.40
8	5.90	7.30	7.30
24	4.30	7.40	7.30
32	4.20	7.30	7.20
48	4.20	7.40	7.30
56	4.10	7.40	7.40
72	4.10	7.40	7.30

Table 3.30. The variation in pH of C. tropicalis GDH 1009 cultured in whole saliva supplemented with 200mM glucose over a 3 day period (Experiment 1 and the two Control experiments 1A and 1B).
Control 1A - Whole saliva supplemented with 200mM glucose only.
Control 1B - Heat killed C. tropicalis GDH 1009 incubated in whole saliva supplemented with 200mM glucose.

Time hours	pH value Experiment 2	Control 2A	Control 2B
0	7.40	7.40	7.40
8	6.10	7.40	7.50
24	4.30	7.40	7.50
32	3.85	7.50	7.60
48	3.90	7.30	7.70
56	4.00	7.40	7.60
72	4.00	7.50	7.60

Table 3.31. The variation in pH of C. tropicalis GDH 1009 cultured in whole saliva supplementd with 200mM glucose over a 3 day period (Experiment 2 and the two Control experiments 2A and 2B).
Control 2A - Whole saliva supplemented with 200mM glucose only.
Control 2B - Heat killed C. tropicalis GDH 1009 incubated in whole saliva supplemented with 200mM glucose.

	Mean pH value		
Time hours	Experiment 1+2	Control 1A+1B	Control 1A+2B
0	7.40	7.35	7.40
8	6.00	7.35	7.40
24	4.30	7.40	7.40
32	4.02	7.40	7.40
48	4.05	7.35	7.50
56	4.05	7.40	7.50
72	4.05	7.45	7.45

Table 3.32. The mean pH of C. tropicalis GDH 1009 cultured in whole saliva supplemented with 200mM glucose over a 3 day period.

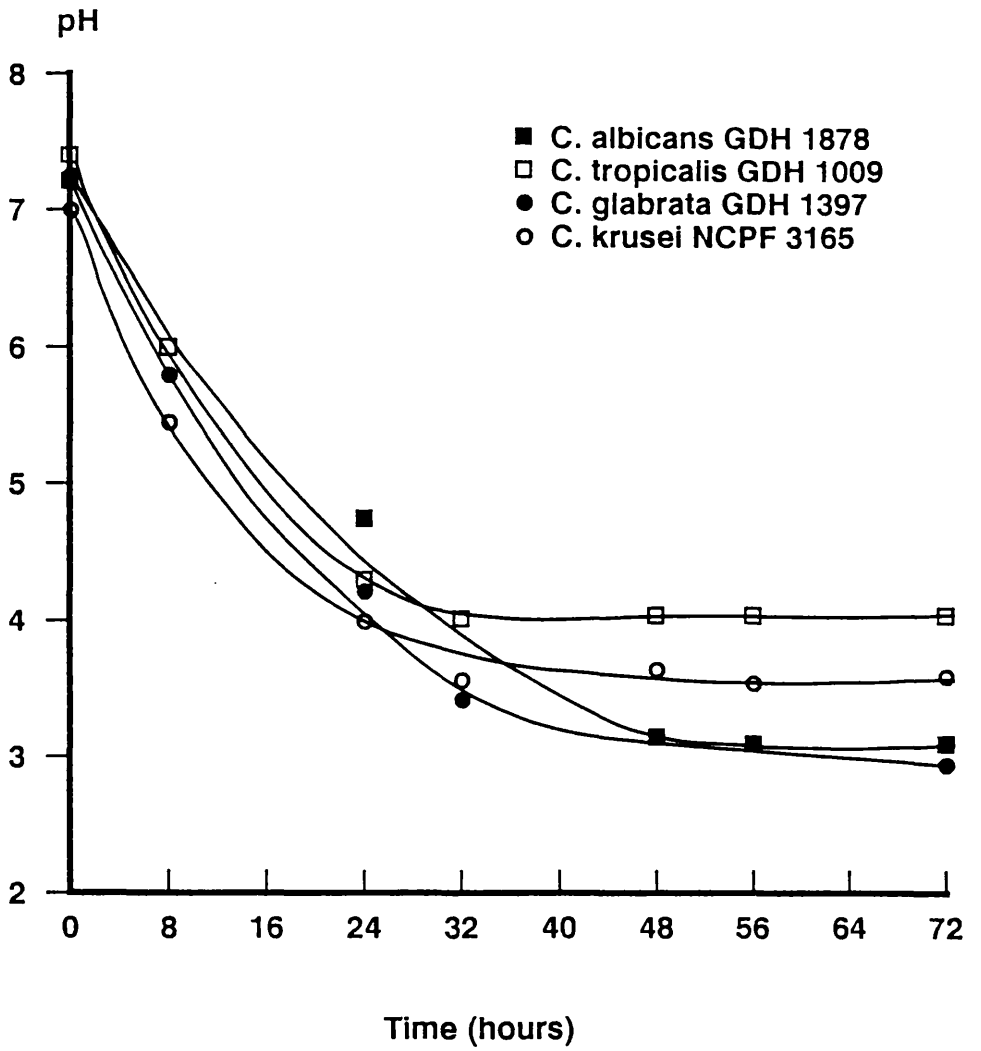

Figure 3.16 The mean pH change of C. albicans GDH 1878; C. tropicalis GDH 1009; C. glabrata GDH 1397 and C. krusei NCPF 3165, cultured in pooled whole saliva supplemented with 200 mM glucose at 37°C and incubated aerobically for 72 hours.

Time	Sample	Formate	Pyruvate	Lactate	Succinate	Acetate	Propionate	Total acids
	T1	0.19*	ND	0.30	0.52	1.91	0.48	3.40
0+	C1A	0.25	ND	0.34	0.29	1.43	0.87	3.18
	C1B	0.26	ND	0.26	0.38	1.98	0.65	3.40
	T1	0.36	0.88	0.69	0.50	2.85	0.80	6.08
8	C1A	0.19	ND	0.27	1.00	2.47	0.58	4.51
	C1B	0.26	ND	0.26	0.33	1.78	0.53	3.16
	T1	0.30	10.35	0.97	1.87	3.98	1.54	19.01
24	C1A	0.29	ND	0.29	1.09	2.12	0.67	4.46
	C1B	0.28	ND	0.30	0.48	1.72	0.64	3.42
	T1	0.31	10.02	0.94	1.39	4.30	1.87	18.83
32	C1A	0.28	ND	0.32	0.42	1.72	0.76	3.50
	C1B	0.19	ND	0.26	0.99	1.83	0.72	3.99
	T1	0.09	7.90	1.02	1.31	3.93	2.01	16.26
48	C1A	0.26	0.32	0.29	0.44	1.86	0.76	3.93
	C1B	0.26	ND	0.34	0.48	1.37	0.91	3.36
	T1	ND	10.90	1.28	2.36	4.00	2.27	20.81
56	C1A	0.26	0.36	0.45	0.46	2.32	0.90	4.75
	C1B	0.28	ND	0.24	0.76	2.32	0.86	4.46
	T1	0.31	9.26	1.35	1.66	3.51	0.78	16.87
72	C1A	0.25	0.22	0.18	0.62	1.92	0.90	4.09
	C1B	0.18	ND	0.31	1.43	2.24	0.60	4.76

Table 3.33. The acid anions produced by C. tropicalis GDH 1009 cultured in pooled whole saliva supplemented with 200mM glucose over a 3 day period. T1 = Test sample 1; C1A = Whole saliva supplemented with 200mM glucose only; C1B = Heat killed C. tropicalis GDH 1009 incubated in whole saliva supplemented with 200mM glucose. + hours; * = millimoles/litre; ND = Not detected.

Time	Sample	formate	Pyruvate	Lactate	Succinate	Acetate	Propionate	Total acids
	T2	0.24*	ND	0.87	0.22	1.08	0.79	3.20
0+	C2A	0.26	ND	0.84	0.27	0.96	0.66	2.99
	C2B	0.25	ND	0.85	0.25	0.98	0.80	3.13
	T2	0.28	0.49	1.15	0.33	1.24	0.92	4.41
8	C2A	0.24	ND	0.80	0.22	1.06	0.70	3.02
	C2B	0.24	ND	0.83	0.22	1.12	0.69	3.10
	T2	0.26	9.94	1.83	0.80	3.24	1.23	17.30
24	C2A	0.25	ND	1.18	0.26	0.92	0.77	3.38
	C2B	0.25	ND	1.23	0.27	0.98	0.79	3.52
	T2	0.23	11.03	1.57	1.19	4.32	1.29	19.63
32	C2A	0.24	0.36	1.02	0.28	1.08	0.67	3.65
	C2B	0.26	ND	0.96	0.27	1.09	0.70	3.28
	T2	0.21	10.29	1.62	1.33	4.27	1.86	19.58
48	C2A	0.24	0.29	1.09	0.28	1.11	0.75	3.76
	C2B	0.25	ND	0.97	0.31	1.11	0.70	3.34
	T2	0.25	9.34	1.60	0.98	3.69	2.06	17.92
56	C2A	0.22	0.26	0.98	0.29	1.07	0.69	3.51
	C2B	0.22	ND	1.06	0.32	1.08	0.71	3.39
	T2	0.28	8.38	1.45	1.09	2.66	2.31	16.17
72	C2A	0.22	0.32	1.06	0.36	1.22	0.65	3.83
	C2B	0.21	ND	0.73	0.34	0.87	0.76	2.91

Table 3.34. The acid anions produced by C. tropicalis GDH 1009 in pooled whole saliva supplemented with 200mM glucose over a 3 day period. T2 = Test sample 2; C2A = Whole saliva supplemented with 200mM glucose only; C2B = Heat killed C. tropicalis 1009 incubated in whole saliva supplemented with 200mM glucose.
+ = hours; * = millimoles/litre; ND = Not detected

Time	Sample	Formate	Pyruvate	Lactate	Succinate	Acetate	Propionate	Total acids
	T	0.21*	ND	0.58	0.37	1.49	0.63	3.30
0+	CA	0.25	ND	0.59	0.28	1.19	0.76	3.08
	CB	0.25	ND	0.55	0.31	1.48	0.72	3.26
	T	0.32	0.68	0.92	0.41	2.04	0.86	5.24
8	CA	0.21	ND	0.53	0.61	1.76	0.64	3.76
	CB	0.25	ND	0.54	0.27	1.45	0.61	3.13
	T	0.28	10.14	1.40	1.33	3.61	1.38	18.15
24	CA	0.27	ND	0.73	0.67	1.52	0.72	3.92
	CB	0.26	ND	0.76	0.37	1.35	0.71	3.47
	T	0.27	10.52	1.25	1.29	4.31	1.58	19.23
32	CA	0.26	ND	0.67	0.35	1.40	0.71	3.57
	CB	0.22	ND	0.61	0.63	1.46	0.71	3.63
	T	0.15	9.09	1.32	1.32	4.10	1.93	17.92
48	CA	0.25	0.30	0.69	0.36	1.48	0.75	3.84
	CB	0.25	ND	0.65	0.39	1.24	0.80	3.35
	T	0.12	10.12	1.44	1.67	3.84	2.16	19.36
56	CA	0.24	0.31	0.71	0.37	1.69	0.79	4.13
	CB	0.25	ND	0.65	0.54	1.70	0.78	3.92
	T	0.29	8.82	1.40	1.37	3.08	1.54	16.52
72	CA	0.23	0.27	0.62	0.49	1.57	0.77	3.96
	CB	0.19	3.59	0.52	0.88	1.55	0.68	3.83

Table 3.35. The mean value of acid anions produced by C. tropicalis GDH 1009 cultured in pooled whole saliva supplemented with 200mM glucose over a 3 day period.

descending order of concentration: pyruvate, acetate, propionate, lactate, succinate and formate. Maximum concentrations of pyruvate (10.52 mmol/l) and acetate (4.31 mmol/l) were produced after 32 hours of incubation (Figures 3.17. & 3.18.). Lactate, succinate and propionate concentrations were similar at 32 hours of incubation ranging from (0.58 - 1.25)mmol/l; (0.37 - 1.29)mmol/l and (0.63 - 1.58)mmol/l respectively (Figure 3.19.), whereas formate showed the lowest concentration (0.27 mmol/l). The acid anion concentrations of the 2 control experiments showed little variation during the course of the experiments.

3.3.14. Change in protein concentration during the growth of *C. tropicalis* GDH 1009 in pooled whole saliva

The change in salivary protein concentrations during the growth of *C. tropicalis* GDH 1009 in pooled whole saliva supplemented with 200mM glucose is shown in Tables 3.36 to 3.38. There was a large reduction in the mean protein concentration from 6.6 to 3.4 mg/ml during the first day, this was further reduced from 3.4 to 1.6 mg/ml during the next day with very little change occurring on the third day (1.6 to 1.5 mg/ml), (Figure 3.20.). Therefore maximum reduction of protein occurred during the first 24 hours of incubation. There were slight variations in the concentrations of protein in the two controls in both experiments (Table 3.36 and 3.37).

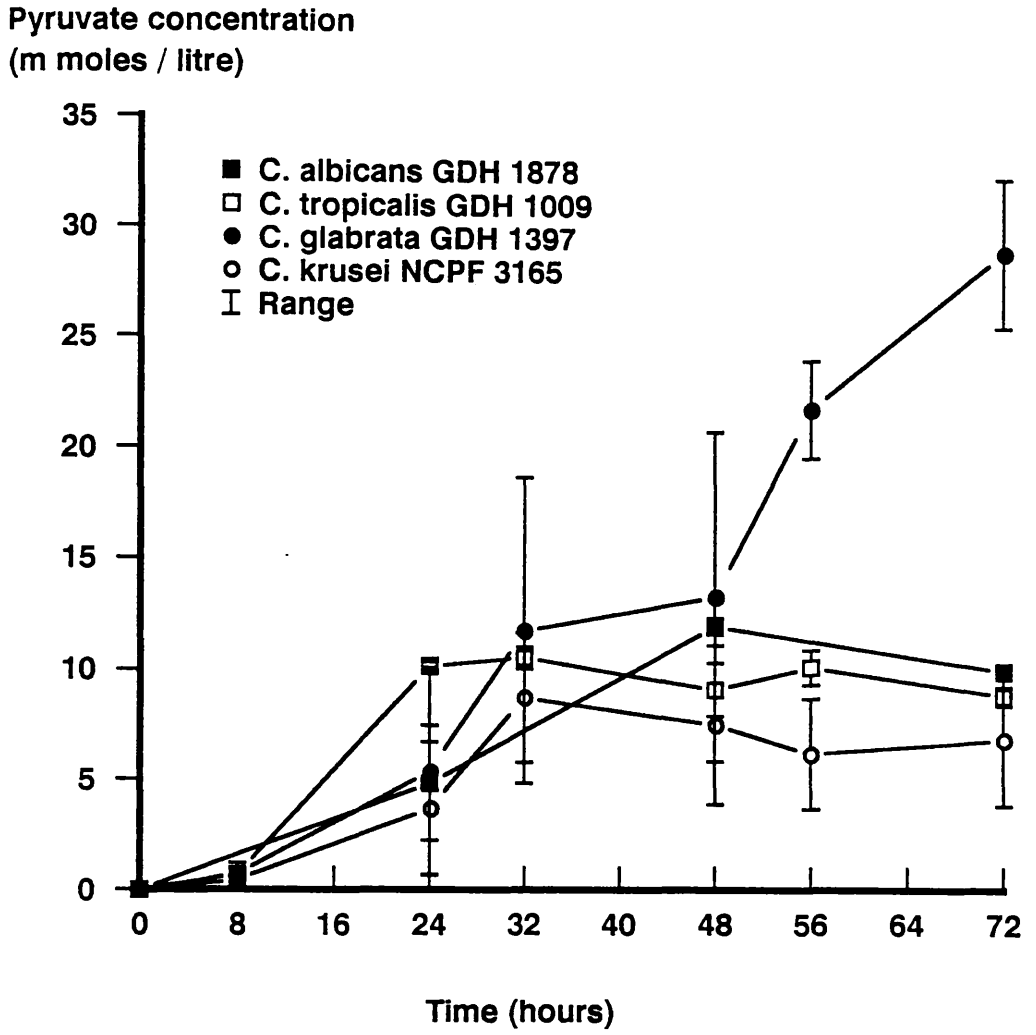

Figure 3.17 Mean pyruvate concentrations produced by C. albicans GDH 1878; C. tropicalis GDH 1009; C. glabrata GDH 1397 and C. krusei NCPF 3165 cultured in pooled whole saliva and incubated at 37°C for 72 hours.

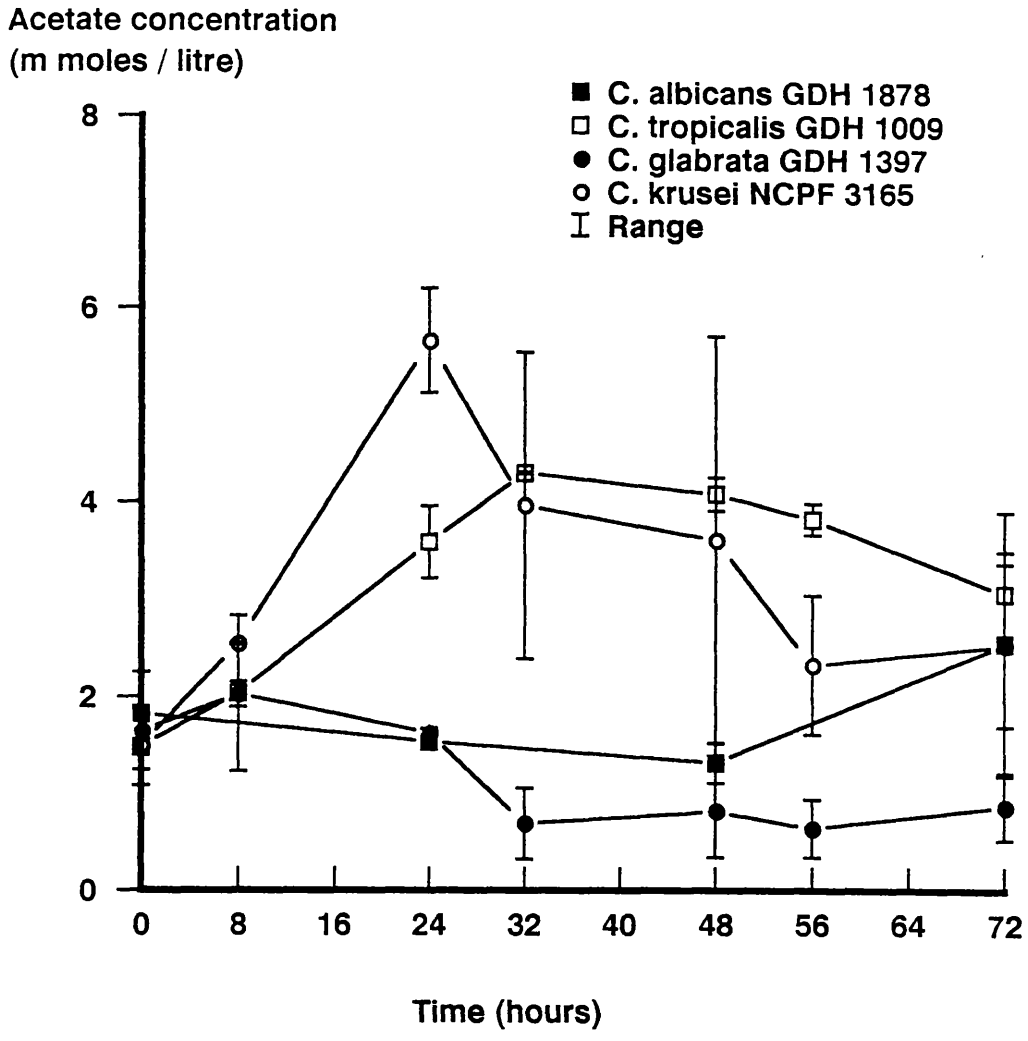

Figure 3.18 Mean acetate concentrations produced by C. albicans GDH 1878; C. tropicalis GDH 1009; C. glabrata GDH 1397 and C. krusei NCPF 3165 cultured in pooled whole saliva and incubated at 37°C for 72 hours.

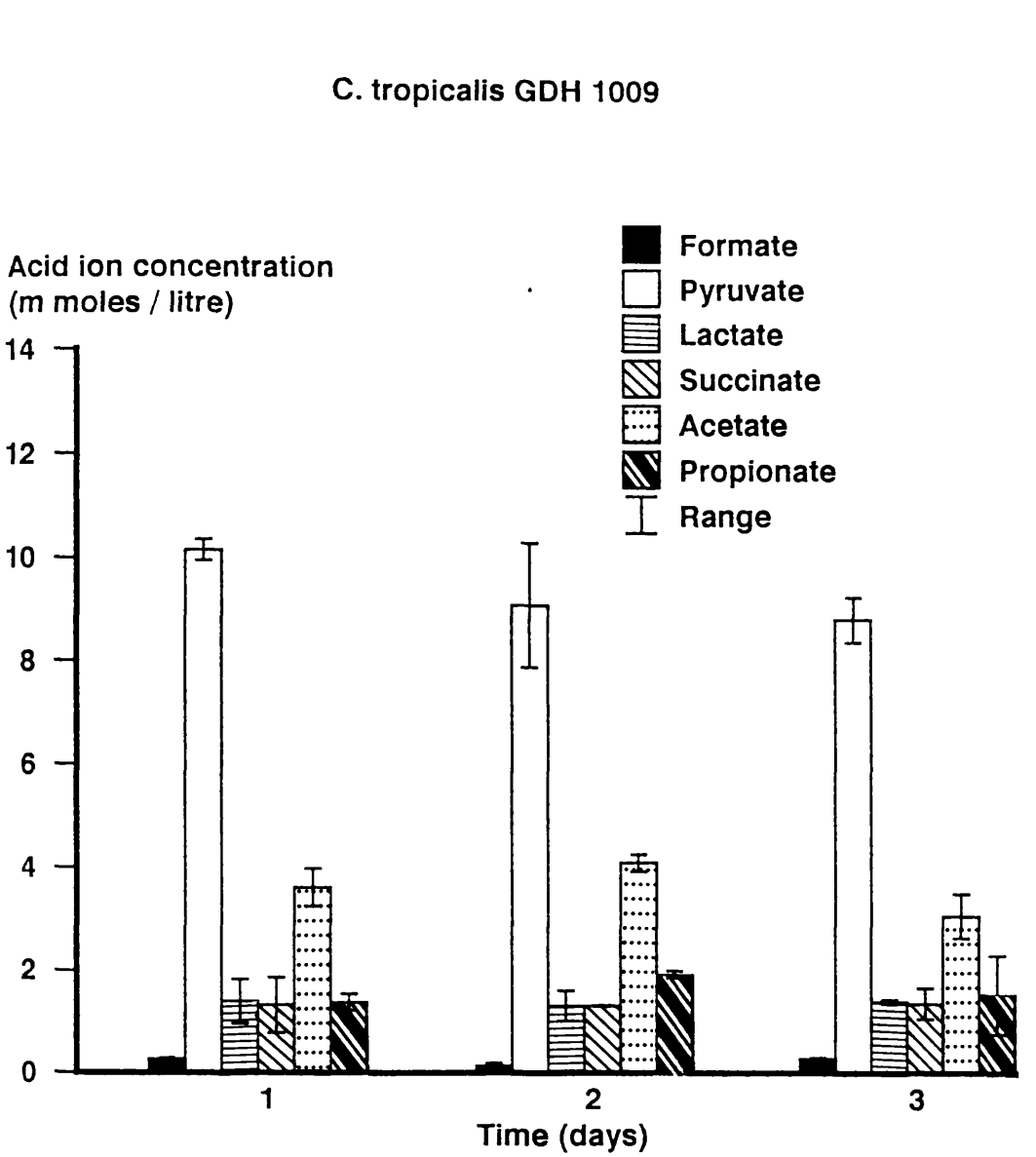

Figure 3.19 The mean total acid anions produced by C. tropicalis GDH 1009 cultured in pooled whole saliva supplemented with 200 mM glucose and incubated aerobically at 37°C for 3 days.

Concentration of protein mg/ml			
Time hours	Experiment 1	Control 1A	Control 1B
0	7.4	7.4	7.0
8	6.6	6.8	7.7
24	4.2	7.4	7.9
32	3.4	7.4	7.4
48	1.9	7.3	7.4
56	1.9	7.3	7.3
72	1.8	7.3	7.3

Table 3.36. The protein concentration at different time intervals when C. tropicalis GDH 1009 was cultured in whole saliva supplemented with 200mM glucose over a 3 day period (Experiment 1 and the two Control experiments 1A and 1B).
Control 1A - Whole saliva supplemented with 200mM glucose only.
Control 1B - Heat killed C. tropicalis GDH 1009 incubated in whole saliva supplemented with 200mM glucose.

Time hours	Concentration of protein mg/ml Experiment 2	Control 2A	Control 2B
0	5.8	5.9	5.9
8	5.3	5.8	5.9
24	2.5	6.0	5.8
32	1.3	5.6	6.1
48	1.2	5.7	6.3
56	1.2	5.9	6.2
72	1.2	6.0	6.3

Table 3.37. The protein concentration at different time intervals when C. tropicalis GDH 1009 was cultured in whole saliva supplemented with 200mM glucose over a 3 day period (Experiment 2 and the two Control experiments 2A and 2B).
Control 2A - Whole saliva supplemented with 200mM glucose only.
Control 2B - Heat killed C. tropicalis GDH 1009 incubated in whole saliva supplemented with 200mM glucose.

Time hours	Mean concentration of protein mg/ml Experiment 1+2	Control 1A+1B	Control 2A+2B
0	6.6	6.6	6.5
8	5.9	6.3	6.8
24	3.4	6.7	6.8
32	2.3	6.5	6.8
48	1.6	6.5	6.8
56	1.5	6.6	6.8
72	1.5	6.6	6.8

Table 3.38. The mean concentration of protein at different time intervals when C. tropicalis GDH 1009 was cultured in whole saliva supplemented with 200mM glucose over a 3 day period.

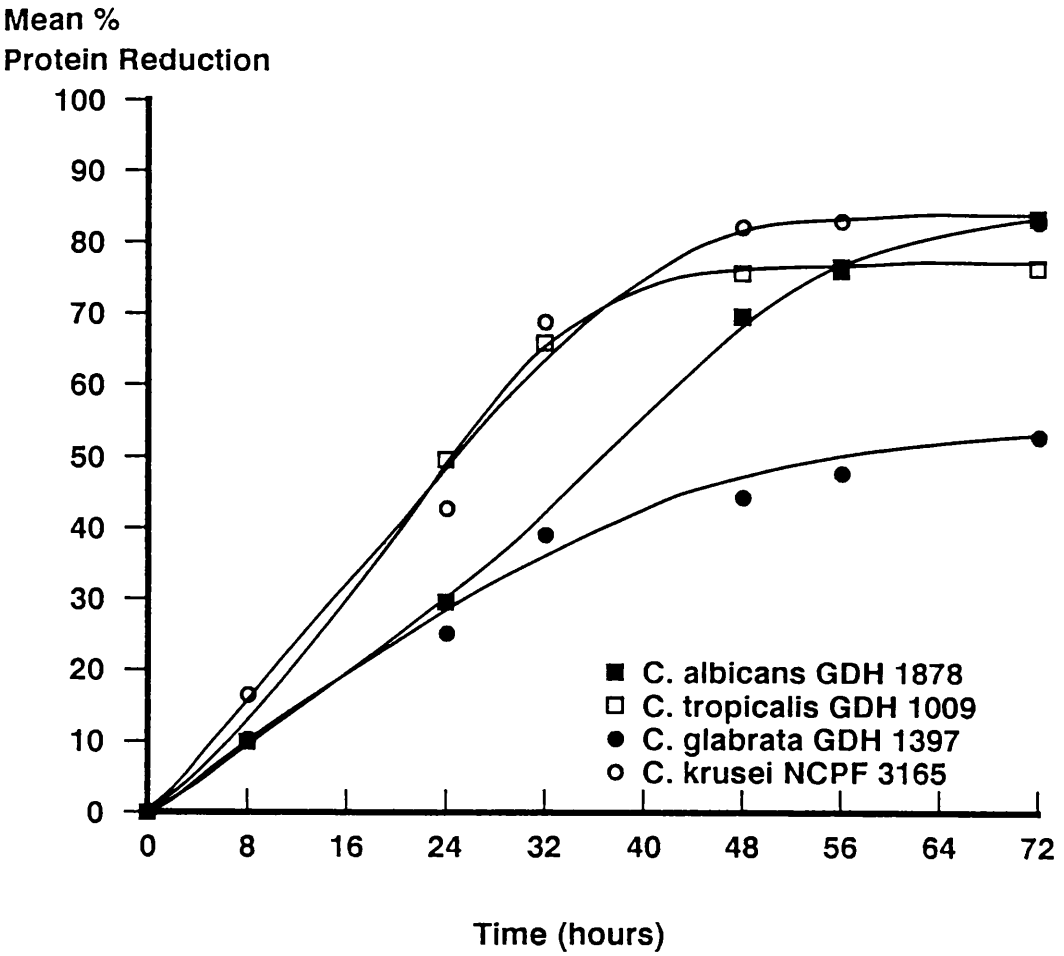

Figure 3.20 The percentage reduction in protein concentration during growth of C. albicans GDH 1878; C. tropicalis GDH 1009; C. glabrata GDH 1397 and C. krusei NCPF 3165 in pooled whole saliva supplemented with 200 mM glucose and incubated aerobically at 37°C for 72 hours.

3.3.15. SDS-PAGE of pooled whole saliva after culture with C. tropicalis GDH 1009

The culture supernatants of the pooled whole saliva samples were examined by polyacrylamide gel electrophoresis for alterations in their salivary protein profiles as described in Sections 3.2.15. to 3.2.17. The SDS-PAGE patterns of the culture supernatant at 24 hours and 48 hours were very similar to SDS-PAGE patterns obtained for C. albicans GDH 1878. As the time of incubation increased the protein bands in the test saliva samples became lighter indicating probable salivary proteolysis. A reduction in the intensity of the protein bands were also seen in the control saliva samples but this was very small, compared with the changes which occurred in the test samples.

3.3.16. Growth of C. glabrata GDH 1397 when cultured in pooled saliva

The growth of C. glabrata GDH 1397 in pooled whole saliva supplemented with 200mM glucose is shown in Tables 3.39 to 3.41. There is rapid growth during the first 32 hours, with a mean increase from 5.60×10^6 to 1.60×10^8 colony forming units per. ml. The yeast reaches the stationery phase of growth at about 32 hours, showing little change in growth during the next 40 hours of incubation (Figure 3.15. presented earlier). On microscopical examination, the yeasts were in the blastospore phase throughout the experiments, and no growth occurred in the control saliva samples.

Time hours	Colony forming units per ml Experiment 1	Control 1A	Control 1B
0	4.60×10^6	0	5.60×10^6
8	1.15×10^7	0	5.00×10^6
24	7.66×10^7	0	3.50×10^5
32	1.88×10^8	0	2.00×10^5
48	2.24×10^8	0	48.00×10^4
56	2.37×10^8	0	20.00×10^4
72	2.26×10^8	0	10.00×10^4

Table 3.39. The growth of C. glabrata GDH 1397 cultured in whole saliva supplemented with 200mM glucose over a 3 day period (Experiment 1 and the two Control experiments 1A and 1B).
Control 1A - Whole saliva supplemented with 200mM glucose, only.
Control 1B - Heat killed C. glabrata GDH 1397 incubated in whole saliva supplemented with 200mM glucose.

Time hours	Colony forming units per ml		
	Experiment 2	Control 2A	Control 2B
0	6.60×10^6	0	7.6×10^6
8	1.54×10^7	0	6.60×10^6
24	7.12×10^7	0	5.60×10^5
32	1.32×10^8	0	3.50×10^5
48	1.79×10^8	0	42.00×10^4
56	2.27×10^8	0	33.00×10^4
72	2.36×10^8	0	25.00×10^4

Table 3.40. The growth of C. glabrata GDH 1397 cultured in whole saliva supplemented with 200mM glucose over a 3 day period (Experiment 2 and the two Control experiments 2A and 2B).
Control 2A - Whole saliva supplemented with 200mM glucose, only.
Control 2B - Heat killed C. glabrata GDH 1397 incubated in whole saliva supplemented with 200mM glucose.

Time hours	Mean colony forming units per ml Experiment 1+2	Control 1A+1B	Control 2A+2B
0	$5.60x10^6$	0	$6.6x10^6$
8	$1.34x10^7$	0	$5.8x10^6$
24	$7.39x10^7$	0	$4.55x10^5$
32	$1.60x10^8$	0	$2.75x10^5$
48	$2.00x10^8$	0	$4.5x10^5$
56	$2.32x10^8$	0	$2.65x10^5$
72	$2.30x10^8$	0	$1.75x10^5$

Table 3.41. The mean growth of C. glabrata GDH 1397 cultured in whole saliva supplemented with 200mM glucose over a 3 day period.

Subculture of the test and control suspensions, on blood agar failed to reveal any bacterial growth, confirming the efficacy of the antibiotic supplements.

3.3.17. The pH changes in pooled whole saliva during the culture of C. glabrata GDH 1397

The pH change of C. glabrata GDH 1397 in pooled saliva supplemented with 200mM glucose is shown in Tables 3.42 to 3.44. There is a continous reduction in pH during the 72 hours (Figure 3.16. presented earlier), with a sharp mean drop from 7.25 to 3.42 during the first 32 hours and then a gradual reduction during the next 40 hours reaching a minimum pH of 2.95. There was no change in the pH of the 2 control saliva samples, during the experimental period.

3.3.18. Production of acids by C. glabrata GDH 1397 when cultured in pooled whole saliva

The acid anions produced by C. glabrata GDH 1397 in whole saliva supplemented with 200mM glucose and the two controls are shown in Tables 3.45 to 3.47. A relatively high concentration of pyruvate is produced by this yeast strain reaching a mean maximum of 28.8 mmol/l after 72 hours of incubation (Figure 3.17. presented earlier). The concentration of succinate was the next highest 2.07 mmol/l, while the concentration of acetate varied from 1.65 to 0.88 mmol/l during the experimental period (Figure 3.18. presented earlier). Propionate, lactate and formate showed little variation during the

Time hours	pH value Experiment 1	Control 1A	Control 1B
0	7.50	7.60	7.60
8	6.10	7.60	7.70
24	4.50	7.50	7.50
32	3.60	7.60	7.60
48	3.40	7.60	7.60
56	3.50	7.60	7.60
72	3.50	7.60	7.60

Table 3.42. The variation in pH of C. glabrata GDH 1397 cultured in whole saliva supplemented with 200mM glucose over a 3 day period (Experiment 1 and the two Control experiments 1A and 1B).
Control 1A - Whole saliva supplemented with 200mM glucose only.
Control 1B - Heat killed C. glabrata GDH 1397 incubated in whole saliva supplemented with 200mM glucose.

Time hours	pH value Experiment 2	Control 2A	Control 2B
0	7.00	7.00	7.00
8	5.55	7.00	7.00
24	3.95	6.90	6.95
32	3.25	7.00	7.00
48	2.90	7.00	7.00
56	2.70	7.00	7.00
72	2.40	7.00	7.00

Table 3.43. The variation in pH of C. glabrata GDH 1397 cultured in whole saliva supplemented with 200mM glucose over a 3 day period (Experiment 2 and the two Control experiments 2A and 2B).
Control 2A - Whole saliva supplemented with 200mM glucose only.
Control 2B - Heat killed C. glabrata GDH 1397 incubated in whole saliva supplemented with 200mM glucose.

Time hours	Mean pH value Experiment 1+2	Control 1A+1B	Control 2A+2B
0	7.25	7.30	7.30
8	5.80	7.30	7.35
24	4.22	7.20	7.22
32	3.42	7.30	7.30
48	3.15	7.30	7.30
56	3.10	7.30	7.30
72	2.95	7.30	7.30

Table 3.44. The mean pH of C. glabrata 1397 cultured in whole saliva supplemented with 200mM glucose over a 3 day period.

Time	Sample	Formate	Pyruvate	Lactate	Succinate	Acetate	Propionate	Total acids
	T1	0.44*	ND	0.31	0.18	1.44	0.96	3.33
0+	C1A	0.44	ND	0.44	0.19	1.31	0.92	3.13
	1B	0.44	ND	0.29	0.19	1.36	0.91	3.19
	T1	0.40	0.22	0.26	0.35	2.16	0.54	3.93
8	C1A	0.38	ND	0.26	0.19	1.32	0.88	3.03
	C1B	0.41	ND	0.40	0.20	1.36	0.90	3.27
	T1	0.23	0.67	0.35	1.62	1.68	0.74	5.29
24	C1A	0.40	ND	0.27	0.20	1.32	0.86	3.05
	C1B	0.43	ND	0.36	0.23	1.35	0.97	3.34
	T1	0.26	4.85	0.25	1.34	1.07	0.70	8.47
32	C1A	0.40	ND	0.44	0.23	1.33	0.79	3.19
	C1B	0.40	ND	0.31	0.20	1.31	0.87	3.09
	T1	0.28	5.84	0.41	1.63	1.31	0.77	10.24
48	C1A	0.41	ND	0.27	0.25	1.26	0.87	3.06
	C1B	0.38	ND	0.30	0.25	1.29	0.80	3.02
	T1	0.21	19.52	0.29	1.35	0.95	0.60	22.92
56	C1A	0.38	ND	0.28	0.25	1.48	0.81	3.20
	C1B	0.40	ND	0.27	0.24	1.42	0.69	3.02
	T1	0.22	25.43	0.48	2.95	1.23	0.74	31.05
72	C1A	0.33	ND	0.24	0.24	1.32	0.72	2.85
	C1B	0.36	ND	0.28	0.35	1.52	0.77	3.28

Table 3.45. The acid anions produced by C. glabrata GDH 1397 cultured in pooled whole saliva supplemented with 200mM glucose over a 3 day period. T1 = Test sample 1; C1A = Whole saliva supplemented with 200mM glucose only; C1B = Heat killed C. glabrata GDH 1397 incubated in whole saliva supplemented with 200mM glucose. + = hours; * = millimoles/litre; ND = Not detected.

Time	Sample	Formate	Pyruvate	Lactate	Succinate	Acetate	Propionate	Total acids
	T2	0.28*	ND	0.37	0.15	1.87	0.80	3.47
0+	C2A	0.33	ND	0.43	0.17	1.87	0.95	3.75
	C2B	0.30	ND	0.38	0.15	1.81	0.77	3.41
	T2	0.28	1.22	0.50	0.22	1.90	0.80	4.92
8	C2A	0.30	ND	0.36	0.15	1.88	0.72	3.41
	C2B	0.28	ND	0.55	0.19	1.59	0.61	3.22
	T2	0.24	10.07	0.65	0.51	1.58	0.95	14.00
24	C2A	0.30	0.29	0.65	0.24	1.81	0.74	4.03
	C2B	0.28	ND	0.50	0.15	1.66	0.65	3.24
	T2	0.19	18.62	0.90	0.81	0.34	1.20	22.06
32	C2A	0.28	ND	0.83	0.25	1.35	0.72	3.43
	C2B	0.26	ND	0.65	0.25	1.71	0.77	3.64
	T2	0.26	20.72	1.09	1.27	0.36	1.74	25.44
48	C2A	0.26	0.39	0.56	0.26	1.66	0.81	3.94
	C2B	0.28	ND	0.55	0.25	1.56	0.71	3.35
	T2	0.26	23.97	0.87	1.10	0.36	1.31	27.87
56	C2A	0.26	0.39	0.58	0.29	1.54	0.74	3.80
	C2B	0.26	ND	0.39	0.28	1.90	0.86	3.69
	T2	0.31	32.26	1.00	1.19	0.54	1.47	36.77
72	C2A	0.28	0.46	0.43	0.28	2.04	0.85	4.34
	C2B	0.25	ND	0.41	0.30	1.99	0.87	3.82

Table 3.46. The acid anions produced by C. glabrata GDH 1397 cultured in pooled whole saliva supplemented with 200mM glucose over a 3 day period. T2 = Test sample 2; C2A = whole saliva supplemented with 200mM glucose only; C2B = Heat killed C. glabrata GDH 1397 incubated in whole saliva supplemented with 200mM glucose. + = hours; * = millimoles/litre; ND = Not detected.

Time	Sample	Formate	Pyruvate	Lactate	Succinate	Acetate	Propionate	Total acids
	T	0.36*	ND	0.34	0.16	1.65	0.88	3.40
0+	CA	0.38	ND	0.43	0.18	1.59	0.93	3.52
	CB	0.37	ND	0.33	0.17	1.58	0.84	3.30
	T	0.34	0.72	0.38	0.28	2.03	0.67	4.42
8	CA	0.34	ND	0.31	0.17	1.60	0.80	3.22
	CB	0.34	ND	0.47	0.19	1.47	0.75	3.24
	T	0.23	5.37	0.50	1.06	1.63	0.84	9.64
24	CA	0.35	0.14	0.46	0.22	1.56	0.80	3.54
	CB	0.35	ND	0.43	0.19	1.50	0.81	3.29
	T	0.22	11.73	0.57	1.07	0.70	0.95	15.26
32	CA	0.34	ND	0.63	0.24	1.34	0.75	3.31
	CB	0.33	ND	0.48	0.22	1.51	0.82	3.36
	T	0.27	13.28	0.75	1.45	0.83	1.25	17.84
48	CA	0.33	0.19	0.41	0.25	1.46	0.84	3.50
	CB	0.33	ND	0.42	0.25	1.42	0.75	3.18
	T	0.23	21.74	0.58	1.22	0.65	0.95	25.39
56	CA	0.32	0.19	0.43	0.27	1.51	0.77	3.50
	CB	0.33	ND	0.33	0.26	1.66	0.77	3.35
	T	0.26	28.84	0.74	2.07	0.88	1.10	33.91
72	CA	0.30	0.23	0.33	0.26	1.68	0.78	3.59
	CB	0.30	ND	0.34	0.32	1.75	0.82	3.55

Table 3.47. The mean value of acid anions produced by C. glabrata GDH 1397 cultured in pooled whole saliva supplemented with 200mM glucose over a 3 day period.

experiments (Figure 3.21.).

The acid anion concentration of the 2 control experiments showedlittle changethroughout the courseofthe experiments.

3.3.19. Change in protein concentration during the growth of C. glabrata GDH 1397 in pooled whole saliva

The change in salivary protein concentration during the growth of C. glabrata GDH 1397 in pooled whole saliva supplemented with 200mM glucose is shown in Tables 3.48 to 3.50. These results indicate a marked reduction in protein concentration during the 72 hour period of incubation changing from an initial mean concentration of 5.4 to 2.5 mg/ml. The reduction in the % reduction of protein during the first, second and third days were 25%, 44%, and 53% respectively. Therefore a very gradual % reduction of salivary protein was observed (Figure 3.20. presented earlier). The protein concentration of control 1A of the 1st experiment (Table 3.48.) is more stable compared with control 2A of the 2nd experiment (Table 3.49.). The protein concentrations of all controls tended to increase overall with time, and this was most marked with control 2A which increased by 1.1 mg/ml over 72 hours.

3.3.20. SDS-PAGE of pooled whole saliva after culture with C. glabrata GDH 1397

The culture supernatants of the pooled whole saliva samples were examined by polyacrylamide gel electrophoresis for alterations in

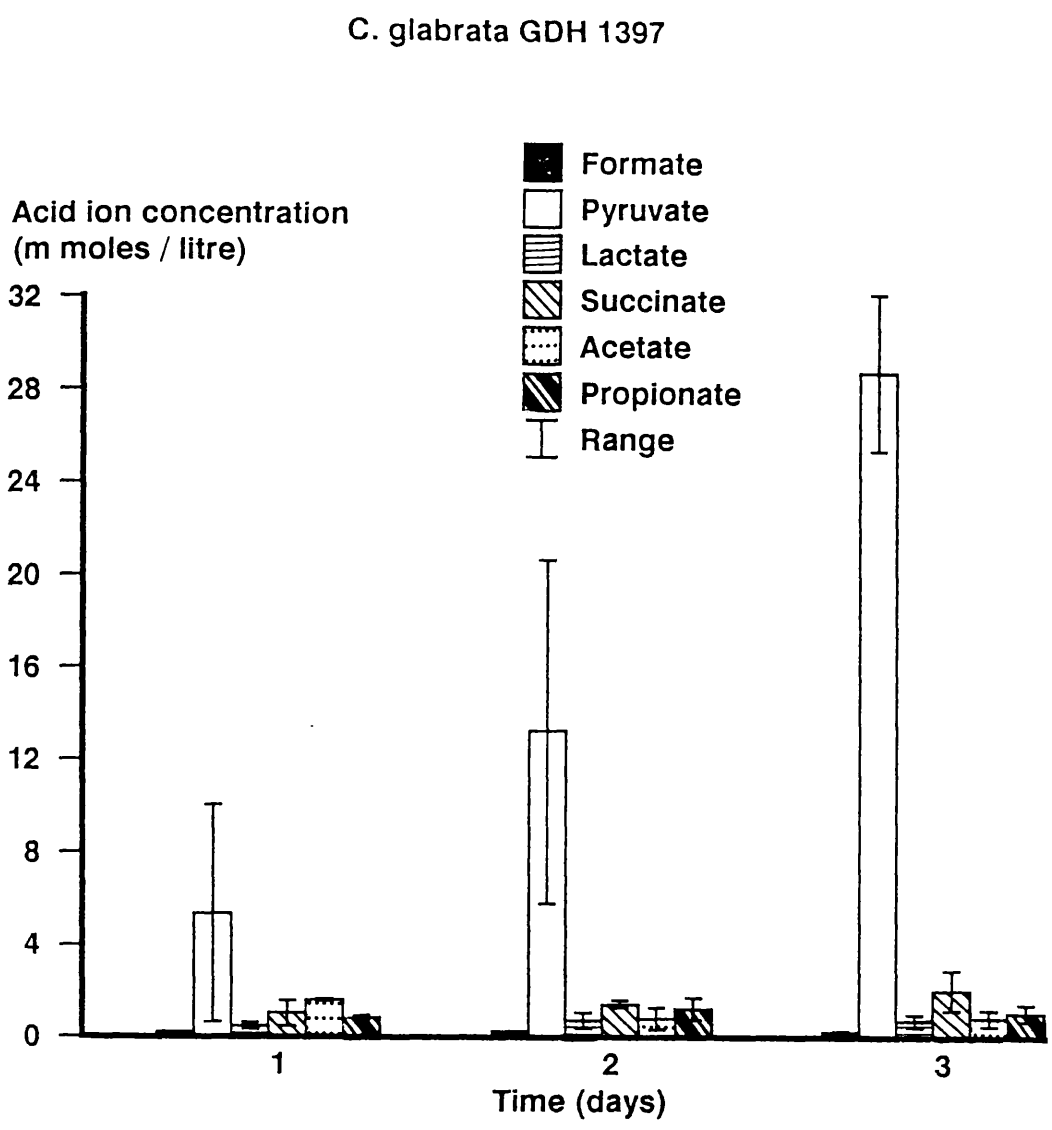

Figure 3.21 The mean total acid anions produced by C. glabrata GDH 1397 cultured in pooled whole saliva supplemented with 200 mM glucose and incubated aerobically at 37°C for 3 days.

Time hours	Concentration of protein mg/ml Experiment 1	Control 1A	Control 1B
0	5.8	5.9	5.8
8	5.2	6.0	5.8
24	4.4	5.8	5.9
32	3.7	5.9	5.9
48	3.1	5.9	6.1
56	3.1	5.8	6.4
72	2.9	6.0	6.5

Table 3.48. The protein concentration at different time intervals when C. glabrata GDH 1397 was cultured in whole saliva supplemented with 200mM glucose over a 3 day period (Experiment 1 and the two Control experiments 1A and 1B).
Control 1A - Whole saliva supplemented with 200mM glucose only.
Control 1B - Heat killed C. glabrata GDH 1397 incubated in whole saliva supplemented with 200mM glucose.

Time hours	Concentration of protein mg/ml Experiment 2	Control 2A	Control 2B
0	5.0	5.0	5.0
8	4.5	5.0	5.0
24	3.7	5.1	5.2
32	2.9	5.1	5.3
48	2.8	5.9	5.5
56	2.5	5.9	6.7
72	2.2	6.1	6.7

Table 3.49. The protein concentration at different time intervals when C. glabrata GDH 1397 was cultured in whole saliva supplemented with 200mM glucose over a 3 day period (Experiment 2 and the two Control experiments 2A and 2B).
Control 2A - Whole saliva supplemented with 200mM glucose only.
Control 2B - Heat killed C. glabrata GDH 1397 incubated in whole saliva supplemented with 200mM glucose.

Mean concentration of protein mg/ml			
Time hours	Experiment 1+2	Control 1A+1B	Control 2A+2B
0	5.4	5.4	5.4
8	4.9	5.5	5.4
24	4.0	5.4	5.5
32	3.3	5.5	5.6
48	3.0	5.9	5.8
56	2.8	5.9	6.6
72	2.5	6.0	6.6

Table 3.50. The mean concentration of protein at different time intervals when C. glabrata GDH 1397 was cultured in whole saliva supplemented with 200mM glucose over a 3 day period.

their salivary protein profiles as described in Sections 3.2.15. to 3.2.17. The SDS-PAGE analysis patterns of the test and control saliva samples at 0, 24 and 48 hours of incubation were very similar to the SDS-PAGE analysis patterns of the test and control salivary protein patterns of C. albicans GDH 1878 (Figures 3.14 (a) & (b) presented earlier) showing a gradual reduction of the protein bands during the course of the experiment.

3.3.21. Growth of C.krusei NCPF 3165 when cultured in pooled whole saliva

The growth of C. krusei NCPF 3165 in pooled wholesaliva supplemented with 200mM glucose is shown in Tables 3.51 to 3.53. The yeast proliferated rapidly during the first 48 hours from a mean of 4.01×10^6 to 1.88×10^8 cfu/ml and reached the stationery phase of growth at about 48 hours (Figure 3.15. presented earlier). On microscopical examination, the yeasts were in the blastospore phase throughout the experiments, and no growth occurred in the control saliva samples.

Subculture of the test and control suspensions, on blood agar failed to reveal any bacterial growth, confirming the efficacy of the antibiotic supplements.

Time hours	Colony forming units per ml		
	Experiment 1	Control 1A	Control 1B
0	2.81×10^6	0	2.80×10^6
8	1.12×10^7	0	1.70×10^6
24	4.08×10^7	0	1.60×10^6
32	6.88×10^7	0	2.60×10^5
48	1.86×10^8	0	2.50×10^5
56	1.20×10^8	0	2.40×10^4
72	1.18×10^8	0	1.00×10^4

Table 3.51. The growth of C. krusei NCPF 3165 cultured in whole saliva supplemented with 200mM glucose over a 3 day period (Experiment 1 and two Control experiments 1A and 1B).
Control 1A - Whole saliva supplemented with 200mM glucose, only.
Control 1B - Heat killed C. krusei NCPF 3165 incubated in whole saliva supplemented with 200mM glucose.

Time	Colony forming units per ml		
	Experiment 2	Control 2A	Control 2B
0	5.21×10^6	0	6.80×10^6
8	3.3×10^7	0	6.30×10^6
24	6.41×10^7	0	4.60×10^6
32	8.82×10^7	0	3.20×10^5
48	1.90×10^8	0	3.00×10^5
56	1.62×10^8	0	2.80×10^4
72	1.21×10^8	0	2.00×10^4

Table 3.52. The growth of C. krusei NCPF 3165 cultured in whole saliva supplemented with 200mM glucose over a 3 day period (Experiment 2 and the two Control experiments 2A and 2B).
Control 2A - Whole saliva supplemented with 200mM glucose, only.
Control 2B - Heat killed C. krusei NCPF 3165 incubated in whole saliva supplemented with 200mM glucose.

Time hours	Mean colony forming units per ml Experiment 1+2	Control 1A+1B	Control 2A+2B
0	4.01×10^6	0	4.8×10^6
8	2.22×10^7	0	4.0×10^6
24	5.24×10^7	0	3.1×10^6
32	7.85×10^7	0	2.95×10^5
48	1.88×10^8	0	2.75×10^5
56	1.41×10^8	0	2.6×10^4
72	1.19×10^8	0	1.5×10^4

Table 3.53. The mean growth of C. krusei NCPF 3165 cultured in whole saliva supplemented with 200mM glucose over a 3 day period.

3.3.22. The pH changes in pooled whole saliva during the culture of C. krusei NCPF 3165.

The pH changes in pooled whole saliva supplemented with 200mM glucose as a result of the growth of C. krusei NCPF 3165 is shown in Tables 3.54 to 3.56. The mean pH of the C. krusei cultures fell from 7.00 to 3.5 over an incubation period of 32 hours. The pH changed very little during the next 40 hours of incubation (Figure 3.16. presented earlier). The pH of the 2 control saliva samples showed very little variation, during the experimental period.

3.3.23. Production of acids by C.krusei NCPF 3165 in pooled whole saliva

The acid anions produced by C. krusei NCPF 3165 in whole saliva supplemented with 200mM glucose and the two controls are shown in Tables 3.57 to 3.59. Pyruvate was produced in highest concentration reaching 8.7 mmol/l after 32 hours incubation while acetate reached a value of 3.9 mmol/l after the same time (Figure 3.22.). Lactate and Succinate were in much lower concentrations, 1.46 and 1.67mmol/l respectively during the first 32 hours of incubation. Generally the acid anions were reduced in concentration during the final 40 hours of incubation (Figure 3.22). However, the concentrations of formate and propionate acid anions increased gradually throughout the 72 hours of the experimental period i.e., the mean formate concentration increased from 0.23 to 0.46 mmol/l and propionate from 0.43 to 1.48 mmol/l, during the same period. The

Time hours	pH value Experiment 1	Control 1A	Control 1B
0	6.70	6.80	6.80
8	5.80	6.80	6.70
24	4.00	6.80	6.80
32	3.35	6.70	6.70
48	3.40	6.70	6.70
56	3.30	6.70	6.70
72	3.40	6.70	6.70

Table 3.54. The variation in pH of C. krusei NCPF 3165 cultured in whole saliva supplemented with 200mM glucose over a 3 day period (Experiment 1 and the two Control experiments 1A and 1B).
Control 1A - Whole saliva supplemented with 200mM glucose only.
Control 1B - Heat killed C. krusei NCPF 3165 incubated in whole saliva supplemented with 200mM glucose.

Time hours	pH value Experiment 2	Control 2A	Control 2B
0	7.30	7.20	7.20
8	5.10	7.30	7.30
24	4.00	7.30	7.40
32	3.80	7.40	7.40
48	3.90	7.40	7.30
56	3.80	7.30	7.30
72	3.80	7.30	7.30

Table 3.55. The variation in pH of C. krusei NCPF 3165 cultured in whole saliva supplemented with 200mM glucose over a 3 day period (Experiment 2 and the two Control experiments 2A and 2B).
Control 2A - Whole saliva supplemented with 200mM glucose only.
Control 2B - Heat killed C. krusei NCPF 3165 incubated in whole saliva supplemented with 200mM glucose.

Time hours	Mean pH value Experiment 1+2	Control 1A+1B	Control 2A+2B
0	7.00	7.00	7.00
8	5.45	7.05	7.00
24	4.00	7.05	7.10
32	3.57	7.05	7.05
48	3.65	7.05	7.00
56	3.55	7.00	7.00
72	3.60	7.00	7.00

Table 3.56. The mean pH of C. krusei NCPF 3165 cultured in whole saliva supplemented with 200mM glucose over a 3 day period.

Time	Sample	Formate	Pyruvate	Lactate	Succinate	Acetate	Propionate	Total acids
	T1	0.29*	ND	1.01	0.40	1.75	0.12	3.57
0+	C1A	0.29	ND	0.80	0.35	2.30	0.11	3.85
	C1B	0.29	ND	0.65	0.46	2.24	0.15	3.79
	T1	0.29	ND	0.76	0.51	2.56	0.26	4.38
8	C1A	0.32	ND	0.82	0.68	2.32	0.19	4.33
	C1B	0.30	ND	0.60	0.42	2.23	0.22	3.77
	T1	0.35	6.69	1.22	0.91	5.14	0.20	14.51
24	C1A	0.29	ND	0.84	0.50	2.06	0.29	3.98
	C1B	0.30	ND	0.57	0.54	2.51	0.23	4.15
	T1	0.36	11.65	1.02	1.26	5.56	0.15	20.00
32	C1A	0.30	ND	1.14	0.49	1.87	0.22	4.02
	C1B	0.30	ND	0.92	0.49	1.88	0.23	3.82
	T1	0.39	11.09	1.52	1.91	5.73	0.37	21.01
48	C1A	0.25	0.46	0.71	0.48	2.73	0.24	4.87
	C1B	0.30	0.29	0.62	0.59	2.45	0.32	4.57
	T1	0.37	8.69	1.19	1.84	3.06	0.37	15.52
56	C1A	0.29	0.32	0.54	0.39	2.14	0.23	3.91
	C1B	0.33	0.26	0.66	0.75	2.51	0.23	4.74
	T1	0.45	9.84	0.92	1.13	3.39	1.35	17.08
72	C1A	0.48	0.12	0.64	0.13	2.38	0.81	4.56
	C1B	0.46	0.10	0.58	0.14	1.98	0.88	4.14

Table 3.57. The acid anions produced by C. krusei NCPF 3165 cultured in pooled whole saliva supplemented with 200mM glucose over a 3 day period. T1 = Test sample 1; C1A = whole saliva supplemented with 200mM glucose only; C1B = Heat killed C. krusei NCPF 3165 incubated in whole saliva supplemented with 200mM glucose. + = hours; * = millimoles/litre; ND = Not detected.

Time	Sample	Formate	Pyruvate	Lactate	Succinate	Acetate	Propionate	Total acids
	T2	0.17*	0.05	0.29	0.38	1.26	0.74	2.89
0+	C2A	0.18	0.12	0.60	0.32	2.05	0.79	4.06
	C2B	0.18	0.05	0.53	0.57	2.07	0.97	4.37
	T2	0.20	0.86	0.68	0.66	2.54	1.21	6.15
8	C2A	0.18	0.09	0.41	0.41	1.66	0.95	3.70
	C2B	0.18	0.09	0.37	0.40	1.67	0.91	3.62
	T2	0.34	0.69	2.29	1.37	6.21	1.41	12.31
24	C2A	0.17	0.12	0.41	0.45	1.51	0.87	9.68
	C2B	0.18	0.12	0.39	0.41	1.62	0.85	3.57
	T2	0.35	5.79	1.90	2.09	2.41	1.66	14.20
32	C2A	0.17	0.09	0.49	0.39	1.43	0.81	3.38
	C2B	0.18	0.09	0.58	0.45	1.53	0.85	3.68
	T2	0.45	3.89	1.33	2.05	1.54	1.81	11.07
48	C2A	0.14	0.19	2.09	0.60	1.50	0.77	5.29
	C2B	0.17	0.15	0.57	0.45	1.56	0.79	3.69
	T2	0.52	3.72	1.25	1.20	1.62	2.09	10.40
56	C2A	0.19	0.12	0.46	0.37	1.42	0.77	3.33
	C2B	0.22	0.19	0.61	0.45	1.58	0.81	3.86
	T2	0.47	3.87	0.77	0.46	1.71	1.62	8.90
72	C2A	0.21	0.01	0.65	0.10	1.59	0.74	3.30
	C2B	0.26	0.08	0.56	0.12	1.77	0.86	3.65

Table 3.58. The acid anions produced by C. krusei NCPF 3165 cultured in pooled whole saliva supplemented with 200mM glucose over a 3 day period. T2 = Test sample 2; C2A = Whole saliva supplemented with 200mM glucose only; C2B = Heat killed C. krusei NCPF 3165 incubated in whole saliva supplemented with 200mM glucose. + = hours; * = millimoles/litre; ND = Not detected.

Time	Sample	Formate	Pyruvate	Lactate	Succinate	Acetate	Propionate	Total acids
	T	0.23*	0.02	0.65	0.39	1.50	0.43	3.23
0+	CA	0.23	0.06	0.70	0.33	2.17	0.45	3.95
	CB	0.23	0.02	0.59	0.51	2.15	0.56	4.08
	T	0.24	0.43	0.72	0.58	2.55	0.73	5.26
8	CA	0.25	0.04	0.61	0.54	1.99	0.57	4.01
	CB	0.24	0.04	0.48	0.41	1.95	0.56	3.69
	T	0.34	3.69	1.75	1.14	5.67	0.80	13.41
24	CA	0.23	0.06	0.62	0.47	1.78	0.58	6.83
	CB	0.24	0.06	0.48	0.47	2.06	0.54	3.86
	T	0.35	8.72	1.46	1.67	3.98	0.90	17.10
32	CA	0.23	0.04	0.81	0.44	1.65	0.51	3.70
	CB	0.24	0.04	0.75	0.47	1.70	0.54	3.75
	T	0.42	7.49	1.42	1.98	3.63	1.09	16.04
48	CA	0.19	0.32	1.40	0.54	2.11	0.50	5.08
	CB	0.23	0.22	0.59	0.52	2.00	0.55	4.13
	T	0.44	6.20	1.22	1.52	2.34	1.23	12.96
56	CA	0.24	0.22	0.50	0.38	1.78	0.50	3.62
	CB	0.27	0.22	0.63	0.60	2.04	0.52	4.30
	T	0.46	6.85	0.84	0.79	2.55	1.48	12.99
72	CA	0.34	0.06	0.64	0.24	1.98	0.77	3.93
	CB	0.36	0.09	0.57	0.13	1.87	0.87	3.89

Table 3.59. The mean value of acid anions produced by C. krusei NCPF 3165 cultured in pooled whole saliva supplemented with 200mM glucose over a 3 day period.

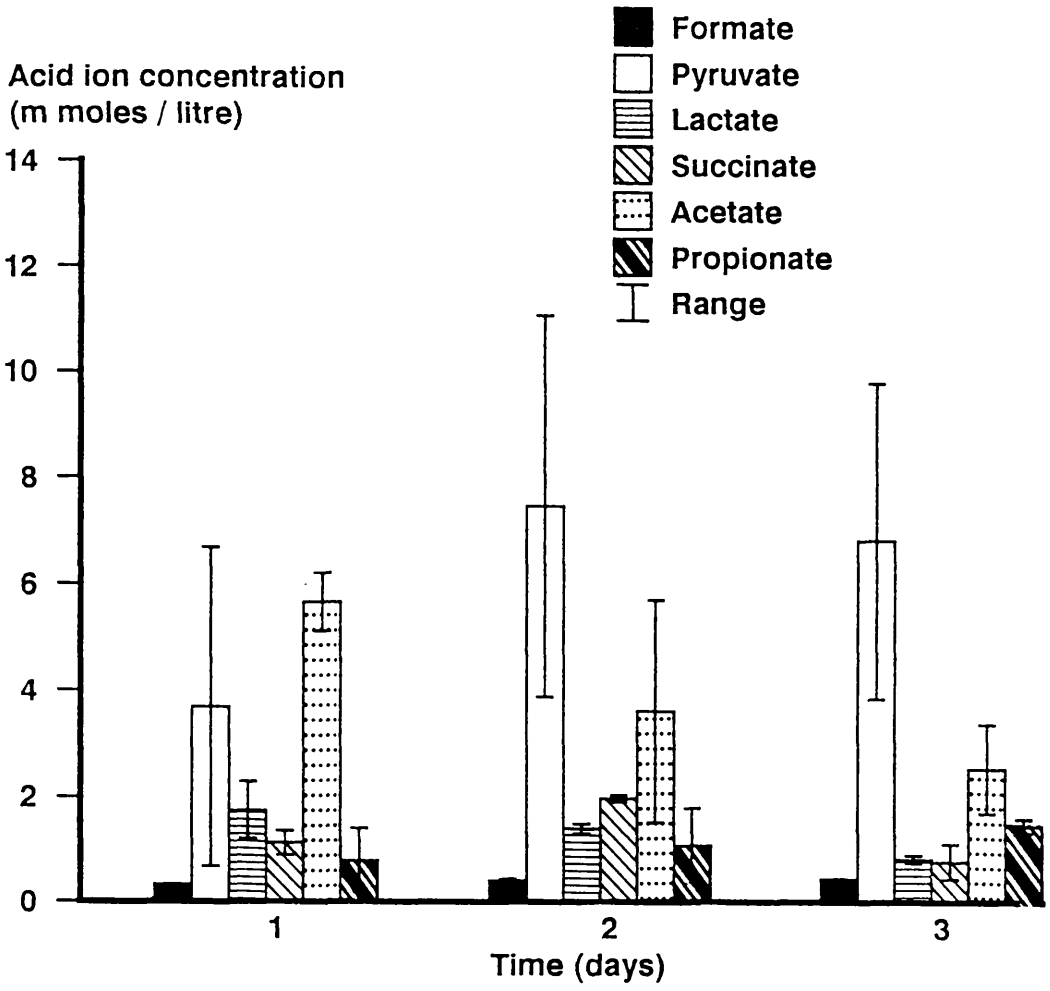

Figure 3.22 The mean total acid anions produced by C. krusei NCPF 3165 cultured in pooled whole saliva supplemented with 200mM glucose and incubated aerobically at 37°C for 3 days.

concentration of acid anions in the two control saliva samples changed only very slightly throughout the experiments.

3.3.24. Change in protein concentration during the growth of C. krusei NCPF 3165 in pooled whole saliva

The change in salivary protein concentrations during the growth of C. krusei NCPF 3165 in pooled whole saliva supplemented with 200mM glucose is shown in Tables 3.60 to 3.62. There was a sharp reduction of protein concentration during the first 48 hours of incubation from 5.5 to 0.9 mg/ml with little degradation of protein during the next 24 hours (Figure 3.20. presented earlier). Little change in the protein concentrations of the two control cultures over the 72 hour period was seen (Tables 3.60 and 3.61).

3.3.25. SDS-PAGE of pooled whole saliva after culture with C. krusei NCPF 3165

The culture supernatants of the pooled whole saliva samples were examined by polyacrylamide gel electrophoresis for alterations in their salivary protein profiles as described in Sections 3.2.15. to 3.2 17. The salivary protein profile of the test pooled whole saliva sample after 0, 24 and 48 hours of incubation with C. krusei NCPF 3165 shows a reduction in the intensity and the number of protein bands as the incubation time increases, which suggests protein degradation. The SDS-PAGE profiles obtained for C. krusei are similar to the SDS-PAGE profiles obtained for C. albicans GDH 1878 (Figure 3.14 (a) &

Time hours	Concentration of protein mg/ml Experiment 1	Control 1A	Control 1B
0	5.9	5.8	5.8
8	4.5	5.8	5.8
24	2.4	5.2	5.7
32	1.6	5.7	5.2
48	0.8	5.2	5.2
56	0.8	5.2	5.2
72	0.8	5.2	5.2

Table 3.60. The protein concentration at different time intervals when C. krusei NCPF 3165 was cultured in whole saliva supplemented with 200mM glucose over a 3 day period (Experiment 1 and the two Control experiments 1A and 1B).
Control 1A - Whole saliva supplemented with 200mM glucose only.
Control 1B - Heat killed C. krusei NCPF 3165 incubated in whole saliva supplemented with 200mM glucose.

Time hours	Concentration of protein mg/ml Experiment 2	Control 2A	Control 2B
0	5.2	5.4	5.4
8	4.7	5.3	5.3
24	3.8	5.4	5.3
32	1.7	5.3	5.4
48	1.0	5.2	5.2
56	0.9	5.2	5.2
72	0.9	5.2	5.2

Table 3.61. The protein concentration at different time intervals when C. krusei NCPF 3165 was cultured in whole saliva supplemented with 200mM glucose over a 3 day period (Experiment 2 and the two Control experiments 2A and 2B).
Control 2A - Whole saliva supplemented with 200mM glucose only.
Control 2B - Heat killed C. krusei NCPF 3165 incubated in whole saliva supplemented with 200mM glucose.

Mean concentration of protein mg/ml			
Time hours	Experiment 1+2	Control 1A+1B	Control 2A+2B
0	5.5	5.6	5.6
8	4.6	5.6	5.6
24	3.1	5.3	5.5
32	1.7	5.5	5.3
48	0.9	5.2	5.2
56	0.9	5.2	5.2
72	0.9	5.2	5.2

Table 3.62. The mean concentration of protein at different time intervals when C. krusei NCPF 3165 was cultured in whole saliva supplemented with 200mM glucose over a 3 day period.

(b) presented earlier). Some reduction in the intensity and number of protein bands was also seen in the control saliva samples, but this was very small compared with the changes noted in the test samples.

3.3.26. SDS-PAGE results of the salivary supernatants from the third control used in experiments with *C. albicans*; *C. tropicalis*; *C. glabrata*; and *C. krusei*

A third set of viable yeast controls was used in experiments with *C. albicans* GDH 1878, *C. tropicalis* GDH 1009, *C. glabrata* GDH 1397 and *C. krusei* NCPF 3165 to test the hypothesis that at least part of the reduction in salivary protein was due to adsorption onto the surface of yeasts. These control experiments with the 4 *Candida* species were carried out separately to the main experiment. These cultures were incubated at room temperature for 30 minutes on each of the 3 days, and stored at $4^{o}C$ during the rest of the experimental period. The analysis and experimental procedures were as described earlier in Sections 3.2.15. to 3.2.17. The SDS-PAGE profiles of the salivary samples obtained for *C. albicans* GDH 1878 at 0, 24, 48 and 72 hours are shown in Figure 3.23.

The visual assessment of these gel profiles indicated that there was no apparent change in the protein bands at 0, 24, 48 and 72 hours. Similar results were also observed for the other three *Candida* species. These results suggest that little or no protein was adsorbed on to the surfaces of viable yeast cells implying that most if not all protein loss in the test saliva sample was due to the proteolytic action of the 4 Candida strains.

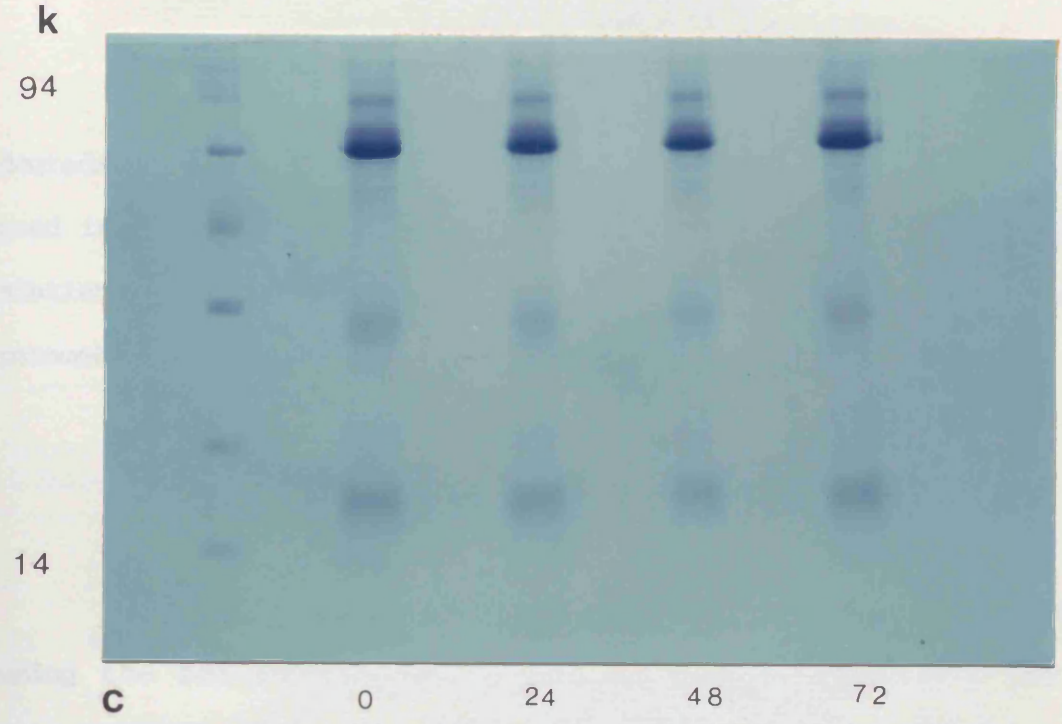

Figure 3.23. The SDS-PAGE analysis patterns of the Third Control obtained at 0, 24, 48 and 72 hours incubation of mixed whole saliva with C. albicans GDH 1878. Incubation was at room temperature for 30 minutes each day and then stored at 4°C for the remaining time.
C0 = 0 hours; C24 = 24 hours; C48 = 48 hours and C72 = 72 hours.

3.3.27. Comparison of growth, pH, acids and proteolytic degradation of pooled whole saliva by Candida species

The tabulated values for growth, pH, acids and protein degradation of the experiments carried out with the 4 Candida species used in this study, are given earlier in this Section. Using the statistical methods described in Section 3.2.20 the following growth parameters were compared.

Growth

The specific growth rates of the 4 strains were calculated using the following formulae: Specific Growth Rate = (MF - MI)2.303/time. (MF = Log_{10} final cell number; MI = Log_{10} initial cell number; 2.303 = a constant; time = in hours) (Stanier et al. 1977). The results were expressed as the rate of candidal growth/hour in saliva and are shown in Table 3.63.

Period of incubation	C.albicans GDH 1878	C.tropicalis GDH 1009	C.glabrata GDH 1397	C. krusei NCPF 3165
0 - 24	0.10	0.12	0.10	0.10
24 - 48	0.05	0.02	0.04	0.05
48 - 72	-0.02	-0.00	0.01	-0.02

Table 3.63. Specific growth rate of Candida species in whole mixed saliva supplemented with 200mM glucose.

Initially on day one, all Candida species showed almost identical growth rates of 0.10. On the second day of incubation C. albicans GDH 1878 and C. krusei NCPF 3165 showed growth rates of 0.05 while the growth rate of C. glabrata GDH 1397 was much lower (0.04) with C. tropicalis GDH 1009 having the lowest growth rate of 0.02. Thereafter the rate of growth declines for the 3 Candida species C. abicans, C. tropicalis and C. krusei while C. glabrata GDH 1397 demonstrated a positive growth rate of 0.01. The rate of growth declines and eventually the growth of Candida stops. This is mainly due to the exhaustion of nutrients or by the accumulation of toxic products of matabolism (Stanier et al., 1977).

When the results were analysed by Repeated Measures Analysis of Variance (Table 3.64.) it was found that growth of all 4 Candida species were significantly different with each other. Further analysis carried out by a One-Way analysis of Variance (Table 3.65) revealed that these differences mainly occurred at the later stages of the experimental period.

pH and Acids

When the pH, and mean production of acids and salivary protein degradation by the 4 Candida species, were analysed by Repeated Measures Analysis of Variance the results indicated that all four species were significantly different from each other (Table 3.64.).

ACIDS

Source	DF	F	P-value
Species	3	6.06	0.004
Time	6	41.24	0.000
Species/Time	18	4.53	0.001
Experiments	4	7.17	0.001
Error	21		

LOG (Protein)

Source	DF	F	P-value
Species	3	58.64	0.000
Time	6	186.96	0.000
Species/Time	18	7.97	0.000
Experiments	4	18.13	0.000
Error	22		

pH

Source	DF	F	P-value
Species	3	18.86	0.000
Time	6	362.88	0.000
Species/Time	18	3.43	0.003
Experiments	4	8.56	0.000
Error	22		

Log (Growth) Source	DF	F	P-value
Species	3	4.64	0.012
Time	6	211.10	0.000
Species/Time	18	2.14	0.045
Experiments	4	3.90	0.015
Error	22		

Table 3.64. **Repeated Measures Analysis of Variance Table. Interaction between time and** *Candida* **species.**
DF = Degrees of freedom
F = Obseved value of Test Statistic

Time (hours)	Acids	pH	Log(Protein)	Log(Growth)
0	0.612	0.602	0.414	0.997
8	0.891	0.341	0.197	0.655
24	0.737	0.106	0.693	0.141
32	0.183	0.628	0.451	0.876
48	0.063	0.075	0.032* G>K	0.987
56	0.559	0.129	0.028* G>K	0.040* G>K
72	0.000* G>A>T,K	0.171	0.013* G>A,K	0.013* G>A,T,K

Table 3.65. One-way Analysis of Variance Table.
* P-value denotes a significant difference
G - C. glabrata GDH 1397
A - C. albicans GDH 1878
T - C. tropicalis GDH 1009
K - C. krusei NCPF 3165

As observed in Table 3.64 there is a dramatic time effect on these 2 parameters. Therefore these results were further analysed by deleting the time effect. A One-Way Analysis of Variance carried out for each distinct time point, indicated that these differences were mainly prominent in the latter stages of the experimental period (Table 3.65). The production of acids by C. glabrata GDH 1397 was significantly different to that of C. albicans GDH 1878, C. tropicalis GDH 1009 and C. krusei NCPF 3165 at 72 hours.

Proteins

The experimental data presented for change in protein concentration in salivary cultures of the 4 strains (Table 3.26, 3.38, 3.50 and 3.62) indicate that there are differences in the protein concentrations of salivary samples collected on different days. Hence to compare changes in protein concentrations in the 4 different species, the % reduction in protein concentration during the experimental period was calculated (Figure 3.20.). When the % reduction of proteins of the 4 species were compared C. albicans and C. krusei showed high proteolytic activity. During the 72 hour period of incubation C. albicans and C. krusei both showed 83% reduction in salivary proteins. C. glabrata was the least proteolytic producing only 53% reduction of protein while C. tropicalis demonstrated intermediate proteolytic activity by producing 76% protein reduction. The statistical analysis of these results appear to indicate highly significant differences among the 4 Candida species. The protein breakdown of C. glabrata was significantly less than that of C. krusei

at 48hrs., 56hrs. and 72 hrs. In addition at 72 hrs., the protein breakdown of C. glabrata was significantly less than the protein breakdown of C. albicans. C. albicans, and C. krusei showed similar (83%) percentage reduction of whole salivary proteins biochemically.

The SDS-PAGE patterns of the test salivary protein profiles of pooled whole saliva samples incubated with C. albicans GDH 1878 (Figure 3.14. (a) and (b) presented earlier) and C. tropicalis GDH 1009 showed a marked difference after 48 hours of incubation. The SDS-PAGE patterns obtained for the other 2 strains, C. glabrata GDH 1397 and C. krusei NCPF 3165 also showed similar results. During the 48 hour incubation period most of the protein bands in the test saliva samples were lost compared with the 2 controls. The intensity of the bands had become less and more diffused. After 72 hours of incubation all protein bands of the test whole saliva samples were almost completely lost. As a result it was not possible to study salivary protein degradation by densitometric analysis of the gels.

3.4. DISCUSSION

3.4.1. Introduction

There is little information regarding glycolysis and proteolysis of saliva by Candida. Therefore in the present study, a preliminary attempt was made to assess growth, pH, acid production and protein degradation of salivary protein by Candida species over a 3 day period. The techniques used for the measurement of growth, pH and acid production were standard and presented no difficulties. The experimental procedures used for the biochemical assay of proteins (BCA) and SDS-PAGE in the present study were relatively new methods. The BCA method was easy to use while the SDS-PAGE technique required slight modification.

3.4.2. Techniques

A. Growth

Candidal growth was measured by counting yeasts in an improved Naeubauer Haemocytometer which is the standard method used for cell counting and is more accurate than measuring yeast numbers by optical density (Germaine et al., 1978). In this study microscopic counting was found to be very satisfactory and gave reproducible results. Previous workers (Samaranayake et al., 1983a, 1986b) also found this method to be reproducible.

B. pH

The pH measurements were read by the use of a E1L 2320 pH meter which is an accurate and standard method of estimating the hydrogen ion concentration of solutions.

C. Acid anion analysis

The acid anions were identified and quantified using isotachophoresis, which is a sensitive and reproducible technique, allowing nanomole levels of acid end products to be detected (Weetman et al., 1985). Isotachophoresis is suitable for the determination of 'aliphatic' series fatty acids C1 to C4 in preference to gas liquid chromatography as the former technique does not require derivatisation of the sample (Weetman et al., 1985). In addition, there are other difficulties in using gas liquid chromatography for the analysis of short chain fatty acids: The interaction of the acids with the column causes loss of sample (Geddes and Gilmour, 1970), and also the volatile nature of formic acid makes it difficult to detect the latter by this method. These can be overcome in isotachophoresis which enables low molecular weight fatty acids including formic acid to be easily detected. However, there are a few drawbacks in using isotachophoresis mainly due to the very small sample volumes used (1 µl) which can result in the evaporation of the sample and possible failure in achieving adequate mixing of the sample. Finally, the presence of impurities in the electrolytes used may cause additional peaks in the acid tracing which can lead to errors. Despite these

shortcomings isotachophoresis is probably superior to gas liquid chromatograhy (Weetman et al., 1985) and hence was employed in the current study.

D. Bicinchoninic acid (BCA) protein assay

The measurement of protein concentration using bicinchoninic acid was first reported by Smith et al. (1985). The water-soluble sodium salt of BCA is stable and is capable of forming an intense purple complex with cuprous ion in an alkaline environment.

$$\text{Protein} + \underset{\text{(green)}}{\text{CuI}} \xrightarrow{\text{BCA}} \underset{\text{(purple)}}{\text{BCA-CuI complex}}$$

Therefore this reagent forms the basis of an analytical method capable of monitoring cuprous ions produced due to the reaction of protein with alkaline Cu^{++} (Biuret reaction). The purple colour is stable and becomes more intense with increasing protein concentration. It has been reported that this is a better method to determine protein concentration in saliva than either Lowry's (Lowry et al., 1951) or Bradford's (Bradford, 1976) methods because it is simple to perform and is more sensitive (Khoo, 1990). Therefore the BCA method was used throughout this study.

The protein concentration of pooled parotid saliva samples was relatively constant with little variation throughout the experiments (Experiment 1 Table 3.12 and Experiment 2 Table 3.13).

However more variation was found with whole saliva protein measurements; for example in Experiment 1 (Table 3.38) the concentration in the test sample was 7.4mg/ml; in the control saliva sample supplemented with 200mM glucose, 5.8 mg/ml, and 6.6mg/ml in the control saliva sample incubated with heat killed Candida cells and 200mM glucose. This tendency to variation in protein levels in mixed but not parotid saliva is difficult to explain but could possibly be due to the more complex composition of whole saliva (see Section 1.4). Saliva was collected and pooled together on 8 separate occasions for the experiments carried out in this study with the 4 Candida species. Therefore it is not surprising that the initial concentration of salivary proteins for each series of experiments was variable. However to minimise this variation saliva was collected from 4 individuals at a specific time of the day and pooled together before clarification.

E. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins can be divided into their individual components (polypeptides) by means of physical, biochemical and immunological methods. Because of the small volumes used in the current experiments a biochemical method was chosen for this study and this involved using an electrophoretic analysis of the salivary proteins.

Proteins carry a net charge at any pH other than their isoelectric points and migrate according to their charge density (the

ratio of charge to mass) when subjected to an electric field (Andrews, 1981). The higher the ratio of charge to mass the faster the protein molecules will migrate. This phenomenon is called electrophoresis. Isoelectric focusing is a technique in which proteins migrate to their isoelectric point in a pH gradient and an optimal gradient can be chosen to yield the maximum information from a single run (Arbuthnott and Beeley, 1975). However iso-electric focusing of salivary proteins is now not widely used, and the method of choice for the analysis of salivary proteins is 1-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (Laemli, 1970).

Polyacrylamide gel electrophoresis (PAGE)

During electrophoresis, proteins of different mobilities travel as discrete zones and these zones separate from each other as electrophoresis proceed. To minimise or exclude adverse effects caused by heating and diffusion, electrophoresis of proteins is always performed in a solution stabilised within a supporting medium. Of these, polyacrylamide is better suited to a size fractionation of proteins since gels with a wide range of pore size can be readily made from highly purified reagents in a reproducible manner. In addition polyacrylamide gels are chemically inert, stable over a wide range of pH, temperature and ionic strength and are also transparent (Hames, 1981). For these reasons polyacrylamide gels have become the medium of choice for salivary protein analysis.

In the analysis of salivary proteins the use of slab gels of acrylamide was preferred since the choice of acrylamide concentration

is critical for optimal separation of protein components by zone electrophoresis. One of the most important advantages of slab gels is that many samples, including molecular weight marker proteins, can be electrophoresed under identical conditions in a single gel such that the band patterns produced are directly comparable (Figure 12 (a) & (b)).

Dissociating buffer system

In zone electrophoresis of proteins in polyacrylamide gels with a denaturing system, the buffer used is designed to dissociate all proteins into their individual polypeptide subunits. The dissociating agent used is the ionic detergent, sodium dodecyl sulphate (SDS). The salivary proteins are denatured by heating the protein mixture at 100^0C for 2 to 3 minutes in the presence of excess SDS and dithiothreitol (DDT) i.e., a reducing agent (Deyl, 1979). Under these conditions most polypeptides bind SDS in a constant weight ratio (1.4 grams SDS per gram of polypeptide). The SDS-polypeptide complexes have identical charge densities and migrate in polyacrylamide gels of the correct porosity strictly according to polypeptide size (Webber and Osborne, 1975). Thus in addition to analysing the polypeptide composition of the sample, the investigator can determine the molecular weight of the sample polypeptides by reference to the mobility of polypeptides of known molecular weight under the same electrophoretic conditions. The simplicity and speed of the method, plus the fact, that only microgram amounts of sample proteins are required, have made SDS-polyacrylamide gel

electrophoresis (SDS-PAGE) the most widely used method for determination of the complexity and molecular weights of constituent polypeptides in a protein sample, including salivary protein.

Discontinous buffer system

SDS-PAGE may be carried out using a continous buffer system in which the pH values of the gel, reservoir buffer and the sample are the same (continous buffer system). However, a discontinous buffer system, was used in the present study, which employed different buffer ions in the gel compared to those in the electrode reservoirs. In this system the sample is loaded on to a large-pore 'stacking gel' which is polymerised on top of the small-pore resolving gel. The discontinous system has the advantage of enabling a larger volume of dilute protein samples to be used. The proteins migrate through the large-pore resolving gel and concentrate into narrow zones (or stacks) before separating out during electrophoresis in the small-pore resolving gel (Hames, 1981). For these reasons SDS-PAGE would appear to be the most suitable method for the analysis of salivary proteins (Khoo, 1990) and therefore was used in this study.

3.4.3. Comparison of growth of Candida in parotid and mixed saliva

The early experiments in this investigation were carried out with parotid saliva mainly to reduce individual variation and also because it can be collected aseptically. In addition it has also been studied in some detail using SDS-PAGE. (Beeley and Beeley,

1987). Therefore if proteolysis occurred in glucose enriched parotid saliva it would be possible to make qualitative and quantitative assessments of the changes in salivary protein patterns that would occur during the culture of Candida.

When C.albicans GDH 1878 was cultured in parotid saliva supplemented with 200mM glucose, an increase in cell numbers from $2x10^4$ to $6x10^7$ cfu/ml, a pH drop of 8.9 to 7.65 and a 43% reduction of protein was observed over the 3 day experimental period. On the other hand when C. albicans GDH 1878 was cultured in mixed saliva enriched with glucose, cell numbers increased from $2x10^6$ to $2x10^8$ cfu/ml, the pH fell from 7.3 to 3.0 and the protein concentration was reduced by 83% over 3 days. The results of the present investigation imply that parotid proteins are not totally degraded by Candida proteinases in-vitro despite the presence of a carbohydrate supplement (200mM glucose) and the long incubation period of 72 hours.

The precise reason for the greater degree of growth, lower pH and increased proteolysis by Candida when grown in whole saliva compared with parotid saliva, is unknown. One explanation may be the alkaline nature of parotid saliva (pH 7 to 9). Most Candida proteinases undergo fast denaturation, under alkaline conditions, e.g., aspartic proteinases of Candida undergo irreversible dimerization at pH 8.4 to 8.5 (Ruchel, 1981) and this mechanism may be involved in the inhibition of Candida proteinases. However it is known that as well as acid proteinases, C. albicans contains at different cellular locations, a variety of intracellular alkaline

proteinases with a pH range of 6 to 8. Although these proteinases have not been studied extensively, a serine proteinase of C. albicans has been well characterized, with an optimum pH of 6.0 to 7.5. Another alkaline proteinase has been obtained from C. tropicalis culture filtrates that specifically cleaves valine residues (Odds, 1988).

The 43% protein breakdown of pooled parotid saliva seen in the present study at an alkaline pH range of 7 to 9 could possibly be due to one or more of the alkaline proteinases of C. albicans. Therefore further work is required to ascertain the activity of this group of proteinases in biological fluids such as saliva. However, the fact that proteolysis was consistently lower under alkaline conditions when compared with acidic conditions (43% in parotid saliva compared with 83% in whole saliva) implies that alkaline proteinases may play a small but not a major role in the growth of Candida in saliva.

The use of whole saliva for metabolic studies has some disadvantages, for it contains variable amounts of submandibular, parotid and minor gland saliva and presents as a viscous fluid rich in mucins. Therefore whole saliva must be centrifuged at about 15,000 rpm and then filter sterilized before use. This pretreatment may remove high molecular weight substances, especially proteins, from mixed saliva as well as most salivary bacteria. Parotid saliva, however does not require clarification before use in the experiments and therefore loss of proteins is not a problem.

Other disadvantages of using whole saliva is the presence of large numbers of dissimilar proteins (Giometti and Anderson, 1979) in variable concentrations together with the difficulties that arise in the analysis of the different protein bands that appear on the SDS-PAGE patterns. In order to minimise individual variation in the composition of saliva, the collection of samples were standardised in this study by defining the time of collection, standardising the method of stimulation and the volume collected from each individual. For the reasons given above it was decided overall that whole saliva was superior to parotid saliva for the current study and hence the former was used extensively in the subsequent studies concerning the growth of Candida species in saliva.

3.4.4. Growth of C. albicans in pooled whole saliva

The degradation of human parotid salivary proteins was first investigated by Germaine, Tellefson and Johnson (1978), who collected saliva samples using a technique similar to that employed in the present study. However, they centrifuged the saliva at 10,000g for 20 minutes while in the present study a speed of 15,000g for 15 minutes was used. Therefore there could be minor differences in the constituents of the supernatants used in the experiments between the two studies. There was agreement between the results of this study and that of Germaine et al. (1978), in that both found that the growth of C. albicans in filter-sterilized whole saliva supernatants supplemented with 0.1% glucose (w/v) was always superior to that obtained in filter-sterilized parotid fluid. However the actual

figures for candidal growth which occurred in the two studies cannot be directly compared, as the growth measurements were obtained in two different ways; Germaine et al. (1978), measured the optical density of the salivary supernatant, while cell numbers were counted microscopically in the present study. The latter method of estimating yeast growth was preferred to the turbidimetric method which can be inaccurate due to the uncontrollable changes in optical density which can occur in saliva due to calcium precipitation (Germaine et al., 1978).

The growth of C. albicans GDH 1878 in pooled mixed saliva obtained in the present study is similar to the results reported by Samaranayake et al. (1983a) and (1986b) who studied the growth, pH and acid production of two other C. albicans strains, GDH 1957 and GDH 1261 using similar experimental methods. In this study C. albicans GDH 1878 grew rapidly in mixed saliva during the first 48 hours then entered the stationery phase of growth which is a similar growth pattern to that described by Samaranayake et al. (1983a) and (1986b) for C. albicans GDH 1957 and GDH 1261. However in the investigations carried out by these workers, both pooled mixed saliva and saliva from separate individuals were also studied. Although C. albicans GDH 1957 grew in whole saliva from one individual, C. albicans GDH 1261 showed very poor growth in the saliva from the same person. Therefore Samaranayake et al. (1986b) suggested that pooled whole saliva should be used for future experiments, advice which was followed in the present study.

pH

A rapid decline in pH was observed in mixed pooled saliva cultures when incubated with C. albicans GDH 1878 which was generally directly related to the increase in yeast numbers. There was a decline in pH from 7.2 to 4.7 within 24 hours and after 48 hours incubation the pH further declined to 3.15. Similar results have been obtained in experiments carried out by earlier investigators; for exmaple Samaranayake et al. (1983a, 1986b) using two C. albicans strains (GDH 1957 and GDH 1261) in pooled mixed saliva observed a rapid decline in pH from 7.5 to 3.7 within 24 hours, with a further fall to 3.2 occurring during the next 24 hours.

The acid anions that were associated with the fall in salivary pH in the present study are pyruvate, acetate, succinate, lactate, propionate and formate. In previous studies (Samaranayake et al. 1986b) the two Candida strains used, (GDH 1957 and GDH 1261) both produced high concentrations of acetate (14 and 13.9 μ moles/ml) and pyruvate (9.6 and 9.8 μ moles/ml) after 48 hours of incubation in whole saliva. In contrast C. albicans GDH 1878 in the present study, produced 12 millimoles/litre of pyruvate and only 1.3 millimoles/litre of acetate acid anions after 48 hours of incubation. The other acid anions (formate, lactate, succinate and propionate) produced in both studies appeared to be similar both qualitatively and quantitatively. The reason for these differences is not clear but veriation in salivary donors and C. albicans strains may have played a role. There are no further studies with which to compare the results

of this investigation since Germaine and associates (Germaine and Tellefson 1981, and Germaine et al., 1978) did not carry out the acid anion profile analysis.

Even in the absence of low pH, and conditions which favour fungal proliferation, Candida species may produce these conditions by the secretion of organic acids consequent to fermentation of dietary carbohydrates (Samaranayake et al., 1983a). Furthermore, it is known that high concentrations of glucose and sucrose, (up to 500mM), can be found in saliva for variable periods, depending upon the frequency and duration of carbohydrate intake (Darlington, 1978). A high carbohydrate diet has been associated with the initiation and aggravation of chronic atrophic candidosis (Neil, 1965; Gentels and La Touche, 1969; Ritchie et al., 1969) and there is evidence that denture wearers consume soft-carbohydrate rich foods. Both Zgraggen and Graf, (1975) and Olsen and Birkeland (1975) have shown that the regular and prolonged intake of carbohydrate food lowered the pH under dentures. Since this low pH is likely to favour the activity of candidal proteinases, several workers, have supported the theory that intra-oral proteolytic activity of C. albicans could contribute to the pathogenesis of chronic atrophic candidosis (Budtz-Jorgensen, 1974; Olsen and Birkeland, 1975; Zgraggen and Graf, 1975).

Other effects of a high carbohydrate intake are enhanced adhesion of Candida to buccal epithelial cells (Douglas et al., 1981; Samaranayake and MacFarlane, 1982b) and acrylic denture surfaces (Samaranayake and MacFarlane, 1980; McCourtie and Douglas 1985) which

in turn may lead to increased numbers of Candida in the mouth, and a subsequent higher risk of developing candidosis.

Salivary protein

Salivary protein degradation was evaluated using the BCA protein assay in the present study (see Section 3.2.11). This is the first time that this assay has been used to investigate salivary protein breakdown by Candida species. The BCA protein assay is a recently developed, very sensitive technique for the analysis of salivary proteins (Khoo, 1990). It was observed that C. albicans GDH 1878 effectively degraded salivary proteins and during the first 24 hours of incubation a degradation rate of 70 µg/ml was observed with a value of 90 µg/ml/hr during the next 24 hours and 30 µg/ml/hr during the last 24 hours of incubation. In an earlier study reported by Samaranayake et al. (1983a) the mixed salivary protein concentration was determined by the spectrophotometric method of Bradford (1976). Both strains of C. albicans (GDH 1957 and GDH 1261) used in the latter study showed less protein degradation during the first 24 hours of incubation (6 and 10 µg/ml/hr respectively) in pooled whole saliva. The rates of protein degradation in the next 24 hours were 5 and 1 µg/ml/hr for C. albicans GDH 1957 and C. albicans GDH 1261 respectively. Therefore it is clear that there are large differences between the rates of protein degradation among the three C. albicans strains. As the initial protein concentrations are not stated in the Samaranayake et al. (1983a) study it is not possible to compare the quantitative differences of salivary protein in these experiments.

However, it was observed that the proteolytic potential of C. albicans GDH 1261 was completely inhibited by the saliva of one individual, but not when the sample was pooled with the salivary samples from other individuals. This indicates that proteolysis by different strains of C. albicans can vary with the source and quality of the saliva samples.

In the present study the overall effect of the proteolytic activity of Candida on salivary proteins was also assessed qualitatively by observing the gel patterns obtained by SDS-PAGE at 24 hours and 48 hours respectively (Figures 3.12 (a) and 3.12 (b)). However, it was difficult to determine the quantitative and qualitative proteolytic breakdown of the individual proteins using these gel profiles due to the generally diffuse patterns produced.

On destaining the SDS-PAGE gels of salivary proteins with 10% acetic acid (see Section 3.2.14.) some of the proteins of whole saliva stained violet-pink with Coomasie brilliant blue R-250 instead of the normal blue coloration. This phenomenon has been previously observed in parotid saliva as 5 distinct bands (Beeley and Beeley, 1987, unpublished results). SDS-PAGE analysis of whole saliva has also been carried out (Marshall and Williams 1987, Minaguchi et al., 1988), but clear resolution of protein bands comparable to those of parotid saliva was not possible. This may be due to the presence of extra proteins in whole saliva derived from salivary secretions, crevicular exudate or microbial degradation products. In this study the mixed salivary samples at 0 hours were found to contain at

least 4 blue-stained and 7 violet-pink stained protein bands (Figure 3.12 (a)). For example, alpha-amylase is clearly seen, appearing as two bands corresponding to molecular weights 62K and 59K Figure 3.12 (a). These bands as well as the other protein bands disappeares after 72 hours, indicating almost total proteolysis of saliva by C. albicans GDH 1878. These data were also confirmed by biochemical estimation of the salivary protein concentrations as discussed earlier. Similar results have been reported by Samaranayake et al. (1983a) who used Iso-electric focusing to study salivary protein breakdown. Iso-electric focusing is used for separating proteins and peptides, and although it is a sensitive technique, operational costs are very high. SDS-PAGE is one of the most widely used methods, for the separation of protein mixtures for the reasons mentioned in Section 3.4.2 (E). The principal difference between the iso-electric focussing profiles of salivary supernates obtained for the control and the test saliva samples which demonstrated proteolysis appeared to be quantitative rather than qualitative. Therefore it appears that the results obtained by the SDS-PAGE and iso-electric focusing show similar results.

Taking into account the available data in the literature it is likely that most if not all strains of C. albicans are capable of using whole saliva as a growth medium, in a situation where there is reduction of the oral commensal microflora and excess glucose conditions.

3.4.5. Growth of C. glabrata in pooled whole saliva

Although the growth, and acid production of a single strain of C. glabrata in pooled whole saliva has been previously studied by Samaranayake et al. (1986b) the present experiments are the first in which the ability of C. glabrata GDH 1397 to break down salivary proteins has been investigated. In the study by Samaranayake et al. (1986b), the C. glabrata strain previously isolated from a patient with chronic atrophic candidosis, showed rapid growth during the first 40 hours of incubation in mixed saliva supplemented with 200mM glucose and then gradually reached stationery phase during the next 24 hours; the pH changed from 7.4 to 4.0 in the first 24 hours and then dropped to 3.2, in the next 24 hours. The results obtained in the present study with C. glabrata GDH 1397 (an isolate from a patient with Sjörgen's syndrome) were generally the same as those described by Samaranayake et al., 1986b. In the experiments reported here, C. glabrata grew rapidly (5.60×10^6 to 1.60×10^8 cfu/ml) in pooled whole saliva supplemented with 200mM glucose and reached the stationery growth phase during the first 32 hours. Little growth occurred during the next 40 hours of incubation. In addition there was a sharp reduction in pH from 7.2 to 3.4 during the first 32 hours and this gradually fell to 2.9 during the following 40 hours.

In the study reported by Samaranayake et al.(1986b) the major acid anion produced by C. glabrata after 48 hours of incubation in glucose supplemented saliva was pyruvate 13.74 mmol/l. The

concentration of the other acid anions was acetate, 6.29 mmol/l; lactate, 0.45; succinate, 0.45 mmol/l; succinate, 0.43 mmol/l; propionate, 0.31 mmol/l and formate, 0.14 mmol/l. Overall similar acid anions were obtained in this study, compared to that of Samaranayake et al. (1986b) with pyruvate (13.28 mmol/l.) being the major acid anion produced after 48 hours. However succinate (1.45 mmol/l) rather than acetate was the next most plentiful anion.

Since no protein analysis was performed by Samaranayake et al. (1986b) there is no data with which the present results could be compared.

3.4.6. Growth of C. tropicalis GDH 1009 and C. krusei NCPF 3165 in pooled whole saliva

This study provides the first set of experimental data on the growth, pH, acid production and protein degradation by a strain of C. tropicalis and C. krusei, cultured in pooled whole saliva. Consequently, it is not possible to compare the present results with previous similar studies.

3.4.7. Comparison of the growth, pH and acid production of four different Candida species in pooled whole saliva

No comparisons of the above mentioned parameters can be made as there are no similar previous investigations in the literature. However, the results of the present study revealed that there were

significant species differences for the parameters of growth, pH and acid production. Of these the pattern of growth between the 4 Candida species showed the least significant difference. C. glabrata GDH 1397 demonstrated the highest growth in saliva, and was also found to be the most significantly different species in relation to acid anion production compared with the other 3 species. These significant differences in growth and acid production were mainly observed during the 48 and 72 hours of incubation.

3.4.8. The relationship between the proteolytic ability of Candida species and their pathogenecity in the oral environment

The importance of secretory proteinases in the development of candidosis has been confirmed by Macdonald and Odds (1983) who compared the pathogenicity in mice of a proteolytic C. albicans strain and a non-proteolytic mutant. It was observed that in a proteinase-inducing medium the proteinase deficient mutant C. albicans strains were phagocytosed and killed by human polymorphonuclear leukocytes while the proteinase-producing strain survived. It is also known that the proteolytic activity of the various Candida species corresponds with the potential to colonise the mucosa and cause acute pseudomembranous candidosis. For example the ability of C. albicans and C. tropicalis to degrade salivary immunoglobulins corresponds with their ability to infect mucosal surfaces (Ruchel, 1986) However, although C. parapsilosis is also proteolytic in vitro this species failed to produce proteinase in vivo (Ruchel, Boning and Borg, 1986).

Germaine et al. (1978), reported that parotid protein degradation is pH-dependent and that the low optimum pH of the enzyme made it unlikely to be a significant virulence factor in Candida-induced oral inflammatory lesions. Later Germaine and Tellefson (1981) confirmed these results and in addition reported that saliva is a possible inhibitor of proteinase synthesis. However these earlier workers failed to take into account the fact that the mouth consists of a range of different ecological niches which can change due to a number of environmental pressures. Thus after the intake of food containing high concentrations of sugar a low pH optimum may occur especially in the protected environment under dentures (Budjtz-Jorgensen, 1974). Also in the present study, degradation of parotid salivary protein was observed in an alkaline environment.

In the present study there is no firm evidence to support Germaine and Tellefson's (1981) hypothesis that saliva is a possible inhibitor of proteinase synthesis since all 4 Candida species actively degraded pooled whole salivary proteins. The reason why Germaine and co-workers failed to detect protein degradation in their studies could be due to a)the low glucose concentration (0.1%) (w/v) used, or b)the methods of protein estimation used, i.e., by absorbance at 280nm or by the method of Lowry et al. (1951). The method used to estimate the salivary protein degradation in the present study (BCA) was a better method (Khoo, 1990) than the two methods mentioned above and discussed earlier in Section 3.4.2. (D). Another reason why neither of these 2 earlier studies detected any measurable proteolysis may be due to the fact that they used a single strain of C. albicans and variation in

the ability of C. albicans strains to grow in saliva samples from different individuals have been reported (Samaranayake et al., 1983a).

In the present study, all 4 Candida species degraded salivary protein effectively. Protein breakdown was significantly different among the 4 Candida species ($P<0.05$); with the difference being more prominent during the latter hours of the experimental period (72 hours only). However, the protein degradation of C. glabrata was significantly lower than C. albicans and C. krusei at 72 hours of incubation. This high proteolytic activity of C. krusei does not seem to coincide with it's low pathogenecity in the oral environment. As there are no previous results concerning the protein degradation by C. tropicalis, C. glabrata and C. krusei no definitive conclusions can be drawn from the limited results of the present data.

In experiments carried out by Ruchel (1984) all the Candida acid proteinases tested were able to cleave IgA, IgA2 and Secretory IgA, which are the major immunoglobulin classes of mucous membranes. Later Ruchel (1986), demonstrated the degradation of high molecular weight salivary immunoglobulins (e.g., IgM and IgA2) when fresh saliva from healthy individuals was infected with blastospores of C. albicans and incubated at 37^0C for 48 hours under gentle agitation. Since sIgA has been shown to prevent the attachment of C. albicans to mucosal epithelium in-vitro (Vudhichamnong et al., 1982), the cleavage of sIgA may facilitate the adherence of yeasts to mucosal

epithelium in-vivo. This mechanism may explain at least in part why individuals with lowered levels of sIgA are more prone to mucosal candidosis (Romero-Piffiguer et al., 1985).

A number of studies have reported the ability of Candida species to hydrolyse BSA (MacDonald, 1984; Schreiber et al., 1985; Ruchel, 1984; Tobgi et al., 1990). MacDonald (1984) reported that proteinases were produced by C. albicans and C. tropicalis but not by C. glabrata and C. krusei. While Schreiber et al. (1985) have observed hydrolysis of BSA by 13% C. glabrata in comparison to 17% C. tropicalis and 67% C. parapsilosis isolates. Tobgi (1990) examined 6 species of Candida for their proteolytic ability in BSA as the source of nitrogen. He observed a range of proteolytic activity for the different species, i.e., C. albicans 68.2%; C. tropicalis 71.4%; C. glabrata 0.0% and C. krusei 0.0%. However when IgA was used as the nitrogen source the proteolytic activity of the different Candida species were as follows: C. albicans 45.5%; C. tropicalis 85.7%; C. glabrata 0.0% and C. krusei 0.0%. The use of IgA as a nitrogen source for Candida has been studied previously (Ruchel, 1984; 1986) who found that the acid proteinases of Candida were able to cleave IgA1, IgA2 and secretory IgA effectively. These immunoglobulins are found abundantly in salivary secretions. However, it was interesting to note that in the present study although C. glabrata and C. krusei were unable to hydrolyse BSA in an in-vitro experimental system, they hydrolysed mixed, pooled salivary proteins. Therefore the proteolytic ability of Candida species appears to vary depending on the protein source.

It is not known if the *in vitro* conditions used in the present studies prevail intraorally and whether proteolytic enzymes potentiate oral candidosis. Whatever the major targets for the enzyme may be *in vivo*, proteinase production is likely to be advantageous to Candida. Niches with low pH values ranging from 4-6 are found within damaged tissues, and in sites protected from the mechanical flushing action of saliva such as the upper denture fitting surface (Butz-Jorgensen, 1974). At lower pH values Candida secrete proteinases of broad substrate specificity which may not only be able to cause salivary proteolysis but also capable of damaging host tissues, e.g., the palatal mucosa under dentures.

3.4.9. Proteins in the control samples

The protein concentrations of the two control mixed saliva samples showed variations during the course of the experimental period with all 4 *Candida* species. These slight differences were also visible in the SDS-PAGE protein profiles. There is no satisfactory explanation for the variations in protein concentration in the control saliva samples supplemented with glucose only, although it may be due to complex chemical interactions of salivary components with time. The variation which occurred in the second control sample supplemented with glucose and dead Candida cells may be due, in addition, to interaction of salivary components with factors related to dead yeasts.

The SDS-PAGE protein patterns of the third control salivary protein samples cultured with viable cells from the four Candida species and incubated at 4^0C and also 30 minutes at room temperature on each of 3 days, tends to confirm that little if any salivary protein is adsorbed on to the yeast cell-surface (Figure 3.23). Certainly, the amount involved, if adsorption occurs, is too small to be measured by detection systems used in this study. Therefore these results agree with the conclusions of Samaranayake et al. (1983a), and support the concept that the extracellular proteinases of C. albicans can hydrolyse salivary proteins.

3.4.10. Conclusions

1. C. albicans GDH 1878 demonstrated moderate growth in pooled parotid saliva ($2.89x10^6$ to $6.90x10^7$) cfu/ml, lowering the pH to 7.6, producing 6 different acid anions and also producing a 43.7% reduction of salivary proteins during an incubation period of 72 hours.

2. C. albicans GDH 1878 grew profusely in glucose supplemented pooled whole saliva ($5.06x10^6$ to $1.39x10^8$) cfu/ml, lowering the pH to 3.10, producing 6 different acid anions and also producing a 83% reduction of salivary proteins during an incubation period of 72 hours.

3. These results indicate that C. albicans uses pooled whole saliva more effectively than pooled parotid saliva.

4. C. tropicalis GDH 1009 grew well in glucose supplemented pooled whole saliva ($3.96x10^6$ to $1.16x10^8$) cfu/ml, lowering the pH to 4.05, producing 6 different acid anions and also producing a 76% reduction of salivary proteins during an incubation period of 72 hours.

5. C. glabrata GDH 1397 grew well in glucose supplemented pooled whole saliva ($5.60x10^6$ to $2.30x10^8$) cfu/ml, lowering the pH to 2.95, producing 6 different acid anions and also producing a 53% reduction of salivary proteins during an incubation period of 72 hours.

6. C. krusei NCPF 3165 grew well in glucose supplemented pooled whole saliva ($4.01x10^6$ to $1.19x10^8$) cfu/ml, lowering the pH to 3.69, producing 6 different acid anions and also producing a 83% reduction of salivary proteins during an incubation period of 72 hours.

7. For all growth parameters, i.e., growth, pH, acid anion production and salivary protein degradation there were significant species variation. Of these, the pattern of growth between the four species showed the least significant difference. C. glabrata GDH 1397 showed slightly higher growth than the other 3 species.

8. Significant differences were found in the pH of saliva, acid anion production and log protein degradation among the 4 species. In the case of pH, C. tropicalis was significantly different than the other 3 species. C. glabrata was found to be the most

significantly different species among the 4 species with regard to acid anion production and log protein degradation. These significant differences occurred mostly at the latter stages of the incubation period i.e., 48 and 72 hours.

9. From the results obtained in this thesis it is very clear that all 4 Candida species C. albicans, C. tropicalis, C. glabrata and C. krusei are able to use whole saliva as a growth medium.

CHAPTER 4

FURTHER STUDIES

Although a large number of investigations have been carried out during the last few decades dealing with host-parasite interactions of Candida, relatively few studies have dealt with Candida-saliva interactions. Many of the studies with Candida have been conducted *in-vitro* using synthetic media, mainly because of the many difficulties that arise when using mixed saliva as a microbiological growth medium. The work presented in this thesis investigated a few interactions of Candida and saliva, and the purpose of this section is to indicate briefly, future lines of research.

The results of Chapter 2 present evidence supporting the role of lysozyme in the regulation of the number of Candida intra-orally, and also suggests that a high dietary intake of sucrose may depress the sensitivity of certain *Candida* species to lysozyme. However it is difficult to extrapolate the results of this thesis directly to the *in-vivo* situation for a number of reasons. Firstly, the work carried out to determine the degree of susceptibility of Candida to the lytic activity of the enzyme used hen egg white lysozyme, because it was commercially available. Ideally human salivary lysozyme should have been used. Selective isolation and purification of lysozyme from it's biological source, saliva, would be ideal to determine it's function as an individual factor for killing Candida. Thus far, human salivary lysozyme has not been isolated in

pure form and it would be desirable to find a suitable method of purifying this enzyme in order to investigate its action on Candida species.

Although the results obtained for growth, pH, acid production and protein degradation for the four Candida species investigated in Chapter 3, can be extrapolated to the in vivo conditions there are a few draw backs. The saliva samples used in the experiments were clarified before use: antibiotics were added to suppress any remaining microorganisms and sugar was added to yield a final salivary concentration of 200mM glucose. These conditions are somewhat artificial as bacteria are always present in-vivo and all individuals do not have glucose concentrations as high as 200mM in their saliva.

As there could be a wide variation in glucose concentration in saliva among individuals who are carriers or non-carriers of Candida it would be useful to find out the glucose concentration of saliva samples prior to starting the experiments. Thereafter a dietary carbohydrate preferably glucose could be added to prepare a range of different concentrations of glucose in saliva samples. Using these saliva samples with different concentrations of glucose it would be possible to determine the minimal concentration of glucose required to obtain maximum growth of Candida.

It would also be of interest to extend the culture studies to include specific oral microorganisms normally found in saliva. This could be done by isolating microorganisms from each salivary donor before clarifying the saliva sample. These isolated bacteria

could then be identified, sub-cultured and inoculated as pure or mixed cultures with Candida to assess their effect on the subsequent growth of Candida in saliva.

The salivary protein degradation studies carried out with sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) do not show any preference or selectivity of the salivary proteins degraded by the Candida proteinases. Due to the poor resolution of the gels they were not very informative. This is probably due to the complex composition of the test samples of mixed saliva. Therefore further attempts should be made to improve the technique of 1-dimensional SDS-PAGE to obtain better resolution of mixed salivary proteins. If this was successful then perhaps visual analysis could be supplemented with accurate laser densitometric quantitation.

Intra-orally, especially in an environment with a high concentration of sucrose, Candida species can proliferate lowering the pH which in turn activates the proteolytic activity of Candida. Under such conditions it is also possible that Candida species hydrolyse lysozyme thus, preventing its candidacidal activity. These data together with the fact that lysozyme may inhibit candidal growth or may be fungicidal imply that while on one hand saliva can be regarded as a growth medium for the proliferation of Candida in the mouth, on the other hand it can also play a protective role by controlling yeast growth and reducing the risk of oral candidosis developing. Nonetheless, the number of Candida isolates which have been studied is relatively small and more

information concerning the variation in glycolytic and proteolytic activity, both for isolates of the same species and between different species, is required before definitive conclusions are drawn. It is, hoped therefore that the basic information provided by this study regarding the interactions of Candida and saliva will assist in planning future investigations to clarify some of the factors involved in this little known area of host-parasite interactions.

APPENDIX A

SABOURAUD'S DEXTROSE AGAR

(Gibco Bio-cult Limited, Paisley, Scotland)

Composition:

	grams/litre
Peptone 180	10.0
Dextrose	40.0
Agar	15.0

Method of preparation

1. Dissolve 65 grams of Sabouraud's dextrose agar in 1 litre of distilled water at 100°C (Koch steamer) with frequent agitation for 2 hours.

2. Aliquot 100ml volumes into Duran bottles.

3. Autoclave at 115°C for 15 minutes.

4. Cool to 50°C and pour plates in a sterile Microflow Cabinet (Microflow, Dent and Hellyer, Hampshire, UK.). Allow to set at room temperature and store at 4°C for up to 10 days, until required. When used for spiral plating the plates were dried at 37°C for 10 minutes in an incubator (Gallenkamp, UK.) prior to use.

COLUMBIA BLOOD AGAR

(Gibco Bio-Cult Limited, Scotland)

Composition:

	grams/litre
Special peptone	23.0
Starch	1.0
Sodium chloride	5.0
Agar No.1	10.0

Method of preparation

1. Dissolve 44grams of Columbia blood agar in 1 litre distilled water at 100^{o}C (Koch steamer).

2. Adjust pH to 7.3 using concentrated HCl or concentrated NaOH.

3. Autoclave at 121^{o}C for 15 minutes.

4. Cool to 50^{0}C and add 50ml sterile defibrinated horse blood (Gibco Europe, Paisley).

5. Aliquot 15ml volumes into sterile petri dishes (3.5 inch in diameter, Sterilin Limited., Teddington) in a sterile Microflow Cabinet (Microflow, Dent and Hellyer, Hampshire, UK.). Plates were allowed to set at room temperature and stored at 4^{o}C for 10 days until required.

SUGAR MEDIUM

1. Molar solutions of glucose sucrose, galactose, maltose, lactose and xylitol were prepared with distilled water in 500ml Duran bottles. All sugars were obtained from BDH Chemicals Limited., Poole, U.K.).

2. The solutions were sterilized by steaming for 30 minutes each day on 3 consecutive days (Tyndalization) in a Koch steamer (BDH Glasgow).

3. Sugar solutions were stored at $4^{o}C$ and used within a month.

YEAST NITROGEN BASE MEDIUM
(Difco, East Molesey, Surrey, U.K.)

Composition:

Yeast Nitrogen Base	1.34 grams	(Twice concentrated)
Distilled water	100ml.	

Composition:

Yeast Nitrogen Base	6.7 grams	(Ten times concentrated)
Distilled water (40^0C)	100ml.	

Method of preparation

1. Dissolve 1.34 or 6.7 grams of YNB in 100ml distilled water.
2. The media were filter sterilized using a 0.2 /um Minisart filter (Satorius, Surrey, U.K.)
3. The concentrated stock solutions of YNB medium were stored at 4^0C, and used within a week.

CONSTITUENTS OF YEAST NITROGEN BASE

Ingredients per liter	
Ammonium sulfate	5g
L-Histidine mono-hydrochloride	10mg
LD-Methionine	20mg
LD-Tryptophan	20mg
Biotin	2µg
Calcium pantothenate	400µg
Folic acid	2µg
Inositol	2000µg
Niacin	400µg
P-Aminobenzoic acid	200µg
Pyridoxine hydrochloride	400µg
Riboflavin	200µg
Thiamine hydrochloride	400µg
Boric acid	500µg
Copper sulfate	40µg
Potassium iodide	100µg
Ferric chloride	200µg
Manganese sulfate	400µg
Sodium molybdate	200µg
Zinc sulfate	400µg
Potassium phosphate (Monobasic)	1g
Magnesium sulfate	0.5g
Sodium chloride	0.1g
Calcium chloride	0.1g
Final pH at 25^0C	5.4

Table 1. Formulae for Bacto Yeast Nitrogen Base. (Difco Manual, 1984).

SUGAR (500mM) IN YEAST NITROGEN BASE MEDIUM

1. Sterile molar solutions of glucose, sucrose, galactose, maltose, lactose and xylitol (BDH Chemicals Limited., Poole, U.K.) were prepared with distilled water in 500ml Duran bottles as described earlier.

2. Within a clear air Microflow Cabinet (Microflow, Dent and Hellyer, Hampshire, UK.) an equal volume of the different sugar solutions were added to the sterile double concentrated YNB (as prepared earlier) to prepare 500mM solutions of glucose and the other sugars.

GLUCOSE (200mM and 50mM) IN YEAST NITROGEN BASE MEDIUM

GLUCOSE (200mM)

1. A sterile molar glucose solution (BDH Chemicals Ltd., Poole, UK.) was prepared as described earlier.

2. A volume of 10ml of ten times concentrated YNB (as prepared earlier) was mixed with 70mls of sterile distilled water and filter sterilized using a 0.2 μm Minisart filter (Satorius, Surrey, UK.). To this solution 20ml of the sterile 1M glucose solution was added to obtain 200mM glucose in YNB liquid medium.

3. A stock solution of glucose in YNB medium (200ml) was prepared and stored at $4^{o}C$, and used within a month.

GLUCOSE (50mM)

1. Yeast nitrogen base (0.67grams) was dissolved in 95ml of sterile distilled water and filter sterilized using 0.2 μm Minisart filter (Satorius, Surrey, U.K.). To this solution 5ml of the sterile 1M glucose solution was added.

2. A stock solution of glucose in YNB medium (200ml) was prepared and stored at $4^{0}C$, and used within a month.

BOVINE SERUM ALBUMIN AGAR

Composition:

	grams/litre
Dextrose	20.0
KH_2PO_4	1.0
$MgSO_4$	0.5
Agar Noble	20.0

Method of preparation

1. Dissolve the constituents above in 1 litre of distilled water at 100°C.

2. Adjust pH to 4.5 using concentrated HCl or concentrated NaOH.

3. Aliquot 100ml in to Duran bottles and autoclave at 110°C for 15 minutes.

4. The bottles of agar were stored at room temperature and used within 2 weeks. When necessary a bottle was melted by placing in a Koch steamer and once the medium was fluid it was cooled to 50°C in a water bath. Twenty millilitres of sterile 1% bovine serum albumin (High Wycombe, U.K.) solution and 1.2ml of vitamin concentrate (Minimum Essential Medium, Gibco, Scotland) were added. The culture medium was mixed slowly to prevent trapping of air bubbles.

5. Aliquots (10ml) were poured into sterile petri dishes, dried for 15 minutes, stored at $4^{o}C$ and used within a week.

Dextrose, KH_2PO_4 and $MgSO_4$ were obtained from BDH Limited; Poole, U.K.

Agar Noble was supplied by Difco, USA.

NAPTHALENE BLACK SOLUTION

Dissolve 1.25 grams of Napthalene black (GURR: BDH Limited, Poole, U.K) in 90 ml methanol (BDH Limited; Poole, U.K.) and 10ml of glacial acetic acid (BDH Limited; Poole, U.K.). This solution was placed on a magnetic stirrer and left to dissolve overnight. The solution was stored at room temperature and used within 1 week.

LYSOZYME (Hen's egg white, three times crystallised)

(Sigma Chemical Co., Poole. UK.)

1. Dissolve 0.04grams of Lysozyme in 10ml of sterile distilled water.

2. From this stock solution serial dilutions of the enzyme ranging from 1000µg/ml to 40µg/ml were prepared using sterile distilled water.

APPENDIX B

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

Preparation of SDS-Polyacrylamide gels

Preparation of all solutions required for SDS-PAGE gel electrophoresis are given below.

STOCK ACRYLAMIDE

Acrylamide	30g
N'-N'-Methylene bis acrylamide	0.8g
Water	100ml

AMMONIUM PERSULPHATE

Ammonium persulphate	1g
Water	10ml

RUNNING BUFFER

Trizma base	9.08g
Water	100ml

pH adjusted to 8.8 with conc. HCl.

STACKING BUFFER

Trizma base	2.06g
Water	100ml

pH adjusted to 6.8 with conc. HCl.

STOCK SDS SOLUTION

Sodium lauryl sulphate	2g
Water	10ml

SAMPLE DISSOLVING SOLUTION

Sodium lauryl sulphate	2g
Dithiothreit	0.385g
Water	10ml

RESERVOIR BUFFER

Trizma base	3.024g
Glycine	14.41g
Sodium lauryl sulphate	1.0g
Water	1000

TRACKER DYE

Bromophenol blue	25mg
Water	10ml

FIXATION AND STAINING

Ethanol	200ml
Acetic acid	40ml
Water	160ml
Coomasie brilliant blue R-250	0.4g

DESTAINING SOLUTION

Acetic acid	10ml
Water	90ml

SUPPLIERS OF REAGENTS

Chemicals:

1. Acrylamide, N'-N'-methylene-bis acrylamide, ammonium persulphate, glycine, acetic acid and ethanol were obtained from BDH (Poole, Dorset, U.K.).
2. Sodium lauryl sulphate, 'Prima grade' was obtained from Fisons plc., U.K.
3. Dithiothreit and Trizma base (hydroxymethyl aminomethane) from Boehringer Manheim Gm bH-W.Germany.
4. Bromophenol blue, glycerol, Coomasie brilliant blue R-250 and N,N.N',N'-tetramethyl-ethylenediamine (TEMED) were purchased from Sigma, St. Lewis, MO.

DECOLOURIZING SOLUTION

1. Phenol crystals (BDH Limited; Poole, U.K.) were placed in a universal container and liquidized by heating in a water bath at $40^{o}C$ for 10 minutes.

2. The final decolourizing solution consisted of 100ml of phenol solution, 300ml glacial acetic acid (BDH Limited; Poole, U.K. and 2000ml distilled water.

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