LABORATORY AND CLINICAL STUDIES ON THE TREATMENT OF
CANDIDA-ASSOCIATED DENTURE STOMATITIS WITH SODIUM
HYPOCHLORITE OR MICROWAVE IRRADIATION

Bettine Constance Webb

M.D.S.

M.H.P. (U.N.S.W.)

A thesis submitted in fulfilment
of the requirements for the degree of

Doctor of Philosophy

Discipline of Removable Prosthodontics

Faculty of Dentistry

The University of Sydney

August, 1996
This thesis is dedicated to my parents Allan and Heather Webb.
STATEMENT OF AUTHORSHIP

The experimental work described in this thesis was performed by the candidate except where stated otherwise. This work has not been submitted in whole or in part for any other degree.

Bettine C. Willis
30/8/96
ACKNOWLEDGMENTS

I would like to express my appreciation to my supervisor Associate Professor C. J. Thomas and co-supervisors Emeritus Professor K. W. Knox and Dr. Mark Willcox for their help, guidance and support throughout my candidature. I am grateful to Dr. Derek Harty for his patient advice and assistance and to Drs. Neil Hunter and Nick Jacques for their interest and encouragement.

Part of this research was conducted at the Electron Microscopy Unit, University of Sydney and my thanks are due to Dr. Clive Nockolds and Mr Tony Romeo for their time and advice.

The clinical studies described in this work were performed at the United Dental Hospital of Sydney and I am grateful to Dr. J. Wilkinson, Director of Dental Services and Dr. H. Champion, Head, Department of Prosthetic Dentistry for permission to use clinic facilities. I am indebted to Dr. M. D. Mercado, for making available her patients for the research on denture plaque and soft denture liners. Thanks are due to all the patients who participated in this study and gave willingly of their time and co-operation.

I am grateful to Ms A. Vincent for provision of library resources, Mrs R. Granger for clerical assistance and Mr R. van Luyn for assistance with the photographic work in this thesis. The support of the Faculty of Dentistry in providing financial assistance towards laboratory expenditure is acknowledged.

Finally, I wish to thank Emeritus Professor K. W. Knox and Associate Professor C. J. Thomas for giving me the opportunity to commence this work which has proved to be an enjoyable and memorable "learning curve".
TABLE OF CONTENTS

STATEMENT ................................................................. ii
ACKNOWLEDGMENTS ...................................................... iii
LIST OF APPENDICES .................................................. viii
PUBLICATIONS ............................................................. ix
LIST OF MICROBIAL NOMENCLATURE ................................ xi
LIST OF ABBREVIATIONS ............................................... xii
SUMMARY ................................................................. xv

CHAPTER 1 ........................................................................... 1
REVIEW OF LITERATURE ................................................ 1
  1.1 Introduction ............................................................ 1
  1.2 Characteristics of Candida species .............................. 2
    1.2.1 Macroscopic characteristics ................................. 3
    1.2.2 Microscopic characteristics ................................. 3
    1.2.3 Identification ................................................. 7
  1.3 Oral microbial ecology and distribution of Candida species 7
  1.4 Factors affecting distribution of oral Candida ............. 8
    1.4.1 Saliva .............................................................. 8
    1.4.2 pH ................................................................. 9
    1.4.3 Adhesion ......................................................... 10
    1.4.4 Mannoprotein .................................................. 12
    1.4.5 Cell surface hydrophobicity ................................. 12
    1.4.6 Oral bacteria ................................................... 13
    1.4.7 Hyphae ........................................................... 14
    1.4.8 Enzymes ........................................................ 16
    1.4.9 Other factors involved in invasiveness ................. 16
  1.5 Oral diseases caused by Candida species ..................... 17
    1.5.1 Classification of oral candidosis ......................... 17
    1.5.2 Host reaction .................................................. 18
    1.5.3 Acute pseudomembranous candidosis .................... 20
    1.5.4 Acute erythematous candidosis ............................ 20
    1.5.5 Chronic hyperplastic candidosis ......................... 20
    1.5.6 Candida-associated denture stomatitis .................. 21
      1.5.6.1 Trauma ..................................................... 25
      1.5.6.2 Bacteria .................................................. 25
      1.5.6.3 Candida species ......................................... 26
      1.5.6.4 Trauma and Candida species .......................... 30
      1.5.6.5 Denture lining materials .............................. 30
      1.5.6.6 Candida species and other oral microorganisms. .. 32
      1.5.6.7 Denture hygiene/denture plaque ..................... 32
      1.5.6.8 Permeability of acrylic resin ....................... 33
      1.5.6.9 Antibodies and immunological factors ............. 34
      1.5.6.10 Multifactorial ......................................... 35
1.5.7 Angular cheilitis ............................................... 35
1.6 Treatment of oral candidosis ...................................... 36
  1.6.1 Antifungals ............................................... 36
  1.6.2 Antiseptics and disinfecting agents ......................... 40
  1.6.3 Sodium hypochlorite ........................................ 43
  1.6.4 Microwave irradiation ....................................... 44
1.7 Aims of the study .................................................. 45

CHAPTER 2
EFFECT OF SODIUM HYPOCHLORITE ON ADHESION OF CANDIDA SPECIES TO SURFACES IN VITRO .............................................. 46
  2.1 Introduction .................................................. 46
  2.2 Materials and methods ......................................... 47
    2.2.1 Microorganisms and growth conditions .................... 47
    2.2.2 Determination of sub-MIC of sodium hypochlorite .......... 48
    2.2.3 Adhesion to polystyrene ................................... 49
    2.2.4 Adhesion to S. gordonii-coated polystyrene ............... 49
    2.2.5 Adhesion to acrylic resin .................................. 50
    2.2.6 Adhesion to BEC ........................................... 51
    2.2.7 Coaggregation with S. gordonii LGR2 ...................... 51
  2.3 Statistical analysis ............................................ 52
  2.4 Results ........................................................ 52
    2.4.1 Adhesion to polystyrene ................................... 53
    2.4.2 Adhesion to S. gordonii-coated polystyrene ............... 53
    2.4.3 Adhesion to acrylic ........................................ 54
    2.4.4 Adhesion to BEC ............................................ 56
    2.4.5 Coaggregation with S. gordonii LGR2 ...................... 56
  2.5 Discussion ..................................................... 57

CHAPTER 3
EFFECT OF SODIUM HYPOCHLORITE ON INVASIVE CHARACTERISTICS OF CANDIDA SPECIES AND HOST INFLAMMATORY REACTION ...................... 61
  3.1 Introduction ................................................... 61
  3.2 Materials and methods ......................................... 63
    3.2.1 Microorganisms and growth conditions .................... 63
    3.2.2 Proteinase production ..................................... 64
    3.2.3 Production of germ tubes .................................. 64
    3.2.4 Examination of Candida cell wall proteins ............... 65
    3.2.5 Aggregation of human platelets ............................ 66
  3.3 Statistical analysis ............................................ 66
  3.4 Results ........................................................ 67
    3.4.1 Proteinase production ..................................... 67
    3.4.2 Germ tube formation ....................................... 67
    3.4.3 Examination of cell wall proteins ......................... 68
    3.4.4 Aggregation of human platelets ............................ 74
  3.5 Discussion ..................................................... 78
CHAPTER 4
IN VITRO EFFECTS OF SODIUM HYPOCHLORITE AND MICROWAVE IRRADIATION ON SURVIVAL OF CANDIDA SPECIES AND OTHER MICROORGANISMS ON DENTURE SURFACES ........................................ 82

4.1 Introduction ........................................ 82
4.2 Materials and methods ................................. 85
  4.2.1 Microorganisms and dentures ....................... 85
  4.2.2 Sodium hypochlorite treatment ....................... 86
  4.2.3 Microwave treatment ................................ 87
    4.2.3.1 Microwave oven ................................ 87
    4.2.3.2 Dimensional stability of dentures ................. 88
    4.2.3.3 Treatment ........................................... 90
  4.2.4 SEM procedures ...................................... 91
4.3 Results .................................................. 93
  4.3.1 Sodium hypochlorite treatment ..................... 93
  4.3.2 Microwave treatment ................................. 94
  4.3.3 SEM procedures ...................................... 95
4.4 Discussion .............................................. 102

CHAPTER 5
STUDIES ON DENTURE PLAQUE: CHARACTERIZATION OF CANDIDA SPECIES AND OTHER MICROORGANISMS ................................................... 105

5.1 Introduction ........................................... 105
5.2 Materials and methods ................................ 106
  5.2.1 Investigation 1 ....................................... 106
  5.2.2 Investigation 2 ....................................... 107
5.3 Microbiological analysis ............................... 107
  5.3.1 Investigation 1 ....................................... 107
  5.3.2 Investigation 2 ....................................... 108
5.4 Results .................................................. 109
  5.4.1 Investigation 1 ....................................... 109
  5.4.2 Investigation 2 ....................................... 114
5.5 Discussion .............................................. 117

CHAPTER 6
MICROBIOLOGY OF DENTURES WITH SOFT LINERS: COLONIZATION BY CANDIDA SPECIES AND AEROBIC BACTERIA .......................................... 122

6.1 Introduction ........................................... 122
6.2 Materials and methods ................................ 123
  6.2.1 Patients ............................................... 123
  6.2.2 Soft denture lining materials ....................... 124
  6.2.3 Procedures ............................................ 124
6.3 Microbiological analysis ............................... 124
6.4 Statistical analysis .................................... 125
6.5 Results .................................................. 126
6.6 Discussion .............................................. 131
CHAPTER 7
CLINICAL STUDY TO TEST THE EFFECT OF SODIUM HYPOCHLORITE SOAKING ON THE MICROORGANISMS OF DENTURE PLAQUE .... 135
  7.1 Introduction ............................................. 135
  7.2 Preliminary study ....................................... 136
  7.3 Main clinical study ..................................... 138
  7.4 Materials and methods ................................ 138
    7.4.1 Group A (sodium hypochlorite) .................. 139
    7.4.2 Group C (control) ................................. 140
    7.4.3 Clinical procedures ............................... 140
  7.5 Microbiological analysis .............................. 142
  7.6 Statistical analysis .................................. 142
    7.6.1 Comprehensive data ............................... 142
    7.6.2 Compliance ......................................... 143
  7.7 Results .................................................. 143
    7.7.1 Patient details .................................... 143
    7.7.2 Aerobic bacterial counts ........................... 144
    7.7.3 Candida counts and species ...................... 147
  7.8 Discussion .............................................. 151

CHAPTER 8
CLINICAL STUDY TO TEST THE EFFECT OF MICROWAVE IRRADIATION ON THE MICROORGANISMS OF DENTURE PLAQUE ............. 156
  8.1 Introduction ............................................. 156
  8.2 Preliminary study ....................................... 156
  8.3 Main clinical study ..................................... 158
  8.4 Materials and methods ................................ 159
    8.4.1 Group B (microwave irradiation) .................. 159
    8.4.2 Group C (control group) ........................... 160
    8.4.3 Clinical procedures ............................... 160
  8.5 Microbiological analysis .............................. 160
  8.6 Statistical analysis .................................. 161
    8.6.1 Overall data ....................................... 161
    8.6.2 Special conditions ................................. 161
  8.7 Results .................................................. 162
  8.8 Discussion .............................................. 168

CHAPTER 9
GENERAL DISCUSSION .......................................... 171

APPENDICES .................................................. 180

REFERENCES .................................................. 208
LIST OF APPENDICES

Appendix A ................................................................. 180

1. Culture media formulae

2. Substances other than media formulae

Appendix B ................................................................. 185

Approvals from Human Ethical Review Committee for use of human subjects, clinical studies Chapters 5, 6, 7, and 8

Appendix C ................................................................. 188

Consent form for clinical studies Chapters 5 and 6

Appendix D ................................................................. 189

Consent form for clinical studies Chapters 7 and 8

Appendix E ................................................................. 190

Consent form for clinical study (Addendum to Chapter 8)

Appendix F ................................................................. 191

Instructions to patients for the use of sodium hypochlorite over-night denture soak

Appendix G ................................................................. 192

Statistical analysis of raw data from Chapter 7, compiled by the Cooperative Research Centre for Eye Research and Technology, University of New South Wales

Statistician’s report ....................................................... 204

Appendix H ................................................................. 206

Addendum to Chapter 8
PUBLICATIONS

All of the following reports contain results described in this thesis.

Journal Articles


Conference Abstracts and Presentations


LIST OF MICROBIAL NOMENCLATURE

The genera of a number of species referred to in the text are abbreviated as follows:

<table>
<thead>
<tr>
<th>Species</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Candida albicans</em></td>
<td><em>C. albicans</em></td>
</tr>
<tr>
<td><em>Candida glabrata</em></td>
<td><em>C. glabrata</em></td>
</tr>
<tr>
<td>(formerly <em>Torulopsis glabrata</em>)</td>
<td>(formerly <em>T. glabrata</em>)</td>
</tr>
<tr>
<td><em>Candida guilliermondii</em></td>
<td><em>C. guilliermondii</em></td>
</tr>
<tr>
<td><em>Candida kefy</em></td>
<td><em>C. kefy</em></td>
</tr>
<tr>
<td>(formerly <em>Candida pseudotropicalis</em>)</td>
<td>(formerly <em>C. pseudotropicalis</em>)</td>
</tr>
<tr>
<td><em>Candida krusei</em></td>
<td><em>C. krusei</em></td>
</tr>
<tr>
<td><em>Candida parapsilosis</em></td>
<td><em>C. parapsilosis</em></td>
</tr>
<tr>
<td><em>Candida tropicalis</em></td>
<td><em>C. tropicalis</em></td>
</tr>
<tr>
<td><em>Candida stellatoidea</em></td>
<td><em>C. stellatoidea</em></td>
</tr>
<tr>
<td><em>Streptococcus anginosus</em></td>
<td><em>S. anginosus</em></td>
</tr>
<tr>
<td><em>Streptococcus gordonii</em></td>
<td><em>S. gordoni</em></td>
</tr>
<tr>
<td><em>Streptococcus milleri</em></td>
<td><em>S. milleri</em></td>
</tr>
<tr>
<td><em>Streptococcus mutans</em></td>
<td><em>S. mutans</em></td>
</tr>
<tr>
<td><em>Streptococcus mitis</em></td>
<td><em>S. mitis</em></td>
</tr>
<tr>
<td><em>Streptococcus oralis</em></td>
<td><em>S. oralis</em></td>
</tr>
<tr>
<td><em>Streptococcus pneumoniae</em></td>
<td><em>S. pneumoniae</em></td>
</tr>
<tr>
<td><em>Streptococcus salivarius</em></td>
<td><em>S. salivarius</em></td>
</tr>
<tr>
<td><em>Streptococcus sanguis</em></td>
<td><em>S. sanguis</em></td>
</tr>
</tbody>
</table>

Since strains within the *Bacteroides* genus are now divided into a number of genera, the name "*Bacteroides*" is used to include these genera, for example species within the *Porphyromonas* group.

The bioMérieux Identification System for yeasts refers to *C. albicans* strains as *C. albicans* 1 and *C. albicans* 2, the former being all strains of *C. albicans* with the exception of "*C. stellatoidea*" which is referred to as *C. albicans* 2. In all experiments described in this thesis where *Candida* species were identified by the bioMérieux ID 32 C system, *C. albicans* refers to *C. albicans* 1 as defined above.
LIST OF ABBREVIATIONS

ADP  adenosine diphosphate
AIDS acquired immune deficiency syndrome
BEC buccal epithelial cell
BHI brain heart infusion
BSA bovine serum albumin
°C degrees Celsius
CFAT cadmium, fluoride, acriflavin, tellurite (selective media)
cfu colony-forming unit
cm centimetre
CMI cell mediated immunity
Co. Company
CSH cell surface hydrophobicity
DNA deoxyribonucleic acid
ed. editor
EDTA ethylenediaminetetraacetic acid
EP extra-cellular polymeric material/polymer
et al. et alii (Latin) = and others
ETSA enriched trypticase soy agar
g gravitational force (9.8 m/s²)
g gram
GSB glucose salts biotin
h hour
HIV human immunodeficiency virus
HOCl hypochlorous acid
Hz hertz
IgA immunoglobulin A
Inc. incorporated
KA kanamycin (selective media)
kBq kiloBecquerel
kDa kiloDalton
l litre
M molar
m metre
mg milligram
MHz megahertz
MIC minimal inhibitory concentration
min minute
ml millilitre
mm millimetre
MRS de Man-Rogosa agar (selective media)
MSA Mitis-Salivarius agar (selective media)
μg microgram
μl microlitre
μm micrometre
n number of subjects in sample
The suffix "s" following an abbreviation indicates the plural, for example, BECs.

Abbreviations are used for frequently mentioned terms including the names of genera, and are listed above and in microbial nomenclature; for example, Sabouraud's broth and *Candida albicans* are referred to as SB and *C. albicans* respectively throughout the text.

The names of genera or species that are referred to only once or infrequently are written in full, for example, *Staphylococcus albus*.

Normal chemical nomenclature is used in this thesis.
In the experiments described in this thesis the term disinfection is used when no microorganisms were detected, and "0" with the appropriate symbol indicating no detectable growth is used in the tables included in the text.

Throughout this thesis the term "oral candidosis" is used in preference to "oral candidiasis" except when the latter is referred to by other investigators.

Discussion arose concerning the use of the terms "sterilization" or "disinfection" within the text of this thesis and it was decided that the more appropriate term was "disinfection". In Chapter 3 (3.2.3; 3.4.2; Table 3.1) the term "germ tubes" has been used in place of "hyphae", as in the method followed in the experiment the former nomenclature was considered technically correct.
SUMMARY

This thesis describes experiments which were carried out at the Institute of Dental Research in Sydney and within the Department of Prosthetic Dentistry at the United Dental Hospital of Sydney between February 1991 and May 1996.

The study is concerned with finding practical means of treating chronic atrophic candidosis, also referred to as Candida-associated denture stomatitis and to this purpose two methods of denture disinfection are investigated, namely, sodium hypochlorite denture soak and microwave irradiation.

Although the aetiology of denture stomatitis is generally considered to be multifactorial, there is sufficient evidence that Candida species and in particular C. albicans play an important role in the aetiology of the condition. In Chapter 1, therefore, the literature review, which provides relevant background information for the experiments to be described in later chapters, is primarily concerned with Candida species. The characteristics and distribution of Candida species are described and factors affecting the distribution of oral Candida are discussed. The literature relating to the cause of chronic atrophic candidosis is vast and consequently a detailed description is given of Candida-associated denture stomatitis in the section concerned with oral diseases caused by Candida and their treatment.

Each of the subsequent chapters, contains a brief literature review of material relevant to the subject of the particular chapter. Chapter 2 describes laboratory work to assess the effect of sodium hypochlorite on the adhesion of Candida species to oral surfaces and the ability of Candida to coaggregate with oral
streptococci. The results showed that sodium hypochlorite decreased the ability of *Candida* species to adhere to both inert surfaces and BECs. However, coaggregation of *Candida* with streptococci was increased. Thus, hypochlorite if used as a denture soak may initially reduce the ability of *Candida* species to adhere to the denture surface and may therefore assist the treatment of denture stomatitis.

The effects of hypochlorite on the characteristics of *Candida* species that are associated with tissue invasion are described in Chapter 3. The production of acid proteinase, the formation of germ tubes and the presence of major cell wall proteins at 43 and 27 kDa are demonstrated. The ability of the whole cells of certain species of *Candida* to aggregate human platelets was assessed. The results showed that sodium hypochlorite did not affect proteinase production by *Candida* species but the rate of germ tube formation and the production of *Candida* cell wall proteins were increased. Hypochlorite did not affect the ability of certain *Candida* species to aggregate human platelets. Mechanisms to defend the host against candidal invasion are discussed and include platelet aggregation where aggregated platelets release antimicrobial factors that are active against *Candida*.

Chapter 4 describes an *in vitro* study to test the effects of sodium hypochlorite and microwave irradiation on the survival of *Candida* species and oral streptococci on denture surfaces. The results showed that 0.02% sodium hypochlorite denture soak for 8 h will eliminate *Candida* species and reduce the growth of streptococci. However, microwaving of dentures at medium setting for 6 min will eliminate both *Candida* and streptococci. This information serves as baseline data for clinical assessments described in Chapters 7 and 8.
Denture hygiene is an important factor in the prevention and treatment of Candida-associated denture stomatitis. Hence, a clinical study to assess the microbiology of denture plaque is described in Chapter 5. The results showed that denture plaque was composed mainly of Gram-positive streptococci with varying proportions of Gram-positive rods, Gram-negative cocci and rods and is similar to dental plaque. Candida was not always isolated and when detected constituted a very small proportion (< 1%) of the total aerobic bacterial count.

The results of an investigation to test the effect of soft denture liners in lower dentures on the colonization of denture surfaces by Candida species and aerobic bacteria are given in Chapter 6. There was no significant difference in Candida/bacterial colonization of dentures with soft denture liners and those without liners.

Chapter 7 describes a clinical study to test the efficacy of sodium hypochlorite (0.02%) over-night denture soak as an effective denture disinfecting agent. Treatment of dentures with hypochlorite over a trial period resulted in reductions of Candida and aerobic bacteria and although the reductions were not significant the effect over the trial period could be assessed. A significant finding was that for the palate, treatment with hypochlorite over the trial period prevented an increase in candidal load. Thus, sodium hypochlorite may function as an effective disinfecting agent when used as a 0.02% denture soak for a prolonged period.

A pilot study to assess the effectiveness of microwaving dentures for ten min (350 W, 2450 MHz) as a potential method of denture disinfection is described in Chapter 8. For practical reasons the dentures were microwaved once only and therefore the effect over a trial period could not be assessed. However, one
treatment resulted in significant reductions in the levels of *Candida* and aerobic bacteria. These findings have indicated that future research should be carried out to test the effect of daily consecutive microwave treatments on candidal and bacterial growth on dentures.

The general discussion in Chapter 9 summarizes the data presented in the previous chapters and from the findings conclusions are made concerning the prevention and treatment of *Candida*-associated denture stomatitis. The limitations of this thesis are recognized and some important aspects of the study are recommended for future research.
CHAPTER 1

REVIEW OF LITERATURE

1.1 INTRODUCTION

In recent years there has been an increasing interest in candidal infections in immuno- and medically-compromised individuals in the community. This has resulted in a large volume of research data which have focused attention on *C. albicans* as the primary aetiologic agent of candidosis, a disease which can vary from superficial mucosal lesions to a life-threatening systemic form. Much interest has been shown in the incidence of oral candidosis as it is frequently an early manifestation of HIV infection in patients with AIDS. Apart from these patients, however, oral candidosis in the form of *Candida*-associated denture stomatitis is a common disease in up to 65% of denture wearers, and *C. albicans*, *C. glabrata* and *C. tropicalis* have been isolated from such cases (Budtz-Jorgensen et al., 1975). This study is concerned with the prevention and treatment of *Candida*-associated denture stomatitis, and investigates the characteristics of *Candida* species that overcome host defences and damage host tissues and the factors that may assist the host to prevent or repel candidal invasion.

This investigation assesses the ability of *Candida* species to adhere to oral surfaces, to coaggregate with oral streptococci and to invade oral epithelium; assays to test the effect of sodium hypochlorite on these parameters will be described. In addition, disinfection of denture surfaces by microwave irradiation is reported. Denture stomatitis has been associated with the presence of denture plaque (Budtz-Jorgensen, 1974), hence, denture hygiene is an important factor
in the prevention and treatment of the disease. This study assesses two methods of denture disinfection which will be of practical use in the clinical environment, namely, sodium hypochlorite and microwaving.

There is an abundance of literature relating to Candida infection within the oral cavity, but the review which follows is orientated to the study of Candida-associated denture stomatitis.

1.2 CHARACTERISTICS OF CANDIDA SPECIES

Yeast cells or blastospores are unicellular, eukaryotic organisms which multiply by a specific process of mitotic cell division known as budding. Odds (1988) has clearly defined a nomenclature for the different morphological stages of Candida development and is of the opinion that budding involves growth of new cellular material from a particular site on the blastospore surface. When the bud has grown to optimal size, nuclear division occurs and a septum is formed between the two cell units. Odds (1988) has defined a hypha as a microscopic tube which contains multiple cell units divided by septa and which may arise from existing hyphae or from blastospores. The hyphae, which develop from blastospores, are known as germ tubes and they grow continuously by apical extension. When blastospores are produced one from another in linear fashion without separating, a structure termed pseudohypha is formed (Warren and Shadomy, 1991). True septate hyphae are produced by some Candida species, such as C. tropicalis, under certain circumstances, but true hyphae are mainly associated with C. albicans. The entire candidal cellular aggregate including hyphae, branches and lateral buds is referred to as mycelium (Odds, 1988).
1.2.1 Macroscopic characteristics

_Candida_ species form soft cream-coloured colonies (Figure 1.1) with a yeasty odour when grown under aerobic conditions on media which have a pH in the range of 2.5 - 7.5, and an incubation temperature in the range of 20°C - 38°C. Growth is usually detected in 48-72 h, and sub-cultures may grow more rapidly. The ability of yeasts to grow at 37°C is an important characteristic to be considered in their identification from clinical specimens as most pathogenic species grow readily at 25°C and 37°C, whereas saprophytes usually fail to grow at the higher temperature (Warren and Shadomy, 1991). In contrast to the convex colonies of other _Candida_ species, _C. krusei_ grows as spreading colonies with a matt or rough whitish yellow surface (Samaranayake and Samaranayake, 1994).

1.2.2 Microscopic characteristics

The gross microscopic appearance of all the species is similar; all yeasts are Gram-positive but sometimes the shapes of the blastospores can vary from ovoid to elongated or spherical (Odds, 1988). Microscopically, _C. albicans_ exhibits dimorphism (Figures 1.2, 1.3) in which there is a transition from ovoid budding blastospores (yeast cells) to parallel-sided hyphae (Odds 1988). Size also varies, with measurements for _C. albicans_ and _C. krusei_ blastospores being given as 2.9-7.2 x 2.9-14.4 μm and 2.2-5.6 x 4.3-15.2 μm respectively (Odds, 1988). The cells of _C. krusei_ appear elongated and have the appearance of "long grain rice", and _C. kefyr_ (_C. pseudotropicalis_) another clinically important species, has a similar microscopic appearance (Samaranayake and Samaranayake, 1994).
Figure 1.1
Colonies of *C. albicans* grown on Sabouraud's medium for 48 h at 37°C
Figure 1.2 Scanning electron micrograph showing dimorphism in *C. albicans* H1 grown on denture acrylic for 48 h at 37°C, budding blastospores and hyphae forming mycelium are clearly visible (bar = 10μm)
Figure 1.3 Scanning electron micrograph showing C. albicans H1 grown on denture acrylic for 48 h at 37°C, budding blastospores and hyphae penetrates the acrylic clearly visible (bar = 10μm).
1.2.3 Identification

The production of pseudohyphae is one of the major differences between *C. glabrata* (a species that cannot form pseudohyphae) and other medically important *Candida* species. Observation of germ tubes and chlamydospores (large thick-walled cells which develop at the tips of pseudohyphae) are also helpful in identifying *C. albicans* (Warren and Shadomy, 1991).

All pathogenic *Candida* species assimilate and ferment glucose as a carbon source. None of them assimilates nitrate as a nitrogen source, but they vary in their abilities to utilize other carbon and nitrogen sources (Odds, 1988). Carbon assimilation and occasionally fermentation studies are needed to differentiate *Candida* species. Of the clinically important *Candida* species, *C. guilliermondii* is the only one to assimilate dulcitol and *C. kefyr* assimilates lactose (Warren and Shadomy, 1991). Certain rare strains of *C. tropicalis* may assimilate cellobiose weakly and show an assimilation pattern similar to that of *C. parapsilosis*. The inclusion of arabinose is useful, since *C. parapsilosis* readily assimilates this carbohydrate whereas most strains of *C. tropicalis* do not (Warren and Shadomy, 1991). The most frequently used yeast identification system is the bioMérieux ID 32 C (bioMérieux Vitek, Inc. Hazelwood, Missouri, USA), and this utilizes the carbohydrate assimilation tests described above.

1.3 ORAL MICROBIAL ECOLOGY AND DISTRIBUTION OF CANDIDA SPECIES

The opportunistic fungus *C. albicans* is the commonest of the *Candida* species found within the oral cavity. Other species which have been isolated include *C.*
*glabrata, C. tropicalis, C. kefyr, C. krusei* and *C. guilliermondii* (MacFarlane and Samaranayake, 1989). *Candida* organisms, as commensal members of the normal oral microbiota, are present on average in 40% (20-60% range) of the human population (MacFarlane and Samaranayake, 1989). The dorsum of the tongue is considered to be the primary oral reservoir of *C. albicans* with other oral sites such as mucosa, plaque-covered tooth surfaces and saliva being colonized secondarily (Arendorf and Walker, 1980). *Candida* species are also found as harmless commensals of the digestive and vaginal tracts. However, in medically compromised and immunosuppressed individuals, when the host’s defence system is impaired, *C. albicans* infection can lead to the establishment of candidosis, which is manifested as superficial, involving the mucosa, or disseminated, which is the more serious invasive form (Shepherd, 1986). Oral candidosis is one of the most common fungal infections associated with HIV infection and *C. albicans* is the species most often isolated, with *C. glabrata, C. tropicalis* and *C. krusei* occasionally detected (Samaranayake and Holmstrup, 1989; MacPhail et al., 1993). A recent review identified *C. krusei* among other species of *Candida* as an emerging nosocomial pathogen in view of its pathogenic and clinical manifestations in hospitalized patients and medically/immuno-compromised persons (Samaranayake and Samaranayake, 1994).

1.4 FACTORS AFFECTING DISTRIBUTION OF ORAL CANDIDA

1.4.1 Saliva

Studies have shown that saliva reduces the adhesion of *C. albicans* to acrylic, whereas serum, which may enter the oral cavity as a result of trauma to the
palatal mucosa, enhances adhesion (Samaranayake et al., 1980; McCourtie and Douglas, 1981). In another study it was suggested that it was the salivary proteins or mucin that act as receptors for mannoproteins on the surface of *C. albicans* (Nikawa and Hamada, 1990). This was confirmed by the work of Edgerton et al. (1993) and Hoffman and Haidaris (1993), who reported that *C. albicans* selectively adsorbs salivary mucins and that mucin-containing saliva enhances adhesion of yeast cells to acrylic.

The reduction or complete absence of salivary secretion in individuals with xerostomia due to Sjogren’s syndrome (a group of symptoms including enlargement of the parotid gland) has a profound effect on the normal oral microbiota. The reduced moisture level favours the growth of bacteria such as *Staphylococcus aureus*, which is resistant to dryness, and inhibits oral commensals adapted to high moisture levels (MacFarlane and Samaranayake, 1989). The study also showed that a low salivary pH and a high oxygen tension alter the oral environment, and, as a result the numbers of *Veillonella*, commensal *Neisseria* and *Micrococcus* species are reduced, while the growth of *Candida* species, *S. mutans* and *Lactobacillus* species is favoured.

### 1.4.2 pH

It has been suggested that *Candida* species tend to favour an acidic environment for colonization (Verran, 1988), and low pH levels have been observed in denture plaque obtained from upper dentures of denture stomatitis patients on sucrose or glucose-rich diets (Samaranayake, 1986).

The effect of pH on the adhesion of *C. albicans* strains to mucosal surfaces has
been shown to vary with the source of the mucosal cell and the strain (Mehentee and Hay, 1989). A recent study by Verran et al. (1991) has shown that *C. albicans* strains appeared to behave differently in response to a change of pH. All strains were capable of adhering to BEC at pH 7.3, 6.0 and 2.6, although adhesion was low. However, adhesion to BEC was increased when stationary phase cells were used instead of early exponential phase cells, with one strain producing hyphae at pH 2.6. In the same study, pH did not significantly influence adhesion to acrylic except in one instance where the strain that adhered best did so at pH 7.3, while two strains isolated from cases of denture stomatitis produced hyphae at lower pH values.

1.4.3 Adhesion

The interactions between *C. albicans* and the host are complex, and several investigators have suggested that the mechanism of attachment involves interactions between *Candida* cell ligands and host cell receptors (Bouchara et al., 1990; Calderone and Braun, 1991). The ligand receptors of *C. albicans* are thought to be mannanproteins, and it has been demonstrated that mammalian cell proteins iC3b, fibrinogen, fibronectin and laminin will bind to *C. albicans* as a result of *Candida* CR3-like recognition (Calderone and Braun, 1991). *C. albicans* produces extra-cellular polymeric material which contains a mannanprotein adhesin. The interaction between *Candida* and epithelial cells is thought to be one involving the protein portion of the mannanprotein adhesin and the fucose or N-acetylglucosamine containing surface glycoproteins of epithelial cells (Critchley and Douglas, 1987).
Recent investigations indicate that the adhesion of *C. albicans* to host cells is
dependent on the type of host cell and strain of the organism, and at least four
different *Candida*-host cell recognition systems have been described (Calderone,
1993, 1994). The first involves yeast cell mannoprotein with lectin-like properties
(lectin being protein that causes agglutination of cells by binding to sugars in
membrane glycoproteins), which recognizes the terminal fucosyl- or N-
acetylglucosaminyl-containing glycosides of host epithelial cells. The second
system involves the CR2/CR3 complement receptor of *C. albicans*, which
recognizes host endothelial cells. A 60 kDa mannoprotein extracted from hyphae
appears to bind both ligands that contain the amino acid sequence arginine-
glycine-aspartic acid (RGD peptides) and non-RGD-containing ligands. The
RGD ligands are important constituents of the extracellular matrix of endothelial
cells with fibronectin being one of the proteins containing the RGD sequence.
Calderone (1993) also mentions two other systems which recognize receptors of
epithelial cells, namely, one involving a mannan oligosaccharide and the other
concerned with the chitin content of *Candida* cell wall. The author has suggested
that the existence of these ligand-recognition systems may explain the wide range
of sites susceptible to invasion by *Candida* species throughout the body.

Odds (1994) has indicated that the production of virulence factors in *Candida*
species may vary according to site, stage of invasion and nature of host response.
Most investigators agree that stationary phase blastospores adhere better to tissue
cells, mucosal cells and acrylic than those at the exponential (log) phase (King
undergoes a change in cell surface composition when grown to the stationary
phase in media containing high concentrations of specific sugars. Modification takes place during the stationary phase of growth and there is the production of an outer fibrillar-floccular layer which is released from the cell surface. This material together with electrostatic forces, is thought to be responsible for enhanced candidal adhesion to acrylic in vitro. In the oral cavity, colonization of the denture requires attachment to the salivary pellicle (consisting of adsorbed proteins and glycoproteins) covering the denture (McCourtie and Douglas, 1981).

1.4.4 Mannoprotein

The cell wall of C. albicans is composed primarily of the polysaccharides mannann, glucan and chitin. The wall is of variable thickness and consists of several layers with differing electron density. The number of layers and their morphology vary and this is related to such factors as stage of growth, yeast form or germ tube, and growth medium but usually there are five layers within the cell wall. The outer fibrillar layer is composed of mannann or mannoprotein which is also found in different locations throughout the cell wall. Mannans represent about 40% of the total cell wall polysaccharide or 15.2%-22.9% of the yeast cell wall (dry weight), while β-1, 3-D-glucans and β-1, 6-D-glucans form 47-60% by weight of the cell wall. Proteins, lipids and chitin account for 6-25%, 1-7% and 0.6-9% by weight of the cell wall respectively (Calderone and Braun, 1991). The role of mannoproteins as Candida cell wall adhesins has been discussed in 1.4.3.

1.4.5 Cell surface hydrophobicity

Studies have shown that CSH is involved in the adhesion of blastospores to
human epithelial cells and plastics. External cell wall protein changes in \textit{C. albicans} are thought to be responsible for changes in hydrophobicity and hydrophilicity (Hazen \textit{et al.}, 1990). The hydrophobic cells of \textit{C. albicans} have been shown to bind diffusely and plentifully to host tissues, whereas the hydrophilic cells' attachment is restricted to specific sites. Hydrophilic cells bind to regions with macrophages in contrast to hydrophobic cells which bind to tissue areas free of macrophages (Hazen \textit{et al.}, 1991), earlier studies agreeing with these findings (Hazen and Hazen, 1988; Antley and Hazen, 1988; Hazen, 1989). This would indicate that hydrophilic cells are more easily removed from the body by phagocytosis than hydrophobic cells, which can colonize epithelial surfaces. Hydrophobic interactions which can take place at long ranges could possibly facilitate specific adhesin-receptor interactions, which take place at short ranges by bringing the surfaces closer together (Israelachvili and McGuiggan, 1988; Hazen \textit{et al.}, 1991).

It has been shown that the adhesion of \textit{Candida} species to plastic surfaces is controlled by attractive London-van der Waals forces (hydrophobic forces) and electrostatic forces. The ability of \textit{Candida} species to adhere to inert polymeric surfaces may give the organisms direct access to the human host (Klotz \textit{et al.}, 1985).

\textbf{1.4.6 Oral bacteria}

Bacteria may contribute to the colonization and proliferation of \textit{Candida} strains in the oral cavity (Hsu \textit{et al.}, 1990). A study by Jenkinson \textit{et al.} (1990) has shown that the coaggregation of \textit{S. sanguis}, \textit{S. gordonii}, \textit{S. oralis} and \textit{S. anginosus}
with *C. albicans* was enhanced by subjecting the blastospores to glucose
starvation. The authors suggested that the coaggregations involved protein-
carbohydrate interactions, and demonstrated that heat or protease treatment of
starved *Candida* cells eliminated their coaggregation with *S. sanguis* and *S.
gordonii*.

Another study demonstrated that *C. albicans* would not readily adhere to
acrylic that had not been preincubated with streptococci, but did adhere to the
adherent *S. sanguis* and *S. salivarius* (Verran and Motteram, 1987). Branting et
al. (1989) showed that adhesion of *C. albicans* to acrylic surfaces was enhanced
when the yeast was incubated simultaneously with *S. mutans*.

### 1.4.7 Hyphae

There is agreement amongst most investigators that the hyphal form of *C.
albicans* is associated with its invasiveness (Kimura and Pearsall, 1980; Cutler et
a strong correlation between germ tube formation and increased adhesion of *C.
albicans* to BEC. The authors showed that germ tubes of *C. albicans* exhibited
enhanced adhesion to human mucosal cells, and it was suggested that this could
be one of the mechanisms related to virulence in *Candida* species. It is thought
that proteinases are produced during hyphal formation which help to disrupt the
integrity of the oral mucosa (Rajasingham and Challacombe, 1987; Borg and

There are a number of factors that regulate the transition of *C. albicans* from
blastospores to hyphae. These include temperature and pH of the growth
medium, the medium containing an inducer such as serum, N-acetyl-D-glucosamine, L-proline, ethanol or a mixture of amino acids, as in Lee's medium and various tissue culture media (Simonetti et al., 1974; Land et al., 1975; Dabrowa et al., 1976; Pollack and Hashimoto, 1985; Shepherd et al., 1985; Odds, 1988). It has been demonstrated in vitro that a temperature of 37-40°C, a pH 6.5-7.0 and an initial blastospore concentration not exceeding $10^6$ ml$^{-1}$ are essential for growth of C. albicans hyphae within several hours (Odds, 1988). The author also showed that at temperatures below 30°C and pH less than 6.0 most C. albicans strains on nutrient-poor media can form very long hyphae over a period of several days. Verran et al (1991) demonstrated that at pH 2.6, adherent C. albicans were able to produce hyphae but suspended Candida cells did not have this ability.

A calcium-calmodulin interaction was shown to induce blastospore-hyphae transition, whereas unrestricted calcium uptake resulted in specific inhibition of C. albicans hyphal growth (Sabie and Gadd, 1989; Holmes et al., 1991). Another study demonstrated that carbon dioxide alone can induce germ tube formation in C. albicans (Mock et al., 1990). Holmes and Shepherd (1988) found that exponential phase blastospores of C. albicans formed germ tubes after a period of glucose starvation followed by transfer to glucose ammonium ion medium at 37°C and pH 6.5. The authors showed that the presence of both a carbohydrate and a nitrogen source were essential for blastospore-hyphae transition.

Other studies have also demonstrated that the nutritional status of the blastospore is an important factor in regulating C. albicans morphogenesis (Holmes and Shepherd, 1987; Paranjape and Datta, 1991). One authority (Odds,
1988) considers that both morphological forms of \textit{C. albicans}, blastospore and hyphae, appear to initiate and sustain pathological responses in mammalian hosts. However, it seems that one form may be better adapted than the other to survive under specific ecological conditions.

1.4.8 Enzymes

Studies of phospholipase A and lysophospholipase activities have shown that \textit{C. albicans} isolates that adhered most strongly to BEC and were the most pathogenic to mice, had the highest phospholipase activities (Barrett-Bee \textit{et al.}, 1985). Some investigators have suggested that hyphae produced by \textit{C. albicans} may possess phospholipase which allows entry to the host epithelial cells (Rajasingham and Challacombe, 1987). Extracellular phospholipases and acid proteases of \textit{C. albicans} were activated by low pH conditions (Samaranayake, 1986).

Proteinase has been shown to have an important influence on candidal adhesion to and invasion of oral epithelium (Borg and Ruchel, 1988). In another study (Borg and Ruchel, 1990) it was demonstrated that blastospores, after ingestion by macrophages, quickly express proteinase. This was followed by increasing proteolytic activity with the formation of germ tubes by the ingested blastospores causing macrophage destruction.

1.4.9 Other factors involved in invasiveness

Recent molecular work has shown that \textit{C. albicans} has the capacity to switch frequently and reversibly between a number of general phenotypes which can be
distinguished by colony morphology (Soll, 1992). Switching in vitro can be initiated by low doses of UV radiation and once in the high-frequency switching mode *C. albicans* exhibits high rates of alterations in colony morphology (Soll, 1992). Kennedy *et al.* (1988) examined one switching system, the white-opaque transition in *C. albicans* where there are two phenotypes and found that the white cells were significantly more adhesive to BECs than the opaque cells.

It is considered that the switching mechanisms of *Candida* may potentiate its pathogenic features, for example, during invasion of different body environments, by eluding the immune system (by altering of its surface antigenicity) and by escaping the action of antifungals (Soll, 1992).

The ability of a pathogen to produce or acquire a surface coat of molecules that mimics host components is a method of evading the host’s immune system (Odds, 1994). Recently, Senet and Robert (1994) presented a theory concerning molecular mimicry as a component of *Candida* virulence and reported that *C. albicans* cells circulating in the blood stream become rapidly coated with host platelets via the fibrinogen-binding ligand.

1.5 ORAL DISEASES CAUSED BY *CANDIDA* SPECIES

1.5.1 Classification of oral candidosis

In view of the difficulty experienced by investigators in identifying the predisposing factors associated with oral candidosis together with the varied clinical appearance, the modified classification proposed by Samaranayake (Scully, El-Kabir and Samaranayake, 1994) is considered to be less confusing and is presented below. Briefly, oral candidosis is categorized as:
Group I. Primary oral candidosis.

This category includes the candidal infections that are confined to oral and perioral tissues such as acute pseudomembranous candidosis, acute erythematous candidosis, chronic hyperplastic candidosis and Candida-associated lesions including denture stomatitis and angular cheilitis.

Group II. Secondary oral candidosis.

This category comprises lesions that are distributed in other parts of the body as well as the oral cavity and includes the oral manifestations of systemic mucocutaneous candidosis. AIDS is included in this group as well as rare disorders such as thymic aplasia (Di George’s syndrome) and candidosis endocrinopathy syndrome (Scully, El-Kabir and Samaranayake 1994).

Other classifications of denture stomatitis have been proposed by previous investigators and are described fully in section 1.5.6. Briefly, the classifications are based on clinical appearance (Newton, 1962), type of inflammation (Budtz-Jorgensen and Bertram, 1970) and inflammatory response (Bergendal and Isacsson, 1983).

1.5.2 Host reaction

Shepherd (1986) considered the T-cell and macrophage mediated activation of the immune system to be critical in the host defence mechanism against C. albicans. T-lymphocytes produce cytokines, which elicit inflammation and the influx of polymorphonuclear neutrophils is the major factor in limiting the spread of infection. Consequently, defects in the cellular immune system predispose individuals to Candida infections. The author found that four factors were
involved in containing candidal infections: the stratum corneum must be intact, the host must generate complement-dependent chemotactic factors, the neutrophils must confine the infection, and epidermal proliferation must occur to clear cutaneous candidosis.

MacFarlane and Samaranayake (1989) noted that systemic conditions that may predispose individuals to oral candidosis included malnutrition, as it occurs in high carbohydrate diets, deficiencies in iron, folate or vitamin B12, hypoendocrine states such as hypothyroidism, Addison’s disease (adrenocortical insufficiency), diabetes mellitus, blood disorders such as acute leukaemia, agranulocytosis, immune disorders such as HIV infection, thymic aplasia, xerostomia due to irradiation, drug therapy, cytotoxic drug therapy and Sjogren’s syndrome (mentioned previously).

The immuno-suppressive states that accompany treatment of malignancies and organ or bone marrow transplants result in changes in the oral mucosa and oral microbiota which favour *Candida* species colonization (Epstein, 1989).

MacFarlane and Samaranayake, (1989) have stated that specific groups such as the very young, the very old and the pregnant are susceptible to oral candidal infections. However, neonates rather than having an age-associated predisposition might merely be given a large inoculum of *Candida* from the mother’s vagina during childbirth. In the case of the elderly this group receives more drugs causing immunosuppression or xerostomia while in pregnancy vaginal infections are relatively more frequent.
1.5.3 Acute pseudomembranous candidosis

Thrush or acute pseudomembranous candidosis occurs in 5% of newborn infants and in 10% of elderly debilitated individuals, and is one of the earliest and sometimes the initial manifestation of AIDS. Clinically there is a creamy white non-keratotic coating on the mucosa of the tongue, soft palate, cheek, gingiva or pharynx. The coating is easily rubbed off to leave a red raw and slightly bleeding surface, and the lesions vary from small discrete areas to confluent white patches covering a wide area (MacFarlane and Samaranayake, 1989).

1.5.4 Acute erythematous candidosis

Acute erythematous candidosis formerly referred to as acute atrophic candidosis (Holmstrup and Axell, 1990) occurs with prolonged steroid or broad spectrum antibiotic therapy and is known as antibiotic sore tongue or "glossodynia". However, any part of the oral mucosa can be affected, including the buccal mucosa and the palate. When the tongue is involved, the dorsum shows marked depapillation and the affected areas become red, shiny and painful (MacFarlane and Samaranayake, 1989).

1.5.5 Chronic hyperplastic candidosis

Chronic hyperplastic candidosis or Candida leukoplakia occurs as chronic, white patches on the oral mucosa and is indistinguishable from leukoplakia due to other causes. The plaques, which are firm, whitish and speckled, and cannot be easily wiped off, usually appear as a single lesion near the commissures or
near the surface of the tongue (MacFarlane and Samaranayake, 1989).

1.5.6 *Candida*-associated denture stomatitis

*Candida*-associated denture stomatitis formerly referred to as chronic atrophic candidosis (Scully, El-Kabir and Samaranayake, 1994) is the commonest form of oral candidosis and is present in 24-60% of denture wearers and may also be associated with orthodontic appliances and obturators. The condition is usually found on the palatal mucosa beneath the fitting surface of the upper denture and both complete and partial denture wearers are affected. It is unusual for denture stomatitis to occur on the lower denture bearing mucosa (MacFarlane and Samaranayake, 1989).

Denture stomatitis has been associated with angular cheilitis, atrophic glossitis, acute pseudomembranous candidosis and chronic hyperplastic candidosis, and has been found to be more common in females than males. The condition is frequently symptomless, but when signs and symptoms are present they may display mucosal bleeding, swelling, burning or other painful sensations, halitosis, unpleasant taste and dryness in the mouth (Arendorf and Walker, 1987).

Denture stomatitis may be classified according to the clinical appearance of the inflamed mucosa under the maxillary complete denture (Newton, 1962) and includes three types. Type I refers to the initial stage of localized pin-point hyperaemia (Figure 1.4) while Type II, which is the most common type of denture stomatitis, is described as having diffuse erythema and oedema of the denture-bearing areas of the palatal mucosa (Figure 1.5). The affected area is confined to the denture-bearing surface and is demarcated at the margins of the
Figure 1.4 *Candida*-associated denture stomatitis, Type I, (indicated with arrows) showing the initial stages of inflammation of the palate

Figure 1.5 *Candida*-associated denture stomatitis, Type II, showing diffuse erythema of the palate
Figure 1.6 *Candida*-associated denture stomatitis, Type III, showing papillary hyperplasia of the palate

Figure 1.7 Angular cheilitis, showing lesions at the angles of the mouth
denture; the condition is not painful and can be associated with angular cheilitis (Figure 1.7). If Type II is untreated for a prolonged period it has been said that Type III may develop, in which a hyperplastic reaction occurs resulting in a nodular lesion of the central palate often with associated atrophic areas, and this is referred to as papillary hyperplasia (Figure 1.6). The view of Thomas et al. (1985), however, is that papillary hyperplasia may form independently and that it may occur in subjects with a genetically sourced papillated palatal mucosa with a superimposed denture stomatitis. All three types, I, II and III may be found simultaneously and in varying combinations.

Budtz-Jorgensen and Bertram (1970) classified denture stomatitis according to the type of inflammation observed on the mucous membrane of the palate under a maxillary denture. There were three classifications, simple localized inflammation (involving a limited area), simple diffuse inflammation (involving the whole area covered by the denture) and granular inflammation (often localized to the central part of the hard palate).

Bergendal and Isacsson (1983) followed Otslund's (1958) classification and used the term local inflammation to describe red spots usually found around the small palatal minor salivary glands; the lesion was thought to be associated with trauma from the dentures. The term diffuse reddening referred to a diffuse hyperaemic, smooth and atrophic mucosa extending over the entire denture area and was associated with increased growth of yeasts. The third type of denture stomatitis was described as granulated and was characterized by hyperaemic mucosa with a nodular appearance in the central part of the palate and both trauma and Candida infection have been linked with this lesion.
The literature contains a large amount of data relating to the cause of denture stomatitis and these are discussed in the following sections.

1.5.6.1 Trauma

Nyquist (1953) was of the opinion that trauma caused by the dentures was the dominant factor in the occurrence of denture stomatitis ("sore mouth"), and he found no association between denture stomatitis and the bacterial microbiota under complete upper dentures. Cawson (1965) found no convincing evidence that denture stomatitis was caused by trauma from the denture, or hypersensitivity to denture base material but in later studies Budtz-Jorgensen (1974) concluded that the significant causes of denture stomatitis were trauma and infection with Candida species. The author considered that the localized simple type of denture stomatitis (mentioned in 1.5.6) was caused by trauma from ill-fitting dentures and would be resolved after proper adjustment of the dentures.

1.5.6.2 Bacteria

Nyquist (1953) also observed that there was no significant difference between the numbers of bacteria counted in cases of denture sore mouth and clinically normal mucosa and also no difference in the types of bacteria. The number of bacteria was generally characteristic of the individual, and there was a significant trend towards larger aerobic than anaerobic populations for any one individual. However, the author found that the bacteria increased in number when the denture was worn for a longer period. He also noted a larger population of aerobic bacteria for a "rubber" (Vulkanite) denture than for an acrylic denture.
although there was no significant difference for the anaerobic population and there was no difference between the types of bacteria occurring in cases of denture stomatitis associated with "rubber" and acrylic dentures. The most common type of bacteria isolated was *Neisseria (Branhamella) catarrhalis*, with *Staphylococcus albus (epidermidis)* (coagulase negative staphylococcus) next in order of frequency, but streptococci and other types of bacteria were sparsely represented. This spectrum of bacteria is unusual, but may reflect the methods of isolation and identification in use over 40 years ago.

In contrast to Nyquist's findings, van Reenen's studies (1973) have given prominence to the detection of bacteria in denture stomatitis lesions. The numbers of Gram-positive cocci including streptococci, pneumococci and staphylococci were more frequently isolated from the palatal mucosa of patients with denture stomatitis. The author suggested that no specific organism was associated with denture stomatitis lesions and that the infection was caused by a community of pathogens. He showed that most bacteria isolated from denture stomatitis lesions were able to adhere to palatal epithelial cells and that in subjects who did not wear dentures, there was a relative absence of bacteria on the palatal mucosa.

1.5.6.3 *Candida* species

One of the earliest studies associating yeast-like organisms with "denture sore mouth" was that of Cahn (1936) who found *Monilia (Candida) albicans* in four patients with compromised health. Both Cahn (1936) and Bartels (1937) were very cautious in their conclusions and emphasized that not all cases of denture...
stomatitis were due to infection by yeast-like organisms. The patients were successfully treated with 5% aqueous solution of gentian violet and they kept their dentures in hypochlorite over-night.

Stenderup and Pedersen (1962) reported on the frequency of isolation of yeast species from various human sites over a two-year period. The frequency with which yeasts were isolated from the oral cavity was 71% and included specimens from the pharynx, tonsils, gingiva and tongue as well as sputum. The two species most frequently isolated were *C. albicans* and *T. glabrata* (*C. glabrata*) and together they formed 87% of all the species from the oral cavity. The authors were of the opinion that *C. albicans* could be considered as part of the normal microbiota in the human body since it was isolated so frequently, and they also isolated *C. krusei*, *C. tropicalis*, *C. pseudotropicalis* (*C. kefyr*) and *C. parapsilosis*.

Early studies on denture stomatitis also included the work by Cawson (1965) who made a number of important observations: *Candida* were isolated much more frequently from cases of denture stomatitis than from healthy denture wearers and the predominant species was *C. albicans*. Three methods were used to isolate *Candida* from patients with denture stomatitis, namely, direct smear from the mucosa, culture of a swab from the mucosa and imprint culture directly from the denture; although *Candida* was isolated by culturing a palatal swab, a saliva specimen was considered to be more sensitive. Hyphae were present as the predominant or only form in most cases of denture stomatitis from which *Candida* had been isolated and it was concluded that hyphae were actively growing in the potential space between denture and mucosa. Cawson was also of the opinion that *Candida* proliferate in this space and when the denture is worn
continuously it eliminates the cleansing effects of tongue, saliva and any antibodies in the saliva. It was emphasized that sampling of the denture-bearing area must take place immediately after the denture is removed as once the mucosa is exposed to the cleansing effect of saliva and tongue movements the microorganisms are easily dislodged, resulting in an inaccurate reading.

Cawson (1965) noted that thrush occasionally developed in association with denture stomatitis and he referred to the "infective origin" of denture stomatitis in the close association with angular cheilitis. It was affirmed that angular cheilitis in denture stomatitis patients was not just a mechanical problem with loss of vertical dimension resulting in the characteristic fold at the angle of the mouth but rather an infection derived from a primary source (Figure 1.7). This theory was confirmed when treatment of denture stomatitis with antifungals resulted in resolution of angular cheilitis without any specific treatment for the latter condition. He also found that nystatin and amphotericin B in the form of tablets dissolved in the mouth were effective in the treatment of denture stomatitis, and emphasized that the medication should be applied topically so that the infected area was exposed to the drug and the dentures removed during treatment.

Davenport (1970) examined fifty denture stomatitis patients and found that greater numbers of Candida cells were recovered from smears prepared from the fitting surface of upper dentures than from those of the palatal mucosa and this was in agreement with the studies of van Reenen (1973) and Buditz-Jorgensen (1990). The author concluded that denture stomatitis was associated with the growth of Candida within the plaque on the denture rather than on the palatal mucosa, and therefore he suggested that treatment be directed to the denture in
preference to the mucosa.

Santarpia (III) et al. (1990) developed a method for the site-specific detection of *C. albicans* on the denture surface and they correlated the distribution of yeast species with the severity of the denture stomatitis lesions. Their study is based on previous findings (Bahn et al., 1962; Davenport, 1972; Santarpia (III), et al., 1988) which showed that *C. albicans* is isolated more frequently from the denture fitting surface than from the corresponding mucosa. The authors used the agar replica system with an amino acid selective synthetic growth medium for *C. albicans*, supplemented with arginine, zinc sulphate and Bacto-agar. The results showed that the severity of palatal inflammation correlated with the number of *C. albicans* colonies on the agar replica, with the greatest number of colonies being observed in patients with Newton type III lesions. However, this clinical correlation was not noted in patients with Newton type I and II lesions. In explaining these findings the authors pointed out that there was a lack of knowledge concerning the specific aetiology of denture stomatitis.

Budtz-Jorgensen (1974) considered that most of the lesions of the generalized simple or granular type of denture stomatitis associated with angular cheilitis or glossitis seemed to be induced by *Candida* species. In common with the findings of Cawson (1965), Budtz-Jorgensen (1974) found that *Candida* species were cultured more frequently and in greater numbers in cases of denture stomatitis than in healthy denture wearers, *C. albicans* being the most common species. The author considered a palatal smear yielding mycelium or pseudohyphae to be sufficient evidence of *Candida*-induced denture stomatitis, and he emphasized the importance of scraping both palate and denture. He concluded that since no
mycelia were observed in scrapings from the local simple lesions, the latter could not be induced by *C. albicans*.

1.5.6.4 Trauma and *Candida* species

Budtz-Jorgensen (1974) considered that treatment of the local simple lesion should include the construction of new properly fitting dentures and the use of tissue conditioners, but the *Candida*-induced lesions were unaffected by prosthetic intervention. Since the dentures seemed to provide an ideal environment for *Candida* growth and infection, Budtz-Jorgensen (1974) and Cawson (1965) both found that removal of the dentures was essential during the treatment period even if this was socially unacceptable to some.

1.5.6.5 Denture lining materials

Denture lining materials, which include tissue conditioners and soft denture liners, are widely used as adjuncts in the prosthodontic treatment and management of traumatized oral mucosa, and are most commonly used in association with the lower mandibular denture. Tissue conditioners are used to assist denture bearing tissues recover from trauma often due to ill-fitting dentures and consist of polyethyl methacrylate polymers with a plasticizer and ethyl alcohol. Resilient or soft denture liners are used on the fitting surfaces of dentures when the patient presents with problems such as sharp or irregular ridges, tissue defects, or thin non-resilient oral mucosa. Current materials are either silicone elastomers, plasticized higher methacrylate polymers or hydrophilic polymethacrylates (Tyas, Atkinson and Harcourt, 1993).
Soft lined mandibular dentures have been associated with candidal growth and, in a study by Makila and Hopsu-Havu (1977), it was shown that Candida species were detected in 85% and 44% of lined mandibular and unlined maxillary dentures respectively. The most commonly detected yeasts were C. albicans, C. glabrata and C. tropicalis. The authors also demonstrated that the liner (Molloplast B, Detax Dental, Karl Huber, Ettlingen, Germany) had no inhibitory effect on candidal growth in vivo but in vitro the uncured material caused a definite inhibition of Candida growth whereas the cured material did not indicate any such inhibition.

However, in another study Wright et al. (1985) found that increased isolation frequency of yeasts on the fitting surface of mandibular dentures lined with either Molloplast B or the tissue conditioner Visco-gel (De Trey Division, Dentsply Limited, Weybridge, Surrey, England) was not associated with an increased inflammatory response of the mandibular denture-bearing mucosa. In their study C. albicans was the most frequently isolated of the Candida species associated with soft denture liners.

Graham et al., (1991) tested two tissue conditioners Veltec, (Teledyne, Elk Grove Village, Illinois, USA) and Coe-Comfort (Coe Laboratories, Inc. Chicago, Illinois, USA) in vivo and found that both materials supported the presence and growth of C. albicans, though there was no significant difference in the prevalence of Candida species between the two materials tested.

Nikawa et al. (1992) studied C. albicans adherence to soft denture-lining materials including Coe-Comfort, Visco-gel and Coe-Soft (Coe Laboratories, Inc. Chicago, Illinois, USA) and found that factors other than hydrophobic action,
such as specific interaction, may be involved in fungal adherence to saliva-coated soft denture liners. Another study by Nikawa et al. (1993) showed that denture pellicle potentiates *C. albicans* colonization and hyphal invasion of denture-lining material and may therefore be a factor in promoting denture stomatitis.

1.5.6.6 *Candida* species and other oral microorganisms

The work of Fouché et al. (1986) paid attention not only to *Candida* species as the causative organism in denture stomatitis but also concentrated on the role played by other oral microorganisms. Their study identified to species level the aerobic microorganisms isolated from a group of subjects with Newton’s type II and III denture stomatitis. The results showed that *C. albicans* was recovered from all participants with one exception when *C. tropicalis* was detected. Three species of lactobacilli, *L. acidophilus*, *L. brevis* and *L. salivarius*, and seven species of streptococci, designated *S. bovis*, *S. mitis*, *S. mutans*, *S. pneumoniae*, *S. salivarius* and *S. sanguis* I and II, (*S. sanguis*, senso stricto and *S. gordonii*) were isolated. *Micrococcus luteus*, *Micrococcus varians* and *Staphylococcus epidermidis* were also recovered. The only Gram-negative microorganisms isolated were *Haemophilus influenza* and *Neisseria meningitidis*.

1.5.6.7 Denture hygiene/denture plaque

Nyquist (1953) investigated the influence of denture hygiene on inflammation of the soft tissues under complete upper dentures and found no evidence that frequency or method of denture cleansing had any effect on the incidence of denture sore mouth. Cleansing of the oral mucosa twice a day was also
ineffective.

Davenport (1972) investigated the porosity and surface texture of acrylic resin and found that the denture surface was of fine texture with an absence of porosity. This did not allow attachment of plaque by penetration of surface defects or by mechanical fixation to surface irregularities. Therefore, the author recommended that microbial plaque on the denture be removed by simple denture hygiene measures such as careful brushing and, as a useful adjunct, 0.1% aqueous chlorhexidine over-night denture soak.

Tarbet (1982) considered the lack of denture cleanliness to be one of the factors involved in the aetiology of denture stomatitis. He emphasized the need to remove the denture plaque at regular intervals especially on the tissue fitting surfaces of dentures. His study demonstrated that when a rigorous denture cleansing regimen was followed, previous denture brushing habits were shown to be totally inadequate.

Schou et al. (1987) have shown that there is a significant relation between plaque formation, soaking habits such as alkaline peroxide and the presence of denture stomatitis. However, they did not find that denture plaque was related to brushing habits or candidal growth. On the other hand, Iacopino and Watthen (1992) noted the presence of C. albicans in microbial denture plaque and emphasized the importance of oral hygiene.

1.5.6.8 Permeability of acrylic resin

Contrary to Davenport’s (1972) findings, van Reenen (1973) showed in vitro that C. albicans penetrated acrylic resin used routinely in denture construction.
Levin (1973) also demonstrated the permeability of acrylic resin to both a fluorescent dye and *C. albicans*. Penetration on the unpolished surface, which is in contact with the mucosa, was greater than that of the polished surface. The author suggested that the unpolished surface of the denture was a suitable site for *Candida* proliferation and recommended sealing of the surface. The effect of acrylic resin on the pH of glucose broth inoculated with *C. albicans* was also studied and it was found that acrylic resin alone did not alter the pH. However, acrylic resin together with *C. albicans* raised the pH and *C. albicans* alone lowered the pH. It was considered possible that a similar interaction between acrylic resin and *C. albicans* could take place in the mouth and the rise in pH could create a favourable environment between denture and mucosa for the growth of microorganisms (Levin 1973).

1.5.6.9 Antibodies and immunological factors

Lehner (1965) found that the antibody titre to *Candida* from serum and saliva by fluorescent antibody technique was significantly higher in patients with denture stomatitis than in healthy denture wearers.

van Reenen (1973) was of the opinion that a low serum antibody titre to *C. albicans* compared with that to other oral pathogens, was an indication that the yeast was not principally involved in denture stomatitis lesions but that the disease was a mixed infection. However, the studies of Iacopino and Wathen (1992) demonstrated that antibodies to *C. albicans* occur later than those to other pathogens, and this may explain why in van Reenen's study significant titres of serum antibodies to *C. albicans* were not found. The authors also suggested that
since the oral mucosa is protected by salivary IgA, this antibody may play an important role in the defence against oral candidosis.

Iacopino and Wathen (1992) were of the opinion that denture stomatitis rarely occurs without at least one predisposing factor that lowers patient resistance and they have presented their immunological theory of patient susceptibility. They observed that candidal infection resulted in both CMI and humoral immune responses, with CMI providing protection against superficial candidal infection. The authors concluded that patients with denture stomatitis who do not suffer any CMI defects may be deficient in migration inhibition factor and may have overactive suppressor T-cells or other T-lymphocyte/phagocyte defects and such immunological factors may predispose the patient to denture stomatitis.

1.5.6.10 Multifactorial

The cause of denture stomatitis is now believed to be multifactorial, and Arendorf and Walker (1987) considered the following factors to be significant: denture trauma, continuous denture wearing, denture cleanliness, dietary factors, *Candida* infections and predisposing systemic conditions. It is generally regarded that *Candida* species, in particular *C. albicans*, are the causative agents of denture stomatitis, although the above mentioned factors and denture plaque bacteria may be involved.

1.5.7 Angular cheilitis

Angular cheilitis (angular stomatitis/perlèche) is frequently a complication of *Candida*-associated denture stomatitis, although it can be associated with any type
of oral candidosis. The lesions occur at the angles of the mouth (Figure 1.7) and vary from initial erythema to ulcerated and crusted fissures (MacFarlane and Samaranayake, 1989). Infection is by Candida and/or Staphylococcus, streptococci and other organisms and can be a varying combination of all. The usual endogenous reservoirs of these organisms are the oral cavity and anterior nares respectively. Beta-haemolytic streptococci, particularly Lancefield Group B, are sometimes identified. Other factors involved in the aetiology of angular cheilitis are iron deficiency anaemia, vitamin B12 deficiency, inadequate vertical dimension of occlusion in dentures and skin creasing at the angles of the mouth due to ageing. Angular cheilitis is also a common oral manifestation of AIDS (MacFarlane and Samaranayake, 1989).

1.6 TREATMENT OF ORAL CANDIDOSIS

1.6.1 Antifungals

A number of in vivo studies have been carried out to investigate the treatment of oral candidosis with antifungal agents. Epstein et al. (1981) showed that treatment with topical nystatin (Mycostatin Oral Suspension, E.R. Squibb Co., Princeton, NJ, USA) as a mouthrinse four times daily for two weeks resulted in a significant reduction in the number of Candida organisms in saliva and a marked improvement in the disease; however, the condition recurred rapidly after cessation of treatment.

Epstein (1989) emphasized the importance of topical antifungal agents for the prevention and treatment of oral candidosis. Amphotericin B and nystatin (Mycostatin) bind to ergosterol in the cell membrane of Candida organisms,
causing changes in permeability, leakage of cell contents and cell death. He noted that nystatin, although useful for topical therapy in oral and pharyngeal candidosis, is poorly absorbed when ingested and most of the drug passes unchanged through the gastrointestinal tract. Amphotericin B, which is an effective topical agent, is the drug of choice for intravenous treatment of progressive and potentially fatal candidal infections. Both amphotericin B and nystatin have an unpleasant taste thus affecting patient compliance, and oral use may sometimes lead to gastrointestinal side effects such as nausea, vomiting and diarrhoea. Renal, bone marrow, cardiovascular or neurological toxicity may result when disseminated candidosis is treated with intravenous amphotericin B.

Epstein (1989) also mentioned that flucytosine, a fluorinated pyrimidine, may be useful for the treatment of disseminated fungal infections; the drug affects protein synthesis and inhibits DNA synthesis of Candida. Also, griseofulvin which is fungistatic, and affects cell wall synthesis, DNA synthesis and mitosis was useful in the treatment of systemic mucocutaneous candidosis (mentioned previously 1.5.1) (Epstein, 1989). The imidazole compounds such as clotrimazole, miconazole, econazole and ketoconazole, are broad-spectrum antifungal agents which affect permeability of Candida membrane by interfering with the synthesis of ergosterol; they also bind more strongly to Candida enzymes than to mammalian enzymes. Clotrimazole, although the most potent agent, is only used topically, because of gastrointestinal and neurological toxicity; econazole exists in topical form only; miconazole and ketoconazole can be used both topically and systemically (Epstein, 1989). However, due to their hepatotoxic and other serious adverse effects, the imidazole compounds have now been largely superseded by
fluconazole.

Recently introduced antifungal drugs include the triazoles, fluconazole and itraconazole both of which inhibit fungal ergosterol production required in cell wall formation. It has been shown that adhesion to epithelial cells is significantly inhibited by fluconazole (Darwazeh et al., 1991). Oral doses of the drug are generally well tolerated and do not seem to suppress the synthesis of corticosteroid hormones. Hay (1990) found fluconazole administered together with the oral antiseptic chlorhexidine to be an effective treatment for denture stomatitis. Itraconazole is eliminated hepatically and hence its use is contraindicated in liver disease.

MacFarlane and Samaranayake (1989) have emphasized that treatment of denture stomatitis involves strict dental hygiene measures and the use of antifungal agents. In particular, patients should be discouraged from wearing their dentures over-night and the dentures should be soaked over-night in an antiseptic solution; they also recommended topical treatment with amphotericin B.

A number of studies have been carried out to test the efficacy of denture lining materials (tissue conditioners/soft liners) containing antifungals in the treatment of denture stomatitis. Odds (1988) considered that denture liners alone usually have no effect on Candida species, though Gruber et al. (1966) had shown that silicone soft liners and tissue conditioners (trade names not supplied) with zinc undecylenate incorporated (1.5% and 1.0% w/w respectively) eliminated candidal growth. Douglas and Walker (1973) demonstrated the inhibitory effect of Tempo (Lang Dental Manufacturing Co., USA) and Coe-Comfort (tissue conditioners) incorporating nystatin (Nystan Bristol-Myers Squibb) and confirmed the results
with an *in vivo* investigation. Thomas and Nutt (1978) showed that Visco-gel (tissue conditioner) combined with nystatin powder was successful in inhibiting the growth of *C. albicans*, *C. krusei* and *C. tropicalis*. However, there was lack of inhibition by Visco-gel alone and a Visco-gel/amphotericin B combination. *In vitro* studies by Carter *et al.* (1986) showed that a ketoconazole-Visco-gel combination used as a tissue conditioner, effectively removed and killed *Candida* associated with dentures, the effect continuing over a long period. Another *in vitro* study by Schneid (1992) investigated a sustained-release delivery system for the treatment of denture stomatitis by using four antifungal agents incorporated into a tissue conditioner Lymal (Dentsply/L.D. Caulk Division, Milford, DE, USA). The antifungal agents were chlorhexidine, clotrimazole, fluconazole and nystatin at low, medium and high concentrations. All drugs were released from the tissue conditioner, with inhibition of candidal growth at each concentration, and nystatin showing the greatest antifungal activity. Inhibition by chlorhexidine and clotrimazole were dose-related and fluconazole samples required pre-incubation at 37°C for activation.

Merkel and Phelps (1989) showed that sub-lethal amounts of amphotericin B inhibited the attachment of *C. albicans* to cultured mammalian cells, and blastospores, which were in their exponential phase of growth or had formed hyphae, were the most sensitive to the drug. Other investigators (Abu-El Teen *et al.*, 1989) demonstrated that sub-inhibitory concentrations of amphotericin B, nystatin, miconazole nitrate and 5-fluorocytosine inhibited adhesion of *Candida* species to BEC. In addition, the outer cell envelope was affected, germ tube formation was suppressed, and the loss of extracellular polymeric material (EP),
known to mediate adhesion, was increased (Abu-El Teen et al., 1989). Mehentee and Hay (1990) showed that sub-inhibitory concentrations of amphotericin B, ketoconazole and itraconazole reduced the adhesion of *C. albicans* to gastric and jejunal mucosa. The effect of the antifungal agent depended on its concentration, susceptibility of the strain and the source of the mucosal surface.

The *in vitro* study of Spiechowicz et al. (1990) was based on the frequently made observation (Davenport, 1972; Budtz-Jorgensen, 1990) that in cases of denture stomatitis *C. albicans* colonies are recovered more frequently from the tissue fitting surface of the acrylic resin denture than from the corresponding palatal mucosa. Hence the need for total removal of the yeast from the dentures of patients with the disease and the prevention of recolonization of the denture by *C. albicans*. The authors evaluated the antifungal effectiveness of nystatin (Oral Suspension, USP Pharmafair, Inc) chlorhexidine gluconate (Peridex, Procter and Gamble, Cincinnati, Ohio, USA) and a histidine polypeptide (poly-L-histidine) (ICN Biomedicals, Costa Mesa, CA, USA) on the surface of acrylic resin discs which were pretreated with the respective antifungal agents for 8 h. They found that pretreatment with poly-L-histidine did not inhibit *C. albicans* adhesion and growth whereas chlorhexidine was completely effective in preventing candidal attachment and growth on acrylic resin. If pretreatment of acrylic resin with nystatin was followed by drying, then the protection was similar to that provided by chlorhexidine.

1.6.2 Antiseptics and disinfecting agents

An *in vivo* programme involving the use of an antiseptic Listerine (Warner-
Lambert Co., Morris Plains, NJ, USA; ethanol 0.26 ml, benzoic acid 1.5 mg, thymol 0.63 mg, and eucalytol 0.9 mg per ml) and Nystatin (E.R. Squibb Co., Princeton, NJ, USA) antifungal as mouth rinses and denture soaks over a period of twenty-eight days resulted in a significant reduction in palatal inflammation and candidal colonization of dentures and palatal mucosa, although denture plaque scores did not differ significantly (DePaola et al., 1986). In another study a 0.2% chlorhexidine gluconate mouth rinse used three times daily significantly reduced plaque, but there was no significant effect on the number of Candida organisms (Addy and Hunter, 1987).

Schwartz et al. (1988) compared antiseptic (Listerine), Nystatin Oral Suspension USP (100,000 units/ml) and control (5% hydroalcoholic) mouth rinses three times per day for 30 sec over a 28-day period. They showed that neither the denture microbial count nor denture stomatitis was reduced by the mouth rinses used. The authors also suggested that the denture may be a reservoir of reinfection and recommended that treatment should include antimicrobial treatment of the denture and removal of the denture for a period of time every twenty-four hours.

Epstein (1989) has described the use of Peridex containing chlorhexidine gluconate, in the treatment of oral candidosis. The drug is a broad spectrum mouth rinse which is adsorbed on the surfaces of microorganisms, increasing permeability of cell membranes and causing precipitation of cytoplasmic contents. He found that chlorhexidine bound to salivary pellicles as well as hard tissues in the oral cavity, resulting in chlorhexidine titres in saliva for twelve hours or more after rinsing. Although effective in the treatment of oral candidosis, unpleasant
side effects included staining of the tooth surfaces and a bitter taste.

Lal et al. (1992) investigated the use of chlorhexidine gluconate in the form of Peridex both as a mouthrinse and a denture soak in the treatment of denture stomatitis. Their study was for a period of 24 days and Peridex oral rinse containing 0.12% chlorhexidine gluconate was used twice daily and dentures soaked overnight in Peridex solution. The authors found that chlorhexidene completely eliminated *C. albicans* on the acrylic resin denture surface and significantly reduced palatal inflammation. However, several weeks after the Peridex treatment was terminated, *C. albicans* recolonized the denture surface and palatal inflammation recurred. This investigation is interesting in that it raises the question as to whether the denture reinfects the palate or whether localized yeast infection of the mucosal surface occurs independently of the presence of *C. albicans* on the denture surface. The authors concluded that palatal inflammation was in response to direct yeast invasion of the mucosa, and recurring infection of the palate by *C. albicans* on the denture surface, and therefore they suggested that treatment of denture stomatitis should include antimicrobial topical application to both denture and mucosa.

As poor denture hygiene and ill-fitting dentures were considered to be the main predisposing factors in the aetiology of denture stomatitis, DePaola et al. (1990) suggested that management of denture stomatitis should be directed at reducing microbial growth and improving adaptation of the denture. Their study compared the effectiveness of Listerine antiseptic mouthrinses and denture soaks, with Coe Comfort maxillary soft denture reliners (tissue conditioners) in reducing denture stomatitis. Over a 28-day period, the patients were asked to use Listerine
antiseptic solution (20ml) as a mouthrinse three times per day and as a denture soak for one hour each evening. The maxillary dentures in the reline group were relined at seven-day intervals and for both groups there was no mechanical cleaning of the dentures during the trial period. The results showed that reduction in inflammation in both Listerine and reline treatment groups was significantly greater than in the control group and soft denture relines significantly improved denture retention and stability. However, denture plaque was not significantly reduced and therefore the authors concluded that in the absence of other mechanical denture hygiene measures, the antiseptic rinses and relines were equally effective in reducing denture stomatitis.

Barkvoll and Attramadal (1989) examined the effect of the combination of nystatin (Mycostatin mixture Novo Industries A/S, Copenhagen, Denmark) and chlorhexidine digluconate (ICI Ltd., Cheshire, England) on \textit{C. albicans in vitro} and found that the combination of the drugs was not effective. Sub-inhibitory concentrations of aqueous garlic extract were shown by Ghannoum (1990) to have inhibitory effects \textit{in vitro} on the adhesion of \textit{Candida} species to human BEC and blastospores treated with the extract had reduced ability to form germ tubes.

1.6.3 Sodium hypochlorite

A number of studies have been carried out to demonstrate the antifungal properties of denture cleansing agents. Ghalichebaf \textit{et al.} (1982) found that those with a high pH and sodium hypochlorite content such as Mersene, pH 11.0 (Colgate-Palmolive Co., New York, NY, USA) were the most effective in removing denture plaque. Basson \textit{et al.} (1992) and Rudd \textit{et al.} (1984) also
demonstrated the disinfecting effect of sodium hypochlorite as a denture soak, while alkaline hypochlorites have been shown to eliminate denture plaque effectively in vitro even after short term exposures (Moore et al., 1984). The effect of hypochlorite is due to the presence of undissociated hypochlorous acid (HOCl) (Hedgcock, 1967), where concentration is dependent on pH, and which oxidizes sulphhydryl groups (-SH) of amino acids and proteins to the disulphide form (S-S) (Cole and Eastoe, 1977; Arnhold et al., 1991). A report by Jagger and Harrison (1995) showed that a large number of people do not know how to clean their dentures satisfactorily and this could account for the deterioration of denture base material due to misuse of chemical cleansing agents.

It is clear from the above review of antimicrobial and antiseptic/disinfecting agents that there is no currently known effective treatment to prevent or reduce the incidence of denture stomatitis. One of the major factors that determines the success of a treatment regimen is patient compliance. Thus, an effective treatment regimen will combine the use of an antifungal agent with an easy-to-use application.

1.6.4 Microwave irradiation

To date there has been only one reported study of the use of microwave irradiation to disinfect denture surfaces (Rohrer and Bulard, 1985). Although other studies have been carried out to investigate the disinfection of dental instruments (Hume and Makinson, 1978), tissue culture vessels (Sanborn et al., 1982), hydrophilic contact lenses (Rohrer et al., 1986), medical instruments and apparatus (Young et al., 1985; Najdovski et al., 1991; Rosaspina et al., 1993) and
polyethylene catheters (Griffith et al., 1993), there is no other report relating to microwave disinfection of dentures. However, these studies provided ample evidence that microwave irradiation at high setting and at specified exposure times is bactericidal and candidacidal. Rohrer and Bulard (1985) showed that microwaving at high setting for eight minutes would disinfect acrylic dentures contaminated with *C. albicans* suspension but if the dentures were contaminated with a mixture of *C. albicans* and aerobic bacteria, a longer period of ten minutes would be required for disinfection. The authors used a modified microwave oven with a three-dimensional rotating device to which the dentures were attached and found no dimensional change in the dentures they microwaved.

1.7 AIMS OF THE STUDY

There is clear evidence from the foregoing literature review that the management of *Candida*-associated denture stomatitis is complex due to its multifactorial aetiology. In this study the effect of sodium hypochlorite on the properties of *Candida* species that are considered to be associated with the aetiology of denture stomatitis will be investigated in a series of *in vitro* experiments. The data obtained will be used to conduct *in vivo* studies to determine means of preventing and treating denture stomatitis. To this purpose two methods of denture disinfection will be assessed, namely, soaking dentures over-night in sodium hypochlorite or microwaving dentures directly.
CHAPTER 2
EFFECT OF SODIUM HYPOCHLORITE ON ADHESION OF CANDIDA SPECIES TO SURFACES IN VITRO

2.1 INTRODUCTION

This chapter will describe a series of in vitro experiments to test the effect of sodium hypochlorite on the adhesion of Candida species to surfaces.

The initial stage of infection in the oral cavity is considered to be adhesion of candidal cells to denture acrylic followed by colonization of epithelial cells (King et al., 1980) and growth and tissue invasion. Attachment to a surface may initially be governed by the effects of non-specific forces such as van der Waals-London forces and hydrophobicity (Hazen, 1989; Klotz et al., 1985). Specific factors, for example, the presence of cell surface mannoprotein (Calderone and Braun, 1991; Critchley and Douglas, 1987), and coaggregation with oral streptococci (Jenkinson et al., 1990; Verran and Motteram, 1987) are also thought to play an important role.

Candida-associated denture stomatitis is believed to be due to a number of factors, including continuous denture wearing, denture cleanliness and candidal infection (Arendorf and Walker, 1987). Alkaline hypochlorites (Clorox-Calgon) have been shown to eliminate denture plaque effectively (Moore et al., 1984) and it can be hypothesized that substances that prevent adhesion and invasion by Candida to oral surfaces may function as effective antifungal agents.

The aim of these investigations was to examine in vitro the effect of sodium hypochlorite on the adhesion of Candida species to surfaces in order that the
data obtained may be utilized in the prevention and treatment of denture stomatitis.

2.2 MATERIALS AND METHODS

2.2.1 Microorganisms and growth conditions

Two strains of *C. albicans*, GRI 682 and GDH 2346 were kindly provided by Dr. J. Verran, Department of Biological Sciences, Manchester Polytechnic, Manchester, England. The strains were originally isolated from a cervical smear (GRI 682) and a case of denture stomatitis (GDH 2346) by Dr. L.J. Douglas, University of Glasgow. Three strains of *C. albicans*, Y5499, H1 and Y523 (the last formerly *C. stellatoidea*), and strains of *C. tropicalis* Y9, *C. parapsilosis* Y316, *C. krusei* Y301, *C. kefyr* Y83 and *C. guillermondii* Y324 were from the freeze-dried culture collection held at the Institute of Dental Research, and were all isolated from the saliva of healthy patients attending the United Dental Hospital of Sydney. *S. gordonii* LGR2 (Kilian et al., 1989) was originally isolated from dental plaque and has been previously designated *S. sanguis* (Wyatt et al., 1988).

*Candida* species/strains were grown in SB (Appendix A) for 48 h at 37°C. and *S. gordonii* LGR2 was grown microaerophilically for 24 h at 37°C in BHI (Appendix A). To radiolabel cells, *Candida* strains were grown in the presence of 74 kBq ³H-uridine (Du Pont NEN Products, Boston, MA, USA) and streptococci were grown in the presence of 74 kBq ³H-thymidine (Du Pont NEN Products).

Cells were harvested by centrifugation and washed three times in PBS and resuspended in PBS to an optical density at 660 nm of 1.0, corresponding to 1.5
x $10^6 \text{ cfu/ml}$ for *Candida* and $1.0 \times 10^9 \text{ cfu/ml}$ for streptococci. Colonies were counted using a colony counter (Gallenkamp, Loughborough, Leicestershire, UK) and the number of cfu/ml measured by taking the mean of $3 \times 20\mu l$ counts (Miles and Misra), and multiplying by the dilution and adjusting to $1 \text{ ml}$ solution, using the following formula:

$$\text{cfu x dilution x 1000/20} = \text{cfu/ml}$$

Samples were either diluted or concentrated by centrifugation to give the required ratios.

### 2.2.2 Determination of sub-MIC of sodium hypochlorite

Milton antibacterial solution (Procter and Gamble, Sydney, Australia) was used throughout the experiments and is a stabilized 1% sodium hypochlorite solution, with standardized salt and free alkali content (pH 10.8 undiluted, pH 8.5 in-use dilution). For the growth of *Candida* species/strains in sodium hypochlorite, Milton solution was filter-sterilized (0.22μm pore size) and serially diluted into SB. The *Candida* were then grown at 37°C for 48 h; $3 \times 20\mu l$ plated onto SA (Appendix A)(SB containing agar at 1% w/v) and the number of cfu/ml at each dilution counted. The sub-MIC was taken as the lowest dilution that did not alter the number of cfu/ml compared to cells grown in the absence of hypochlorite. *Candida* strains, grown in the presence or absence of hypochlorite were tested in the reactions detailed below. Two cultures of each *Candida* strain were used for all assays which were performed in triplicate unless otherwise stated, and the mean of the results obtained.
2.2.3 Adhesion to polystyrene

Wells in Linbro/Titertek 96 well microtitre plates (Flow Laboratories, Mclean, VA, USA) were washed with PBS and radiolabelled *Candida* cell suspension (100µl)(1.5 x 10^6 *Candida* cells/ml) added in columns 2-12. Following incubation for 15 min at ambient temperature, the wells were washed three times with PBS and then 30% hydrogen peroxide (100µl) plus 70% perchloric acid (50µl) added to the wells in columns 1-12 (Kotarski and Savage, 1979), hence providing a control in column 1 of wells with no *Candida* cells attached. After incubation for 30 min at 60°C, the contents of each well were counted by liquid scintillation using the Liquid Scintillation Analyzer (1500 Tri-Carb, Canberra Packard, USA). The counts per well were calculated as the number of cells adhering to polystyrene. The results for columns 2-12 were compared with those of the control (column 1). The number of cells adhering to the polystyrene was calculated by measuring the radioactivity associated with 100 µl of *Candida* suspension (in hydrogen peroxide: perchloric acid: scintillant), and comparing to the counts obtained after adhesion of the *Candida* to polystyrene.

2.2.4 Adhesion to *S. gordonii*-coated polystyrene

Microtitre plates were washed in PBS and unlabelled *S. gordonii* cell suspension (100µl)(1.0 x 10^9 *S. gordonii* cells/ml) added to wells in columns 2-12. After 15 min at ambient temperature, wells were washed three times with PBS and radiolabelled *Candida* cell suspension (100µl) added to wells in columns 1-11, thus allowing controls of *Candida* adhering to naked polystyrene (column 1) and streptococci adhering alone to polystyrene (column 12). The plates were then
incubated (15 min, ambient temperature), washed with PBS and hydrogen peroxide (100µl) plus perchloric acid (50µl) added to the wells (Kotarski and Savage, 1979). Following incubation for 30 min at 60°C, the radioactivity associated with the wells was measured and the number of adherent Candida cells calculated (2.2.3). Adhesion of Candida to S. gordonii-coated polystyrene was calculated, together with adhesion of streptococcal cells only; the latter was subtracted from the former and the result compared with that of the uncoated polystyrene control.

2.2.5 Adhesion to acrylic resin

Methylmethacrylate squares (1cm x 1cm x 2mm; presenting both polished and unpolished surfaces) were washed in PBS and radiolabelled Candida cell suspension (0.5ml)(1.5 x 10⁶ Candida cells/ml) added. Following incubation at ambient temperature for 2 h with rotation, the acrylic pieces were washed three times in PBS to remove unattached cells, and subjected to liquid scintillation. The amount of radioactivity associated with the acrylic pieces was measured and numbers of adherent Candida cells calculated (2.2.3).

The adhesion of Candida cells per mm² of acrylic resin piece was calculated as follows:

Number of Candida cells/mm² for 1 acrylic piece:

horizontal surface area = 10 x 10 mm = 100mm², as there are 2 sides = 200mm²
vertical surface area = 10 x 2 mm = 20mm², as there are 4 sides = 80mm²
∴ total surface area = 280mm²
2.2.6 Adhesion to BEC

The method of Willcox and Knox (1991) was used with minor modifications. BECs were collected (from BCW), washed twice in PBS and resuspended in PBS to an optical density at 550 nm of 0.2 (1.5 x 10⁶ BEC/ml). Following the original protocol (Willcox and Knox, 1991), equal volumes (100μl) of BEC and radiolabelled candidal cells (1.5 x 10⁶ cfu/ml) were mixed together in microtitre plate wells to give a ratio of Candida to BECs of 1:1 and incubated with shaking for 2 h at 37° C. After incubation, BECs plus attached candidal cells were separated from unattached candidal cells by filtering (polycarbonate filter of 8.0μm pore size; Nucleopore Corp. Pleasanton, CA, USA). Unattached candidal cells were washed through the filters leaving the larger BECs plus attached Candida on the filter. The radioactivity associated with the filters was then measured; controls of the candidal cells alone were also calculated. Adhesion was expressed as the number of Candida cells adhering to 100 epithelial cells and calculated from the radioactivity associated with 100μl of original candidal suspension.

The adhesion of Candida cells per mm² BEC was calculated as follows:

Number of Candida cells/mm² of BEC:
Assume BEC are flat circles with 2 sides
average diameter = 12μm = 0.012mm
area of 1 BEC = πr² = (3.1416 x 0.006²) x 2 (as 2 sides) = 0.0002mm²

2.2.7 Coaggregation with S. gordonii LGR2

The coaggregation experiment follows the method outlined by Jenkinson et al.
(1990) with some modifications. Radiolabelled *S. gordonii* cell suspension of 1.0 \( \times 10^9 \) cfu/ml (16\( \mu \)l) plus non-radiolabelled *Candida* cell suspension of 1.5 \( \times 10^4 \) cfu/ml (300\( \mu \)l) in PBS (148\( \mu \)l) were mixed together at 10 rpm for 20 min at ambient temperature. The ratio of *Candida* to streptococcal cells was 1:36. The coaggregated cells were pelleted by low speed centrifugation (400 x g; 1 min), the resulting supernatant was removed and subjected to high speed centrifugation (12,000 x g; 5 min), and the radioactivity associated with the supernatant and pellet after each centrifugation counted. *S. gordonii* cell suspensions alone (16\( \mu \)l) in PBS (484\( \mu \)l) were used as a control with every assay, and the radioactivity of the assays compared to that of the control. Adhesion was measured by using the following formula: 

\[
c - s \times \frac{100}{c} = \% \text{ coaggregation}
\]

\[(c = \text{control} \ s = \text{sample})\]

2.3 STATISTICAL ANALYSIS

The Wilcoxon signed rank test was used to assess differences between data sets and to determine whether sodium hypochlorite had a significant effect on properties of the *Candida* cells. Pearson’s and Spearman’s correlation coefficients were used to measure whether there were correlations between experiments.

2.4 RESULTS

The sub-MICs of sodium hypochlorite for the *Candida* strains tested were determined and found to be: 0.06% for *C. albicans* H1; 0.05% for *C. albicans* Y5499, GRI682, GDH2346 and Y523, *C. tropicalis* Y9 and *C. guilliermondii* Y324; and 0.02% for *C. kefyr* Y83 and *C. parapsilosis* Y316. In all subsequent
experiments 0.02% sodium hypochlorite was used as the sub-MIC for all Candida species. However, this did not alter the effect of hypochlorite in any adhesive assay as strains with a sub-MIC of 0.02% were equally likely to show an effect of hypochlorite as strains with a sub-MIC of 0.06%.

2.4.1 Adhesion to polystyrene

The results of the polystyrene adhesion assay are given in Table 2.1; the standard deviation about the mean was always less than 10%. Sodium hypochlorite significantly reduced the adhesion of all Candida species (p<0.01) with the exception of C. guilliermondii Y324. Candidal cells were generally not very adherent as the maximum value was $1.38 \times 10^5$ adherent cells per well for C. albicans Y523, compared with $7 \times 10^6$ adherent cells for S. gordonii LGR2. However, these differences may be due to the differences in size of candidal and streptococcal cells. Analysis of the results showed that strains of C. albicans had a range of values similar to the other candidal species where the maximum value was $1.2 \times 10^5$ adherent cells per well for C. krusei.

2.4.2 Adhesion to S. gordonii-coated polystyrene

When S. gordonii LGR2 was allowed to adhere to polystyrene prior to Candida cells (Table 2.1), the biofilm of S. gordonii decreased the adhesion of most Candida species/strains (p<0.025) compared to adhesion to naked polystyrene. All strains of C. albicans and all of the other Candida species with the exception of C. tropicalis Y9 showed reduced adhesion to S. gordonii-coated polystyrene. Except for one strain of C. albicans, Y523, sodium hypochlorite had no significant
effect on the ability of *Candida* to adhere to *S. gordonii*-coated polystyrene.

Table 2.1. Adhesion of *Candida* species to polystyrene and *S. gordonii*-coated polystyrene and the effect of sub-MIC sodium hypochlorite

| *Candida* species     | Adhesion to polystyrene 
| (x10^6)* | Adhesion to *S. gordonii*
|                   | coated polystyrene 
|                   | (x10^6)* |
|----------------------|---------|-------------------------|
|                      | C†      | Hy‡                     |
| *C. albicans* Y5499  | 1.6     | 1.0                     |
| *C. albicans* GDH2346| 4.6     | 4.0                     |
| *C. albicans* GR1682 | 2.8     | 2.0                     |
| *C. albicans* H1     | 5.4     | 2.0                     |
| *C. albicans* Y523   | 13.8    | 8.2                     |
| *C. tropicalis* Y9   | 6.8     | 3.4                     |
| *C. parapsilosis* Y316| 3.4   | 1.8                     |
| *C. krusei* Y301     | 12.0    | 5.6                     |
| *C. kefyr* Y83       | 10.4    | 3.0                     |
| *C. guilliermondii* Y324| 2.9 | 3.3                     |
|                      | C       | 0.5                     |
|                      | Hy      | 0.1                     |
|                      | C       | 0.3                     |
|                      | Hy      | 0.5                     |
|                      | C       | 0.1                     |
|                      | Hy      | 0.1                     |
|                      | C       | 0.4                     |
|                      | Hy      | 0.2                     |
|                      | C       | 13.0                    |
|                      | Hy      | 8.3                     |
|                      | C       | 11.2                    |
|                      | Hy      | 12.0                    |
|                      | C       | 0.8                     |
|                      | Hy      | 0.7                     |
|                      | C       | 10.3                    |
|                      | Hy      | 9.4                     |
|                      | C       | 3.4                     |
|                      | Hy      | 2.5                     |
|                      | C       | 1.1                     |
|                      | Hy      | 1.6                     |

* Number of cells adhering to substratum
† Control, cells grown in absence of sodium hypochlorite
‡ Hypochlorite, cells grown in presence of sodium hypochlorite
(Standard deviation about the mean always < 10% of the mean value)

2.4.3 Adhesion to acrylic

Adhesion to acrylic was correlated with adhesion to polystyrene (r=0.3, p=0.017). *C. albicans* strains and the other *Candida* species showed varying degrees of adhesion to acrylic surfaces (Table 2.2), ranging from 1.5 - 11.0 x 10^4 and 2.5 - 10.0 x 10^4 cells/acrylic piece respectively. The maximum values were recorded for *C. albicans* Y523 and *C. krusei* respectively. Sodium hypochlorite
reduced adhesion for most strains of *C. albicans*, but increased adhesion for most other candidal species with the exception of *C. krusei* which showed a decrease.

The adhesion of *Candida* cells per mm² of acrylic resin piece was calculated (method, 2.2.5):

- maximum adhesion = $11.0 \times 10^4$ cells per acrylic piece
  
  $\therefore$ cells per mm² = $11.0 \times 10^4 / 280 = 393$

- minimum adhesion = $1.5 \times 10^4$ cells per acrylic piece
  
  $\therefore$ cells per mm² = $1.5 \times 10^4 / 280 = 54$

Table 2.2. Adhesion of *Candida* species to acrylic resin and BEC and the effect of sub-MIC sodium hypochlorite

<table>
<thead>
<tr>
<th><em>Candida</em> species</th>
<th>Adhesion to acrylic (x10⁴)*</th>
<th>Adhesion to BEC (cells/100 BEC)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C†</td>
<td>Hy‡</td>
</tr>
<tr>
<td><em>C. albicans</em> Y5499</td>
<td>4.5</td>
<td>3.0</td>
</tr>
<tr>
<td><em>C. albicans</em> GDH2346</td>
<td>4.0</td>
<td>0.5</td>
</tr>
<tr>
<td><em>C. albicans</em> GRI682</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td><em>C. albicans</em> H1</td>
<td>4.0</td>
<td>3.5</td>
</tr>
<tr>
<td><em>C. albicans</em> Y523</td>
<td>11.0</td>
<td>3.4</td>
</tr>
<tr>
<td><em>C. tropicalis</em> Y9</td>
<td>4.5</td>
<td>8.0</td>
</tr>
<tr>
<td><em>C. parapsilosis</em> Y316</td>
<td>2.5</td>
<td>7.0</td>
</tr>
<tr>
<td><em>C. krusei</em> Y301</td>
<td>10.0</td>
<td>7.5</td>
</tr>
<tr>
<td><em>C. kefyr</em> Y83</td>
<td>4.0</td>
<td>9.0</td>
</tr>
<tr>
<td><em>C. guilliermondii</em> Y324</td>
<td>7.0</td>
<td>12.5</td>
</tr>
</tbody>
</table>

* Number of cells adhering to substratum
† Control, cells grown in absence of sodium hypochlorite
‡ Hypochlorite, cells grown in presence of sodium hypochlorite
§ No detectable result
(Standard deviation about the mean always < 10% of mean value)
2.4.4 Adhesion to BEC

Most *Candida* strains adhered to BEC (Table 2.2), with *C. albicans* GDH 2346 showing the highest value of 5.3 candidal cells per 100 BEC. Most *C. albicans* strains adhered better than the other candidal species (p<0.05), where *C. kefyr* showed the highest value of 3.7 cells per 100 BEC followed by *C. krusei* with 1.7 cells per 100 BEC. Sodium hypochlorite reduced adhesion in all cases where more than 1 candidal cell/100 BEC adhered (p < 0.025).

The adhesion of *Candida* cells per mm² BEC was calculated (method, 2.2.6):
maximum adhesion = 5.3 *Candida* cells/100 BEC

:. cells per mm² = 0.053/0.0002 = 265

minimum adhesion = 0.4 *Candida* cells/100 BEC

:. cells per mm² = 0.004/0.0002 = 20

2.4.5 Coaggregation with *S. gordonii* LGR2

All *Candida* species/strains coaggregated with *S. gordonii* LGR2 (Table 2.3). *C. albicans* strains showed varying degrees of coaggregation, ranging from 18.3% ± 15.1 to 53.7% ± 16.4. Values for the other *Candida* species were generally lower and ranged from 11.8% ± 6.3 to 33.0 ± 3.8. Growth of *Candida* strains in sodium hypochlorite significantly increased coaggregation except in the case of *C. tropicalis* (Table 2.3).
Table 2.3. Coaggregation of *Candida* species with *S. gordonii* LGR2

<table>
<thead>
<tr>
<th><em>Candida</em> species</th>
<th>Coaggregation % *</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td><em>C. albicans</em> Y5499</td>
<td>21.1 (7.1)†</td>
</tr>
<tr>
<td><em>C. albicans</em> GDH2346</td>
<td>53.7 (16.4)</td>
</tr>
<tr>
<td><em>C. albicans</em> GRI682</td>
<td>18.3 (15.1)</td>
</tr>
<tr>
<td><em>C. albicans</em> H1</td>
<td>26.4 (0.9)</td>
</tr>
<tr>
<td><em>C. albicans</em> Y523</td>
<td>22.0 (14.3)</td>
</tr>
<tr>
<td><em>C. tropicalis</em> Y9</td>
<td>33.0 (3.8)</td>
</tr>
<tr>
<td><em>C. parapsilosis</em> Y316</td>
<td>13.8 (11.3)</td>
</tr>
<tr>
<td><em>C. krusei</em> Y301</td>
<td>18.0 (12.8)</td>
</tr>
<tr>
<td><em>C. kefyr</em> Y83</td>
<td>16.1 (10.9)</td>
</tr>
<tr>
<td><em>C. guillermondii</em> Y324</td>
<td>11.8 (6.3)</td>
</tr>
</tbody>
</table>

* Ratio *Candida : S. gordonii* LGR2 = 1 : 36
† Standard deviation about the mean

2.5 DISCUSSION

This study has demonstrated that *Candida* species and strains differ in their ability to adhere to surfaces that may be present in the oral cavity, and that sodium hypochlorite in sub-MIC can affect certain adhesive events. Adhesion to polystyrene (Table 2.1) was shown to correlate with the ability of *C. albicans* strains to adhere to acrylic surfaces (Table 2.2)(p =0.017). This is in agreement with other studies that have demonstrated that adhesion to inert surfaces is mediated by non-specific forces including hydrophobicity (Klotz et al., 1985; Klotz, 1989; Miyake et al., 1990; Samaranayake et al., 1994). Most species grown in hypochlorite were less adhesive to polystyrene, and for *C. albicans* strains this reduction was paralleled by a reduction in their ability to adhere to acrylic. Of the other *Candida* species, *C. krusei* showed the greatest ability to adhere to
polystyrene and acrylic surfaces, and this ability was only somewhat less than that of *C. albicans* Y523 which adhered best of the *C. albicans* strains. With one exception, growth of other *Candida* species in hypochlorite reduced their ability to adhere to polystyrene but, excluding *C. krusei*, did not reduce their ability to adhere to acrylic.

Coating polystyrene with *S. gordonii* cells reduced the adhesion of *Candida* although studies on *S. crista* (*sanguis*) (CR311) and *S. salivarius* have demonstrated that they may increase candidal adhesion (Verran and Motteram, 1987). In the present study, reduced adhesion in the presence of *S. gordonii* may indicate that the streptococcal cells are occupying similar or adjacent sites to those that *Candida* cells would normally adhere. Previous studies (Theilade *et al.*, 1983; Koopmans *et al.*, 1988) have shown that denture plaque is primarily composed of streptococci and therefore the current study is based on the assumption that *S. gordonii* will influence adhesion.

Overall, most *C. albicans* strains were better at adhering to BEC than the other species; adhesion of *C. albicans* GDH 2346 to BEC was three-fold greater than that of the single strain of *C. krusei*, although Samaranayake *et al.* (1994) reported that the adhesion of *C. albicans* to BEC was twelve-fold greater than that of *C. krusei*. However, different strains were used in each study which may explain the differences. The present data are generally in agreement with previously published results (McCourtie and Douglas, 1984) but other studies have shown that strains GDH 2346 and GRI 682 are capable of adhering to BEC in much higher numbers, namely twenty-fold and eighty-fold respectively (Verran *et al.*, 1991). This discrepancy may be attributed to the different sources of BEC.
used in the studies. Adhesion to polystyrene did not correlate with adhesion to BEC, which agrees with the conclusion that hydrophobicity is not a dominant mechanism for adhesion of *Candida* to BEC (Hazen, 1989). Adhesion to BEC is mediated by mannoprotein on the cell surface of *Candida* (McCourtie and Douglas, 1981; 1984) and it appears that it is the protein component of the mannoprotein, which recognizes L-fucose or N-acetyl-D-glucosamine-containing receptors (Chapter 1, 1.4.5) on epithelial cells (Calderone and Braun, 1991; Calderone, 1993), that is important in adhesion. Hypochlorite significantly reduced adhesion of most species to BEC probably by oxidizing this protein component.

*Candida* adhered better to acrylic than to BEC, when allowance is made for differences in surface areas. This is in accord with the findings of previous investigators (Chapter 1, 1.5.5), who showed that greater numbers of *Candida* cells were isolated from the fitting surfaces of acrylic dentures than from the palatal mucosa. However, the results do indicate that treatment of *Candida*-associated denture stomatitis should give attention to both the denture and the underlying palatal mucosa.

Although *C. albicans* was no better at adhering to surfaces than the other *Candida* species examined, this species showed greater coaggregating abilities with *S. gordonii* but did not adhere well to *S. gordonii*-coated surfaces. This suggests a mechanism of adhesion whereby *Candida* cells coaggregate with streptococci and the coaggregates then adhere to surfaces in the mouth. Growth in hypochlorite increased coaggregation and since protease treatment of *Candida* cells abolished their ability to coaggregate with *S. sanguis* and *S. gordonii*
(Jenkinson et al., 1990), coaggregation is probably mediated by a protein on the surface of *Candida*.

Treatment of dentures with hypochlorite may reduce *Candida* colonization even though *Candida* cells may already be present in the denture plaque. Hypochlorite treatment may affect subsequent *Candida* colonization by reducing the ability of daughter cells to adhere to the denture.

In general, hypochlorite decreased adhesion to inert surfaces and the ability of *Candida* to adhere to BEC, but increased coaggregation of *Candida* with streptococcal cells. Thus sodium hypochlorite may initially reduce the ability of *Candida* species to adhere to oral surfaces, and, when used as a denture soak, may be a useful adjunct in the treatment of denture stomatitis.
CHAPTER 3
EFFECT OF SODIUM HYPOCHLORITE ON INVASIVE
CHARACTERISTICS OF CANDIDA SPECIES AND HOST
INFLAMMATORY REACTION

3.1 INTRODUCTION

This chapter will describe a series of in vitro experiments to investigate the effect of sodium hypochlorite on the characteristics of Candida species that are associated with tissue invasion and inflammatory reaction by the host.

Invasion of the host appears to be dependent on the production of acid proteinase and the transition of blastospore to hyphal form, with host mucosal proteins being disrupted by Candida proteinases prior to invasion of the mucosa by growing hyphae (Calderone, 1993). Hyphae have been mainly associated with C. albicans and are considered to be the initiators of epithelial cell penetration (Odds, 1988). C. albicans germ tubes and hyphae have been found to adhere better than yeast cells to human buccal epithelia (Kimura and Pearsall, 1980) (Chapter 1, 1.4.7). The mechanisms controlling virulence and the factors stimulating hyphal formation have been discussed in Chapter 1 (1.4.7). Serum is a consistently successful medium for stimulation of C. albicans hyphae, and is used for growth of C. albicans germ tubes for identification purposes.

Colonization and invasion of different areas in the body such as oral epithelium and other mucosal surfaces, or normal and sometimes damaged endothelium are due to different Candida-host cell recognition systems. These systems involve the presence of specific cell surface mannoprotein adhesins that can recognize
specific types of host cells (Calderone, 1993).

Candida species can be associated with localized inflammatory conditions such as denture stomatitis or disseminated blood borne diseases such as endocarditis. Once Candida cells have entered the blood stream they may interact with a variety of cell-mediated or humoral immune mechanisms. Platelet aggregation is part of the normal host defence system and can be involved in the inflammatory response to candidosis. Recent unpublished studies by M.D.P. Willcox and co-workers (personal communication, 1996) have indicated that mechanisms exist to remove Candida from the blood stream by a combination of phagocytosis, release of anti-candidal proteins from platelets and aggregation of platelets.

Skerl et al. (1981) demonstrated the aggregation of human platelets by C. albicans and C. krusei cell walls. They suggested that Candida species mediate a platelet aggregation response through a complement-dependent mechanism and that platelets stimulate the formation of germ tubes. Holder and Nathan (1973) used cell-free sonic extracts of C. albicans to aggregate mice platelets while Koltz et al. (1989) showed that only yeast cytosol and not whole yeast cells could induce platelet aggregation. Maisch and Calderone (1981) suggested that cell surface mannan may influence the adhesion of C. albicans to fibrin-platelet matrices.

The aim of these investigations was to examine in vitro the effect of sodium hypochlorite on the characteristics of Candida species that are associated with invasion and inflammation of host tissues.
3.2 MATERIALS AND METHODS

3.2.1 Microorganisms and growth conditions

The *Candida* isolates used in this study were previously described in Chapter 2 (2.2.1), and comprised five strains of *C. albicans*: GRI 682, GDH 2346, Y5499, H1 and Y523 (formerly *C. stellatoidea*), and strains of *C. tropicalis* Y9, *C. parapsilosis* Y316, *C. krusei* Y301, *C. kefyr* Y83, *C. guillermondii* Y324 and, in the case of the platelet aggregation assay, *C. glabrata* (isolated from the denture of a healthy patient attending the United Dental Hospital of Sydney and identified by bioMérieux ID 32 C, no strain notation available).

All *Candida* strains were grown under conditions similar to those described in Chapter 2 (2.2.1), namely, in SB for 48 h at 37°C, and after harvesting were resuspended in PBS to an optical density at 660 nm of 1.0, corresponding to 1.5 x 10^6 cfu/ml.

Milton antibacterial solution was used throughout the experiments; as the sub-MIC had been determined previously (Chapter 2, 2.2.2), the dilution was set at 0.02% to cover all *Candida* strains for growth in SB. Since it was necessary to standardize the nitrogen source, experiments were repeated using GSB medium to determine the sub-MIC of all strains in that medium.

*Candida* strains, grown in the presence or absence of hypochlorite, were tested in the experiments which are described below. Two cultures of each *Candida* strain were used in all assays which were performed in triplicate unless otherwise stated and the mean of the results obtained.
3.2.2 Proteinase production

Using a modification of published methods (Banerjee et al., 1991; Remold et al., 1968; Shimizu et al., 1987), Candida cells were grown for 48 h at 37°C in GSB, pH 5.2, modified to contain 0.2% (w/v) BSA (2mg/ml) as a nitrogen source (Shepherd and Sullivan, 1976). Cells were removed by centrifugation (7,000 x g; 10min), and the supernatant filtered through 0.22μm filters. Aliquots of supernatant (0.5ml) were incubated for 24 h at 37°C with 2.0ml bovine haemoglobin (6mg/ml; Sigma-Aldrich, Sydney, Australia) resuspended in 0.05 M citric acid-sodium buffer (pH 3.2) as substrate. The addition of 5% TCA stopped the reaction and precipitated undegraded haemoglobin. After centrifugation (10,000 x g; 10min), the supernatant was assessed for protein using the Lowry protein assay. Cells were tested after growth in the presence or absence of hypochlorite, with a 0.001% dilution of hypochlorite being the sub-MIC in GSB. Hypochlorite, 0.001% dilution, was also added to the culture supernatants of cells grown in its absence to test whether hypochlorite affected the activity of the proteinase. Aliquots of culture supernatant (0.5ml) incubated with 2.0 ml 0.05 M citric acid-sodium citrate buffer (pH 3.2) were used as controls with every assay.

3.2.3 Production of germ tubes

The method of Barlow et al. (1974) was used. Washed Candida cells (4 x 10⁴ cfu/ml) were added to 0.5 ml of human serum (Red Cross Blood Bank, Sydney, Australia) and incubated for 4 h at 37°C; microscopic observations were recorded for germ tube production every 30 min.
3.2.4 Examination of *Candida* cell wall proteins

Using modifications of published methods (Hazen *et al.*, 1990; Zeuthen and Howard, 1989), proteins were extracted from *Candida* cells suspended in digest buffer (1 mM phenylmethylsulphonyl fluoride plus 0.5 M MgSO₄ in 0.1 M Trizma hydrochloride, pH 8.1) and incubated overnight at 35°C, with 35 shakes/min in the presence of lyticase solution (1000 units/ml digest buffer) (Sigma Chemical Co., St. Louis, MO, USA). Cell debris was removed by centrifugation and the Coomassie protein assay (Pierce Chemical Co., Rockford, IL, USA) was used to measure the protein content of the supernatant, which was then precipitated overnight with 80% (v/v) ethanol at 4°C. Following centrifugation, the pellet was dissolved in double strength disaggregation buffer containing 0.125 M-Tris hydrochloride (pH 6.8), 2% (w/v) SDS, 5% β-mercaptoethanol, 10% (v/v) glycerol and 0.001% (w/v) bromophenol blue, and boiled for 3-5 min. Separation of *Candida* cell wall proteins was achieved with SDS-PAGE, 12.5% (w/v) polyacrylamide gel (Appendix A). The amount of protein per lane was 70μg; samples were electrophoresed and then visualized by staining with 0.1% Coomassie brilliant blue R-250 (Bio-Rad Laboratories Pty. Ltd. North Ryde, Australia) containing 0.1% Coomassie R-250, 40% methanol and 1% acetic acid. The following molecular mass markers were used as standards: phosphorylase *b* 94 kDa, albumin 67 kDa, ovalbumin 43 kDa, carbonic anhydrase 30 kDa, trypsin inhibitor 20.1 kDa, and α-lactalbumin 14.4 kDa. Densitometer scans of the stained gels were performed, utilizing the LKB 2222-020 UltraScan XL Laser Densitometer (Pharmacia LKB Biotechnology, Bromma, Sweden).
3.2.5 Aggregation of human platelets

Fresh whole human blood was kindly donated by the Red Cross Blood Bank, Sydney. PRP was obtained by centrifugation of blood for 10 min at 250 x g and removal of the supernatant, and PPP was obtained by recentrifugation of the pellet from the PRP for 10 min at 2,500 x g and removal of the supernatant. PRP was then adjusted, with PPP, to give a platelet concentration of 2.5 x 10⁸ platelets/ml. Washed Candida cells were resuspended in PBS to an optical density at 660 nm of 1.0 corresponding to 1.5 x 10⁶ cfu/ml. Using a two-channel recording platelet aggregometer (Chrono-log Corporation, Havertown, PA, USA), the optimal ratio of Candida to platelets was determined by testing various ratios ranging from 1:833 to 1:8, to obtain the maximum percentage aggregation. Candida and platelets were mixed together and allowed to interact at 37°C for 25 min. Aggregometer readings were obtained by adding whole yeast cells in suspension (50 μl) in PBS to PRP (250 μl) and PPP (250 μl). Aggregation was recorded as a decrease in optical density. ADP, a natural agonist, was used to measure platelet function and also the degree of aggregation caused by Candida cells.

To confirm that platelet aggregation and not agglutination was being observed, platelet aggregation inhibitors (25 μl) EDTA (7.7 mM), imipramine (150 μM), quinacrine (77 μM) and apyrase (0.76 mg/ml) were added to PRP, PPP (250 μl) and C. tropicalis (50 μl) and tested against a control aggregation without inhibitor.

3.3 STATISTICAL ANALYSIS

The Wilcoxon signed rank test was used to assess differences between data sets
and to determine whether sodium hypochlorite had a significant effect on properties of the *Candida* cells. Pearson’s and Spearman’s correlation coefficients were used to measure whether there were correlations between experiments.

### 3.4 RESULTS

#### 3.4.1 Proteinase Production

Only three of the five strains of *C. albicans*, GDH 2346, GRI 682, H1, and the single strain of *C. parapsilosis*, Y316, produced proteinase (Table 3.1). Sodium hypochlorite produced no significant difference in proteinase production by *Candida* species or strains (GDH 2346, p = 0.8182; GRI 682, p = 0.4848; H1, p = 0.2222; Y316, p = 0.2222) nor was the proteinase that had been produced significantly affected by the presence of hypochlorite (GDH 2346, p = 0.8182; GRI 682, p = 0.4848; H1, p = 0.8413; Y316, p = 1.0000).

#### 3.4.2 Germ tube formation

All strains of *C. albicans* produced germ tubes with the exception of strain Y523. (Table 3.1). *C. parapsilosis* Y316, *C. kefyr* Y83 and *C. guillermondii* Y324 produced pseudohyphae, which could be distinguished from true hyphae as these did not produce septae or mycelia after 24 h incubation in serum. The same strains of *C. albicans* that produced proteinase were associated with germ tube formation whereas *C. parapsilosis* produced pseudohyphae. Sodium hypochlorite reduced the time for germ tube formation by *C. albicans* strains from 2 h to only 30 min. Sodium hypochlorite had no effect on the production of pseudohyphae by *C. parapsilosis*, and for *C. kefyr* and *C. guillermondii*, production of
pseudohyphae was eliminated.

Table 3.1. Proteinase production and germ tube formation by *Candida* species and the effect of growth in hypochlorite

<table>
<thead>
<tr>
<th>Candida species</th>
<th>Proteinase production* (μg protein/ml)</th>
<th>Germ tube formation (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Hypochlorite</td>
</tr>
<tr>
<td><em>C. albicans</em> Y5499</td>
<td>0†</td>
<td>0</td>
</tr>
<tr>
<td><em>C. albicans</em> GDH2346</td>
<td>59 (25)‡</td>
<td>69 (35)</td>
</tr>
<tr>
<td><em>C. albicans</em> GRI682</td>
<td>91 (48)</td>
<td>78 (46)</td>
</tr>
<tr>
<td><em>C. albicans</em> H1</td>
<td>111 (29)</td>
<td>137 (21)</td>
</tr>
<tr>
<td><em>C. albicans</em> Y523</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>C. tropicalis</em> Y9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>C. parapsilosis</em> Y316</td>
<td>98 (18)</td>
<td>113 (19)</td>
</tr>
<tr>
<td><em>C. krusei</em> Y301</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>C. kefyr</em> Y83</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>C. guilliermondii</em> Y324</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Substrate: bovine haemoglobin, 6 mg/ml (600 μg/ml)
† No detectable result
‡ Standard deviation in parenthesis
§ Pseudohyphae

3.4.3 Examination of cell wall proteins

Cell wall proteins were extracted from strains that produced germ tubes, namely, *C. albicans* GDH 2346, GRI 682 and H1, from Y523, which did not produce germ tubes, and *C. parapsilosis*, Y316 which produced pseudohyphae. Protein profiles of strains grown in the absence of hypochlorite and scanned
following SDS-PAGE showed that two major protein components of 43 kDa and 27 kDa were common to all strains except Y523, where the 27 kDa component predominated and the 43 kDa component could not be detected. A comparison between GRI 682 and H1 (typical strains) and Y523 is shown in lanes 1, 3 and 5 respectively of Figure 3.1. Cells grown in the presence of hypochlorite showed an increase in the amount of 43 kDa protein when compared with growth in its absence, and for H1 an additional band at 47 kDa was detected; as shown in lanes 2, 4 and 6 respectively of Figure 3.1. Scans of gels showing GRI 682 and H1 grown in the absence of hypochlorite demonstrated similarities in protein profiles and are shown in Figures 3.2 A and 3.3 A respectively. Scans of gels showing GRI 682 and H1 grown in the presence of hypochlorite are shown in Figures 3.2 B and 3.3 B respectively. However, for Y523 the protein profile for organisms grown in hypochlorite showed an increase in the amount of protein in bands at 56, 48, 28 and 26 kDa, with the dominant one being 48 kDa, and additional bands at 41, 38, 36 and 35 kDa (Figure 3.4).
Figure 3.1 Characterization of cell wall proteins by SDS-PAGE from *Candida* strains GRI682, H1 and Y523; M, molecular mass standards; lane 1, strain GRI682 grown in absence of sodium hypochlorite; lane 2, strain GRI682 grown in presence of hypochlorite; lane 3, strain H1 grown in absence of hypochlorite; lane 4, strain H1 grown in the presence of hypochlorite; lane 5, strain Y523 grown in absence of hypochlorite; lane 6, strain Y523 grown in presence of hypochlorite (each lane loaded with 70μg protein).
Figure 3.2 The effect of growth with sodium hypochlorite on the cell wall proteins of *C. albicans* GRI682. A, growth in absence of hypochlorite; B, growth in presence of hypochlorite. Numerals at top of peaks indicate protein bands at specified kDa.
Figure 3.3 The effect of growth with sodium hypochlorite on the cell wall proteins of *C. albicans* H1. A, growth in absence of hypochlorite; B, growth in presence of hypochlorite. Numerals at top of peaks indicate protein bands at specified kDa.
Figure 3.4 The effect of growth with sodium hypochlorite on the cell wall proteins of *C. albicans* Y523 (formerly *C. stellatoidea*). A, growth in absence of hypochlorite; B, growth in presence of hypochlorite. Numerals at top of peaks indicate protein bands at specified kDa.
3.4.4 Aggregation of human platelets

The optimal ratio of *C. tropicalis* to platelets for maximum percentage aggregation, was approximately 1:80, with no aggregation occurring at 1:40 (or below) or 1:100 (or above). A ratio of 1:80 was used for the platelet aggregation assays described below.

When cells grown in the absence of hypochlorite were tested, platelets were aggregated by one strain of *C. albicans* Y523 and strains of *C. tropicalis* Y9, *C. parapsilosis* Y316, *C. krusei* Y301, *C. kefyr* Y83, *C. guilliermondii* Y324 and *C. glabrata*. The other strains of *C. albicans* failed to aggregate platelets. The plot for strain Y9 is shown in Figure 3.5. The results shown in Table 3.2 are the means of values obtained from assays performed in triplicate. There was considerable variation between strains in the lag phase that preceded aggregation, ranging from 6.5 ± 1.0 to 15.7 ± 2.6 min. The percentage aggregation ranged from 83.0 ± 15.1 to 100%, and the time taken to reach aggregation varied from 9.7 ± 2.2 to 21.8 ± 4.1 min, with *C. tropicalis* Y9 having the shortest lag phase of 6.5 ± 1.0 min and reaching 100% aggregation in the shortest time of 9.7 ± 2.2 min.

Taking *C. tropicalis* grown in the absence of hypochlorite as the test organism, the addition of EDTA, which reduces the availability of divalent cations (in particular Ca$^{2+}$ and Mg$^{2+}$), or imipramine, which affects platelet membrane stability, inhibited aggregation completely. Quinacrine, which inhibits the arachidonate cascade, and apyrase, which affects the availability of ADP, reduced aggregation to 60% and 35% respectively. These data indicate that true platelet aggregation and not agglutination was observed.
Figure 3.5  Aggregation of human platelets by *Candida* species; *C. tropicalis* Y9 aggregation curve, Y9 grown in absence of sodium hypochlorite
In general, sodium hypochlorite increased the lag phase time as shown for *C. tropicalis* Y9 in Figure 3.6 and in Table 3.2. The exceptions were *C. guilliermondii* which showed a decrease, and *C. glabrata* where there was no difference.

Also, hypochlorite increased the time taken to reach final aggregation for most *Candida* species, with the exception of *C. albicans* Y523, and *C. glabrata* which showed a decrease and *C. guilliermondii* where there was no difference. However, for percentage aggregation which was generally in the vicinity of 100%, there were varying responses to hypochlorite. Overall, sodium hypochlorite had no significant effect on the ability of *Candida* species to aggregate human platelets.

Table 3.2. Aggregation of human platelets by *Candida* species/strains

<table>
<thead>
<tr>
<th>Candida species</th>
<th>Control</th>
<th>Hypochlorite</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lag phase</td>
<td>Final aggregation</td>
</tr>
<tr>
<td></td>
<td>(min)</td>
<td>(min)</td>
</tr>
<tr>
<td><em>C. albicans</em> Y523</td>
<td>8.0</td>
<td>21.5</td>
</tr>
<tr>
<td></td>
<td>(3.0)*</td>
<td>(5.8)</td>
</tr>
<tr>
<td><em>C. tropicalis</em> Y9</td>
<td>6.5</td>
<td>9.7</td>
</tr>
<tr>
<td></td>
<td>(1.0)</td>
<td>(2.2)</td>
</tr>
<tr>
<td><em>C. parapsilosis</em> Y316</td>
<td>10.4</td>
<td>16.1</td>
</tr>
<tr>
<td></td>
<td>(10.4)</td>
<td>(3.8)</td>
</tr>
<tr>
<td><em>C. krusei</em> Y301</td>
<td>15.7</td>
<td>21.8</td>
</tr>
<tr>
<td></td>
<td>(2.6)</td>
<td>(4.1)</td>
</tr>
<tr>
<td><em>C. kefyr</em> Y83</td>
<td>7.7</td>
<td>19.4</td>
</tr>
<tr>
<td></td>
<td>(7.0)</td>
<td>(4.9)</td>
</tr>
<tr>
<td><em>C. guilliermondii</em> Y324</td>
<td>14.0</td>
<td>18.2</td>
</tr>
<tr>
<td></td>
<td>(6.8)</td>
<td>(7.6)</td>
</tr>
<tr>
<td><em>C. glabrata</em></td>
<td>8.5</td>
<td>20.4</td>
</tr>
<tr>
<td></td>
<td>(2.4)</td>
<td>(3.8)</td>
</tr>
</tbody>
</table>

* Standard deviation in parenthesis
Figure 3.6 Aggregation of human platelets by *Candida* species; *C. tropicalis* Y9 aggregation curve, Y9 grown in presence of sodium hypochlorite.
3.5 DISCUSSION

Once they have adhered to surfaces in the oral cavity, Candida cells may invade tissues. This process has been linked to the ability of C. albicans strains to produce hyphae and proteinase (Negi et al., 1984; Ruchel et al., 1986; Cutler, 1991; Wright et al., 1992). Other investigators (Rajasingham and Challacombe, 1987) have demonstrated that hyphae produce proteinases at their growing tips. Yamamoto et al. (1992) and Banerjee et al. (1991) found that all C. albicans strains and the majority of C. parapsilosis and C. tropicalis produced acidic proteinase. In this study, the majority of C. albicans strains and the strain of C. parapsilosis also produced an acidic proteinase, but growth in the presence of hypochlorite did not affect the production or activity of proteinase.

Hyphal production depends on a number of factors, including nutrition (Holmes and Shepherd, 1988) or the presence of serum (Barlow et al., 1974), and hyphal production by C. albicans in response to serum has been used for many years in identification of this species (Taschdjian et al., 1960). The exact nature of the stimulant in serum is not known nor is the precise mechanism of hyphal formation. However, it has been shown that 'heat-shock' or stress-proteins may be involved in the blastospore to hyphal transition, as a mutant lacking in 'heat-shock' protein HSP 47 (47 kDa protein) was found to be germination defective (Zeuthen and Howard, 1989). In this study, it is possible that growth in the presence of hypochlorite induced stress proteins that primed the cells for germ tube formation and this may explain the increased rate of germ tube formation.

The analysis of cell wall proteins demonstrated the presence of major components of 43 and 27 kDa in C. albicans strains GDH 2346, GRI 682, H1
and in *C. parapsilosis* Y316, and a major protein band at 27 kDa in *C. albicans* Y523. Another study showed that lyticase digestion of *Candida* cells yielded proteins in the range of 34-38 kDa (Hazen *et al.*, 1990). In this study, growth in hypochlorite resulted in an increase in the amount of protein at band 43 kDa, for strains GDH 2346, GRI 682, H1 and Y316, with an increase in a 47 kDa band for H1, and more general changes in the case of Y523. It is noteworthy that some published data (Kwon-chung *et al.*, 1990) consider Y523, which was previously designated *C. stellatoidea*, to be taxonomically indistinguishable from *C. albicans*.

It could be suggested that the increased presence of the 43 kDa protein is linked with the reduction in time of germ tube production in serum. The mechanism for signalling blastospores to form germ tubes is complex and multifactorial, involving external and internal signals. The 43 kDa surface protein may be the receptor molecule and/or signal molecule that signals the blastospores to produce germ tubes. Perhaps, interactions of one or more serum proteins with the 43 kDa candidal protein, triggers the transformation events. Further work on the exact mechanisms of germ tube production may shed further light on the above hypothesis. Although *C. parapsilosis* Y316 also had a 43 kDa cell surface protein, this strain did not produce germ tubes, and thus this strain may lack other important elements that are essential for germ tube formation.

The induction of germ tube formation by hypochlorite is intriguing from another perspective. PMNs are at the front-line of the body’s cell-mediated defence system and release hypochlorite upon activation (Arnhold *et al.*, 1991), and it has been shown that *Candida* cells can activate PMNs (Palma *et al.*, 1992). It may be that in vivo this activation can trigger the production of germ tubes.
Production of large hyphae by *Candida* cells appears to be one mechanism this yeast uses to evade phagocytosis by PMNs (Odds, 1988).

While there is published evidence that *C. albicans* can interact with aggregated platelets and platelet-fibrin clots (Maisch and Calderone, 1981; Klotz *et al.*, 1989), previous investigators have concluded that whole cells do not aggregate platelets (Skerl *et al.*, 1981; Klotz *et al.*, 1989). However, their conclusions may be due to the very high (1:1000) ratio of *Candida* to platelets used in their experiments (Klotz *et al.*, 1989). In the current study a ratio of *Candida* to platelets of 1:80 was optimal for platelet aggregation. The majority of strains aggregated human platelets, and this ability was not significantly affected by sodium hypochlorite.

Aggregation was not dependent on blastospore-hyphal transition since the four strains of *C. albicans*, that did produce germ tubes did not aggregate platelets, and three of the six other strains of *Candida*, which aggregated platelets, produced pseudohyphae only. It could be suggested that the data demonstrated a pathogenic trait for one strain of *C. albicans* (Y523) and strains of *C. tropicalis*, *C. parapsilosis*, *C. krusei*, *C. kefyr*, *C. guilliermondii* and *C. glabrata*. However, *C. albicans* is the causative organism in the majority of *Candida* endocarditis cases (Odds, 1988) and this experiment has shown that four strains of *C. albicans* did not aggregate human platelets. This would indicate that platelet aggregation may be a mechanism to defend the host against candidal invasion since aggregated platelets release antimicrobial factors which assist the host’s defence system and recent studies have shown that these may be active against *Candida* (Willcox, 1996). In these studies the *Candida* strains were isolated as commensals from healthy mouths rather than strains isolated from infections such as endocarditis,
and this may explain why *C. albicans* was unable to aggregate platelets. However, it is likely that strains causing endocarditis were originally commensals in the oral cavity.

The results of this investigation have shown that sodium hypochlorite increased the production of *Candida* cell wall proteins and the rate of germ tube formation in serum, but did not affect proteinase production by *Candida* strains. The data also showed that hypochlorite did not affect the ability of whole cells of certain *Candida* species to aggregate human platelets.

It may be concluded therefore that platelet aggregation by *Candida* species and subsequent release of vasodilator and inflammatory mediators may produce symptoms of inflammation associated with candidosis. However, sodium hypochlorite may not function in an anti-invasive capacity when used as a denture soak in cases of denture stomatitis.
CHAPTER 4

IN VITRO EFFECTS OF SODIUM HYPOCHLORITE AND MICROWAVE IRRADIATION ON SURVIVAL OF CANDIDA SPECIES AND OTHER MICROORGANISMS ON DENTURE SURFACES

4.1 INTRODUCTION

Thus far it has been demonstrated that growth in the presence of sodium hypochlorite has a number of effects on Candida, particularly with reference to their ability to adhere to polymeric substrata and epithelial cells. This is significant and indicates that sodium hypochlorite used as a denture soaking agent may be effective in reducing the presence of C. albicans by preventing cells adhering either to the denture or to the palate surface.

This chapter will describe an in vitro study to test the effect of two procedures, namely sodium hypochlorite soak and microwave irradiation, on the control of Candida species and other microorganisms associated with denture surfaces. As already described, species of Candida and in particular C. albicans may be involved in the aetiology of denture stomatitis. Studies have also shown that Candida species and other oral microorganisms, including species of Streptococcus, Lactobacillus, "Bacteroides" and Actinomyces are associated with denture plaque (Theilade and Budtz-Jorgensen, 1988; Koopmans et al., 1988), and, accordingly, denture hygiene is an important factor in the prevention and treatment of denture stomatitis.

Minagi et al. (1987) who investigated the efficacy of three denture-cleansing agents, considered that they should have high antifungal activity for the
prevention of denture stomatitis. Evidence for the usefulness of sodium hypochlorite is provided by Ghaliachebaf et al. (1982) who tested four immersion-type denture cleansers and found that hypochlorite-containing cleansers such as Mersene and Clorox/Calgon were the most effective. Similar observations were made by Basson et al. (1992) who tested the disinfecting effect of five different household products and found 4.0% Milton (sodium hypochlorite 0.04% plus sodium chloride 0.66% v/v) to be the most effective. Rudd et al. (1984) also demonstrated the disinfecting effects of 5.25% sodium hypochlorite as a denture soak. However, Moore et al. (1984) found that, although hypochlorites eliminated \textit{C. albicans in vitro}, they were not effective under clinical conditions.

Jagger and Harrison (1995) have recommended that acrylic dentures be soaked in Milton solution for 20 minutes in the evening, rinsed thoroughly with cold water and then soaked in cold water overnight. Although concentration of the Milton solution was not mentioned this procedure would overcome the problem of corrosion to metal parts because of brevity of exposure.

There have been a number of studies carried out by other investigators on disinfection of other surfaces by microwave irradiation and these are summarized in Table 4.1. The importance of Rohrer and Bulard's study (1985) has been mentioned in Chapter 1 (1.6.4) as their study was the only one to test the effect of microwave irradiation on the disinfection of microorganisms on denture surfaces. However, these authors emphasized that consistent disinfection could only be accomplished if the dentures were rotated in three dimensions within the microwave oven.
Table 4.1. A summary of published studies on disinfection of surfaces by microwave irradiation

<table>
<thead>
<tr>
<th>Investigators</th>
<th>Experiment</th>
<th>Surfaces</th>
<th>Procedure</th>
<th>Microwave oven</th>
<th>Inclusions/ Modifications</th>
<th>Watts, time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hume and Makinson, 1978</td>
<td>Dental instruments</td>
<td><em>Staph. aureus, Herpes simplex</em></td>
<td>&quot;Sharp&quot; *</td>
<td>&quot;Sharp&quot; *</td>
<td>Details not given</td>
<td>12 min</td>
</tr>
<tr>
<td>Sanborn * et al.*, 1982</td>
<td>Plastic tissue culture vessels</td>
<td>Gr. neg, Gr. pos., bacteria, viruses</td>
<td>&quot;Kenmore&quot;, home-type †, 2450 MHz</td>
<td>Rotating platter, parallel load</td>
<td>650 W, 3 min</td>
<td></td>
</tr>
<tr>
<td>Rohrer and Bulard, 1985</td>
<td>Dentures</td>
<td><em>C. albicans, C. albicans, aerobes</em></td>
<td>&quot;Toshiba&quot;, 2450 MHz ‡</td>
<td>3-dimensional rotating device parallel load</td>
<td>720 W, 8 min</td>
<td>720 W 10 min</td>
</tr>
<tr>
<td>Young * et al.*, 1985</td>
<td>Nasal hoods</td>
<td>Viruses</td>
<td>&quot;Toshiba&quot;, 2450 MHz</td>
<td>3-dimensional rotating device parallel load</td>
<td>720 W, 4 min</td>
<td></td>
</tr>
<tr>
<td>Rohrer * et al.*, 1986</td>
<td>Hydrophilic contact lenses</td>
<td><em>C. albicans, bacteria, viruses</em></td>
<td>&quot;Toshiba&quot;, 2450 MHz</td>
<td>3-dimensional rotating device parallel load</td>
<td>700 W, 4 min</td>
<td></td>
</tr>
<tr>
<td>Najdovski * et al.*, 1991</td>
<td>Infective waste</td>
<td>Vegetative bacteria Spores</td>
<td>1) &quot;Litton&quot;, 2) &quot;Riviera&quot; 2450 MHz, neon-type §</td>
<td>1) Max. power 650 W 2) Max. power 1400 W</td>
<td>650 W, 5 min</td>
<td>1400 W, 20 min</td>
</tr>
<tr>
<td>Griffith * et al.*, 1993</td>
<td>Polyethylene catheters</td>
<td><em>Proteus sp. bacteria</em></td>
<td>Standard household 650 W</td>
<td>Rotating platter, parallel load</td>
<td>650 W, 6 min</td>
<td></td>
</tr>
<tr>
<td>Rosaspina * et al.*, 1993</td>
<td>Scapel blades, cover glasses</td>
<td>Enterobacteria</td>
<td>&quot;Ghimas Sterivelox&quot; ‡, S90S, 2450 MHz, 600 W</td>
<td>Instrument containers</td>
<td>600 W, 2.5 min</td>
<td></td>
</tr>
</tbody>
</table>

* Sharp Microwave (Australia) † Kenmore model 99601, Oklahoma, USA ‡ Toshiba, Japan § Neon-type Litton, type Anita, Neon-type Riviera, Yugoslavia ‡ Sterivelox S90S, Ghimas-Bologna, Italy
Further evidence of the value of microwave disinfection is shown in the study by Griffith et al. (1993) who used a standard household 650 W microwave oven to sterilize polyethylene catheters at high setting (650 W) for 2, 4, 6 and 8 min, with sterility being achieved at 6 min. They stressed that only microwave ovens with rotating platters should be used to minimize the effect of "cold" areas where no bactericidal effect is achieved, and recommended the use of the "heat sink" (a small beaker of water) to prevent overheating. Similar observations were made by Sanborn et al. (1982) who used a domestic microwave oven at high setting to sterilize tissue culture vessels from 0 to 4 min with sterility at 3 min.

The in vitro experiments to be described below were designed to investigate the effect of sodium hypochlorite and microwave irradiation on the control of Candida and streptococci associated with denture surfaces. The aim of these investigations was to obtain data which will provide baseline information for a clinical project on the control of microorganisms associated with denture stomatitis.

4.2 MATERIALS AND METHODS

4.2.1 Microorganisms and dentures

The strains of C. albicans H1 and S. gordonii LGR2 used for inoculation of dentures, were described previously in Chapter 2 (2.2.1). C.albicans H1 was grown in SB (Appendix A) for 48 h at 37°C and S.gordonii LGR2 was grown for 24 h at 37°C in BHI (Appendix A).

The dentures used in this experiment were fabricated in a standardized procedure, and had been stored dry for one year and then soaked in distilled
water for one week prior to the commencement of this study. Between runs, the
dentures were rinsed in distilled water and autoclaved at 121°C for 15 min and
re-used for the succession of experiments described below.

Aseptic techniques were used for the procedures that are described in the
following assays which were always performed in triplicate.

4.2.2 Sodium hypochlorite treatment

Milton antibacterial solution at sub-MIC of 0.02%, (determined previously,
Chapter 2, 2.2.2) or 0.0125% was used throughout the experiments for the 8 h
sodium hypochlorite denture soaks.

Ten dentures were selected for inoculation with *C. albicans* H1; SB (110 ml)
was dispensed into each of five beakers (Pyrex 250 ml) to cover two upper acrylic
dentures; beakers and contents were sealed with foil and autoclaved at 121°C for
15 min. An inoculum of *C. albicans* H1 (11 ml)(1.5 x 10^6 Candida cells/ml), grown
for 24 h at 37°C, was transferred to each autoclaved beaker containing dentures
and SB. Beakers were then placed in a shaking water bath (80 strokes/min) for
48 h at 37°C.

Following incubation, all dentures were rinsed with gentle agitation for 10 sec
in 0.9% (w/v) NaCl solution (100 ml) to remove non-adherent microorganisms
and media; each rinsed denture was then transferred to a beaker covered with
foil. Five dentures were selected for soaking (8 h) in sodium hypochlorite solution
(Milton), either 0.02% (sodium hypochlorite 2 ml/distilled water 98 ml) or
0.0125% (sodium hypochlorite 1.25 ml/distilled water 98.75 ml). Five controls
were soaked in distilled water for a similar period. Following the 8 h soak, all
dentures were rinsed as before in 0.9% (w/v) NaCl solution (100 ml) to remove non-adherent microorganisms and sodium hypochlorite solution, thus ensuring that no sodium hypochlorite was carried over into the culture with the effect of reducing survival. After rinsing, 0.9% (w/v) NaCl solution (75 ml) was added to each beaker to cover the denture; the soaked denture was then sonicated (Douglas and Lamb, 1988) for 60 sec to remove adherent cells, using an ultrasonic water bath (Branson 12, Branson Instruments Company, Shelton, Ct, USA).

Using 0.9% (w/v) NaCl solution as diluent, serial tenfold dilutions, $10^{-1}$ to $10^{-4}$, were plated onto SA medium, incubated for 48 h at 37°C and cfu/ml calculated. To check for contamination, similar dilutions were also plated onto Columbia blood agar (Appendix A) and incubated for 48 h at 37°C. The control dentures, which were not treated with hypochlorite, were also sonicated and the sonicate treated as outlined above.

The same procedures were repeated using S. gordonii LGR2 as the inoculum.

To ensure that sonication was controlled C. albicans H1 and S. gordonii LGR2 in solution were tested and the sonication procedure did not reduce the viability of the eluted organisms.

4.2.3 Microwave treatment

4.2.3.1 Microwave oven

The use of a modified microwave oven such as the one used by Rohrer and Bulard (1985) was considered impractical, as such a modification would not be available to the average householder or institution. Microwaving of acrylic
dentures was therefore deliberately performed in an unmodified domestic oven with a rotating table (Thomas and Webb, 1995); details are given in 4.2.3.2.

The microwave oven used is shown in Figure 4.1 and the specifications were: Model N.603M, input 240 volts, AC 50 HZ 1200W 5.3A, output 650 W, frequency 2450 MHz (NEC Corporation Japan). To ensure accurate results, the centigrade method of power calibration was used in which the power outlets of the microwave oven at high, medium and low settings were checked against standards calculated by a method suggested by the manufacturer. The initial temperature of 1000 ml of water (which should be between 17-27°C) was taken, the water heated for 60 sec at the specific setting, and the final temperature of the water taken after heating. The standards were calculated by subtracting the initial water temperature from the final water temperature and multiplying the temperature rise by a factor of 70.

Power output checks at high, medium and low settings were carried out three times, and the mean determined as 604 W (±92), 331 W (±34) and 70 W (±0) respectively. The manufacturer considered these figures to be within an acceptable range of accuracy. The microwave oven clock was also checked against a laboratory timer for accuracy.

4.2.3.2 Effect of microwave irradiation on dimensional stability of dentures

It was essential to establish the effect of microwaving on the dimensional stability of acrylic dentures to ensure that the procedures aimed to control denture plaque would not be harmful to the denture itself. Using the microwave oven described above (4.2.3.1) an exposure of 10 min at high setting (Rohrer and
Figure 4.1 The unmodified domestic microwave oven (NEC Model N. 603 M) used in this study, note rotating table and inclusion of parallel load.
Bulard, 1985) was used as a baseline from which other exposures were selected. Ten experimental dentures were exposed to 604 W, 2450 MHz of full radiation for 10 min on fifteen occasions while ten control dentures were left in water at 37°C. The control dentures were then microwaved fifteen times at medium setting (350 W, 2450 MHz) for 6 min. Measurements were made before and after microwaving and non-microwaving (control) at six points: inter-molar (IM), inter-canine (IC), antero-posterior (AP); flange heights at right premolar (RP), left premolar (LP) and anterior centre (CE). The results showed that after microwaving at 604 W for 10 min the vertical measurements (RP, LP and CE) all increased (only CE, p< 0.05) but the horizontal measurements (IM, IC and AP) decreased. However, reduced exposure at medium setting (331 W) for 6 min brought about minimal changes (range, -0.11 to +0.04%).

It was concluded therefore, that microwaving of acrylic dentures at high setting for 10 min causes unacceptable dimensional changes while microwaving of dentures at medium setting for 6 min results in minimal dimensional change which was considered to be sustainable in the long term (denture lifespan, 5-7 yr).

4.2.3.3 Treatment

Following the same procedure as in 4.2.2, ten dentures in SB were inoculated with *C. albicans* H1, incubated in a shaking water bath for 48 h at 37°C and then rinsed with gentle agitation for 10 sec in 0.9% (w/v) NaCl solution (100 ml). Five dentures were selected for microwaving and five controls were not microwaved. Each rinsed denture was then transferred to a beaker and for the dentures
selected for microwaving, foil covers were replaced by plastic wrap; the experimental dentures were then individually exposed to microwave radiation. In accordance with the protocol of Rohrer and Bulard (1985), a glass beaker containing 150 ml water (replenished for each run) was placed in the microwave oven during all exposures as an absorber of microwaves along with the denture to be disinfected.

A range of exposure times and settings of 1, 2, 4, 6, 8 and 10 minutes at high (604 W, 2450 MH₂) and similar times at medium (350 W, 2450 MH₂) and low (70 W, 2450 MH₂) setting were selected. After microwaving, 0.9% (w/v) NaCl solution (75 ml) was added to each beaker to cover the denture; the microwaved denture was then sonicated as described in 4.2.2. Tenfold serial dilutions 10⁻¹ to 10⁴ from the sonicate were plated onto SA medium, incubated for 48 h at 37°C and cfu/ml calculated. To check for contamination, similar dilutions were also plated onto Columbia blood agar and incubated as previously.

The control dentures which were not treated with microwave irradiation, were also sonicated and the sonicate treated as outlined above.

The above procedures were repeated using S. gordonii LGR2 as the inoculum.

4.2.4 SEM procedures

Four methylmethacrylate resin specimens (1 cm x 1 cm x 2 mm) were inoculated with C. albicans H1, following the procedures outlined for the denture assay. Two glass bottles (25 ml), each containing two acrylic pieces in SB (10 ml) were autoclaved at 121°C for 15 min. The inoculum of C. albicans H1 (1 ml)(1.5 x 10⁶ Candida cells/ml) grown for 24 h at 37°C, was transferred to each bottle
and incubated in a shaking water bath (80 strokes/min) for 48 h at 37°C.

Following incubation, all samples were rinsed with gentle agitation in 0.9% (w/v) NaCl solution (3 ml). Two of the four samples were selected for 8 h soaking in 0.02% or 0.0125% sodium hypochlorite solution and two control samples were soaked in distilled water for 8 h. After soaking, samples were rinsed again in 0.9% (w/v) NaCl solution (3 ml) and prepared for SEM by fixing for 2 h in 2.4% glutaraldehyde (v/v) in cacodylate buffer (pH 7.4) and then dehydrating for 15 min at each concentration in a graded ethanol series (50, 70, 90, 95 and 100 %) (Sutton et al. 1994).

The dry specimens were mounted on stubs, sputter coated with platinum in a planar magnetron sputter coater to a depth of 20 nm (Nockolds et al., 1982), and samples examined with a Jeol 35C scanning electron microscope.

The above procedures were repeated using S. gordonii LGR2 as the inoculum.

Following the protocol outlined above, another four methylmethacrylate resin specimens (1 cm x 1cm x 2mm) were autoclaved in SB (10 ml) and then inoculated with C. albicans H1 (1 ml)(1.5 x 10⁶ Candida cells/ml). The samples were then incubated for 48 h at 37°C with vibration as described above, removed and rinsed in 0.9% (w/v) NaCl solution (3 ml). After rinsing, two samples were selected for microwaving and two control samples were not microwaved. Each rinsed resin specimen was transferred to a bottle, and individually exposed to 6 min microwave radiation at medium (350 W) setting. A similar bottle containing 10 ml of water was placed in the oven together with the sample to be microwaved during all exposures. After microwaving, the samples were rinsed again in 0.9% (w/v) NaCl solution (3 ml) and prepared for SEM according to the
protocol outlined above.

The same procedures were repeated using \textit{S. gordonii} LGR2 as the inoculum.

Analysis of the data from the above assays involved the following calculations: for \textit{C. albicans} HI and \textit{S. gordonii} LGR2, the survival rate expressed as a percentage was calculated by dividing the number of cfu/ml at the specified sodium hypochlorite concentration at 8 h for the experimental group, by the corresponding values for the control group, and the result multiplied by 100. The same method was used to calculate the survival percentage for the above strains at specified microwave settings and exposure times.

4.3 RESULTS

4.3.1 Sodium hypochlorite treatment

Dentures inoculated with \textit{C. albicans} H1 or \textit{S. gordonii} LGR2 were soaked in 0.02% and 0.0125% sodium hypochlorite for 8 h. Under neither condition were viable \textit{Candida} cells detected though \textit{S. gordonii} LGR2 showed a survival rate of 3.7% for 0.02% sodium hypochlorite and 50.1% for 0.0125% hypochlorite. These data indicate that 0.02% sodium hypochlorite 8 h soak is not only candidacidal in its effect, but also results in less streptococcal survival than a similar soak in 0.0125% hypochlorite. The raw data for this experiment are given in Table 4.2.
Table 4.2. The effect of sodium hypochlorite (NaOCl) solutions 0.02% and 0.0125% on the survival of *C. albicans* H1 and *S. gordonii* LGR2, expressed as mean cfu/ml

<table>
<thead>
<tr>
<th>Hypochlorite concentration</th>
<th><em>C. albicans</em> H1 (cfu/ml)</th>
<th><em>S. gordonii</em> LGR2 (cfu/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>NaOCl 8 h soak</td>
</tr>
<tr>
<td>0.02%</td>
<td>1.4 x 10^6 (5.2 x 10^5)*</td>
<td>0</td>
</tr>
<tr>
<td>0.0125%</td>
<td>8.0 x 10^6 (2.0 x 10^6)</td>
<td>0</td>
</tr>
</tbody>
</table>

* Standard deviation in parenthesis

4.3.2 Microwave treatment

Microwaving of dentures for 10 min at low setting resulted in 100% survival of *C. albicans* H1 and consequently no further tests at low setting were carried out.

Microwaving of dentures at medium setting showed a survival rate for *C. albicans* decreasing to 1.3% at 4 min and none detectable with longer periods (Table 4.3). Not unexpectedly, the high setting proved more effective with no detectable survival of microorganisms at 2 min of microwaving (Table 4.3).

Similar overall results were obtained with *S. gordonii*, with none being detectable at 6 min of microwaving at medium setting and 2 min at high setting. There were minor differences in the survival percentages for the two microorganisms after shorter periods of microwaving.
Table 4.3. The effect of exposure time at high and medium microwave settings on the survival of *C. albicans* H1 and *S. gordonii* LGR2

<table>
<thead>
<tr>
<th>Exposure time (Min)</th>
<th><em>C. albicans</em> H1 Survival (%)</th>
<th><em>S. gordonii</em> LGR2 Survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Medium</td>
<td>High</td>
</tr>
<tr>
<td>1</td>
<td>46.4</td>
<td>11.0</td>
</tr>
<tr>
<td>2</td>
<td>22.2</td>
<td>0*</td>
</tr>
<tr>
<td>4</td>
<td>1.3</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* No detectable microorganisms

4.3.3 SEM procedures

The whole resin piece was initially scanned at low magnification (x 200) and any areas showing particulate matter were viewed under high magnification. As shown by SEM, *C. albicans* H1 readily adheres to methylmethacrylate resin (Figure 4.2) but there were no cells detectable after 0.02% hypochlorite treatment (Figure 4.3); microwaving of resin pieces at medium setting for 6 min did not, however, remove the presumably non-viable cells (Figure 4.4). Similar results were obtained with *S. gordonii* as shown by a comparison of Figures 4.5, 4.6 and 4.7.
Figure 4.2 Scanning electron micrograph showing intact *C. albicans* H1 blastospores and hyphae adherent to acrylic resin prior to treatment with sodium hypochlorite (bar = 10μm)
Figure 4.3 Scanning electron micrograph of acrylic resin formerly with *C. albicans* H1, after treatment with sodium hypochlorite (note scarcity of *C. albicans* H1 blastospores) (bar = 10μm)
Figure 4.4 Scanning electron micrograph showing damaged, non-viable C. albicans H1 blastospores and hyphae still adherent to acrylic resin after microwave treatment (bar = 10μm)
Figure 4.5 Scanning electron micrograph showing intact *S. gordonii* LGR2 adherent to acrylic resin prior to treatment with sodium hypochlorite (bar = 1μm)
Figure 4.6 Scanning electron micrograph of acrylic resin formerly with *S. gordonii* LGR2, after treatment with sodium hypochlorite (note scarcity of *S. gordonii* LGR2) (bar = 10μm)
Figure 4.7 Scanning electron micrograph showing damaged, non-viable *S. gordonii* LGR2 still adherent to acrylic resin after microwave treatment (bar = 1μm)
4.4 DISCUSSION

The study has demonstrated that 0.02% sodium hypochlorite used as a denture soak for 8 h will eliminate the growth of *C. albicans* H1 and reduce the growth of *S. gordonii* LGR2 by removing the microorganisms from the surface of the denture. It has also been demonstrated that microwaving of acrylic dentures for 6 min at medium setting will eliminate the growth of *C. albicans* H1 and *S. gordonii* LGR2, although the procedure does not remove presumably non-viable microorganisms from the denture surface.

Thus, 0.02% sodium hypochlorite used as an 8 h denture soak meets the criterion established by Minagi *et al.* (1987) (Chapter 4, 4.1), namely, that a denture cleansing agent should have a high antifungal activity for the prevention of denture stomatitis. The data concerning the use of sodium hypochlorite generally agree with those reported by previous investigators (Chapter 1, 1.6.3; Chapter 4, 4.1)

Soaking of dentures in sodium hypochlorite has not always been recommended because of the possibility of a bleaching effect on the denture base. Although some investigators have recommended methods to counteract this disadvantage (Chapter 4, 4.1), no detrimental effect on the denture base material was observed either at 0.02% or 0.0125% sodium hypochlorite, but in the long term (7+ yr) adverse effects could manifest themselves. One of the known side-effects of hypochlorite is its action on metals (McGowan *et al.*, 1988; Cawson and Spector, 1989) and although this study was confined to complete maxillary acrylic dentures, in one denture where metal pins of anterior teeth were exposed, corrosion of the pins was observed after soaking in 0.02% sodium hypochlorite.
solution.

Disinfection of acrylic dentures by microwave irradiation has been clearly demonstrated in this study, and the results generally agreed with those of Rohrer and Bulard (1985) (Chapter 1, 1,6,4). Contrary to the procedures carried out by Rohrer and Bulard (1985) (Chapter 1, 1,6,4; Chapter 4, 4.1), in this study an unmodified domestic microwave oven with a rotating table was used as this is what is commonly available, and this agreed with the views of Sanborn et al. (1982) and Griffith et al. (1993) (Chapter 4, 4.1; Table 4.1). Irrespective of the type of microwave oven used, namely, standard household or modified, previous investigators emphasized that a parallel load should be included, and in this study, this was consistently complied with. Denture base acrylic resins are transparent to microwaves, and if placed in the oven without a separate container of water (microwave absorbent) it would be equivalent to operating an empty oven with consequent damage to the magnetron. This may be the reason for the unusually negative results of Hume and Makinson (1978) (Chapter 1, 1.6.4; Chapter 4, Table 4.1), who did not mention the method of exposure, nor the inclusion of a parallel load.

Although Rohrer and Bulard (1985) recorded no dimensional changes in dentures exposed to microwaves for up to 16 min at 720 W, they were using a modified microwave oven. At lower values the dimensional changes may be of no clinical significance as shown by the microwaving of acrylic resin specimens for 3-15 min at 500 W (Polyzois et al., 1995) or acrylic dentures in an unmodified oven for 6 min at 350 W (Thomas and Webb, 1995) (4.2.3.2). A summary of eight studies by other investigators (Table 4.1) concerning disinfection of surfaces
by microwave irradiation showed that all studies, with one exception where details were not given, used a high setting for disinfection. However, this investigation has included assays at medium setting and by doing so has demonstrated an optimal microwave exposure time which ensures denture disinfection with minimal dimensional change.

SEM procedures indicated that treatment of resin specimens with 0.02% sodium hypochlorite resulted in removal of candidal cells and streptococcal cells from the surface, although total disinfection was never achieved. SEM also showed that alterations in cell morphology occurred following microwave irradiation of resin specimens, and this is consistent with the findings of Rosaspina et al. (1993) who showed that irradiation induced a progressive series of alterations which were proportional to exposure time, with the final stage being complete destruction of bacterial cells.

In conclusion, the results of this in vitro study have indicated that microwaving dentures at medium setting for 6 min may be a more effective method of controlling the growth of Candida and streptococci on denture surfaces than denture soaking for 8 h in 0.02% sodium hypochlorite. This work has been extended to a clinical study (described in Chapter 7) to test the efficacy of sodium hypochlorite denture soaking in the control of denture plaque and, ultimately, denture stomatitis. Another study (described in Chapter 8) tests the efficacy of microwave irradiation as a method of denture plaque control.