Oral Yeast Carriage and Salivary Protein Profiles in Xerostomia Subjects and in Age-and Gender-Matched Controls

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BMD BDS

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This thesis represents the culmination of work and learning that has taken place over a period of almost three years (2010 - 2012). Using saliva rinse samples collected from a group of 20 xerostomia subjects and age- and gender-matched controls, oral yeast carriage and the saliva protein profiles of those subjects were studied.

The body of this thesis is organized into seven chapters. Chapter 1 and 2 introduce the project and explore the scientific basis for the study. Following the literature review, Chapter 3 describes the methods used, including equipment and chemicals, and the experimental approaches for each study. General data for the study participants are presented in Chapter 4. The first part of the experimental study measured oral yeast carriage in xerostomia subjects and matched controls and the results are reported in Chapter 5. The second part of the study investigated the saliva protein profiles of the study participants. Initially in Chapter 6 a method for analyzing protein profiles was developed. In the second part of Chapter 6 the results of the subjects protein profile analysis are presented. Chapter 7 contains a discussion of the results obtained from the yeast and protein studies, including the conclusions, the limitations of the current project and possible future research directions.
Abstract

Aims: To investigate if there is a significant association between increased oral colonisation by yeast species, such as *C. albicans*, and a sensation of dry mouth, and if there is a difference in the saliva proteins detected in xerostomia subjects from those in age- and gender-matched controls.

Methods: This was a cross-sectional study (ethical approval: LRS/10/09/034) in which oral yeast carriage and saliva proteins were investigated in saliva rinse samples. Mouth rinses (10 ml bottled water for 30 s) were obtained from 20 individuals attending Oral Medicine clinics; inclusion criteria included self-reported dry mouth. Samples were also obtained from 20 age- and gender-matched controls. Study participants completed a medical questionnaire. Yeast numbers and species were presumptively identified using chromogenic agar plates (CHROMagar™ *Candida*). Saliva samples were subjected to SDS-PAGE analysis of protein content. Salivary proteins were visualized by two staining techniques; a Coomassie Blue-based commercial stain (EZBlue™) and the more sensitive silver stain. Protein profiles were analyzed and compared both by visual comparison to an internal standard and by using an automated image analysis system (Gel Doc™ EZ).
**Results:** Examination of the patient data revealed that there were several possible causes of the self-reported dry mouth including use of medications linked to xerostomia and denture wearing. Sixteen of the 20 dry mouth patient had a salivary flow rate \( \leq 0.1 \, \text{ml/min} \). Five patients met the criteria for Sjögren’s syndrome.

The prevalence of yeasts in saliva samples from individuals reporting a dry mouth was significantly greater \( (p < 0.05) \) than in control samples. There was also a greater number of yeast species and a greater total number of yeast cells in the xerostomia saliva samples. Interestingly, although 18/20 xerostomia subjects were colonised by yeast presumptively identified as *C. albicans* six of these subjects were also colonised by a yeast presumptive identification as *Candida glabrata*. *C. glabrata* is an emerging opportunistic pathogen, which shows inherent resistance to theazole class of antifungal drugs. There was a weak negative correlation between the number of yeast present in the rinse sample and the saliva flow rate for xerostomia subjects.

Protein profile analysis confirmed the utility of an automated image analysis system. Although the system allowed rapid comparison of gels and determination of the molecular weights of individual proteins, it had less sensitivity than use of a non-automatic digital camera and visual analysis of images. However, neither method revealed any consistent differences between the salivary protein profiles of the two subject groups.
Conclusions: The study showed that oral colonisation by yeasts in individuals with xerostomia occurred at a greater frequency than in healthy individuals and thus individuals with xerostomia may be more susceptible to oral yeast infections. No significant differences in the salivary protein profiles of xerostomia subjects and healthy controls were observed, and therefore it was not possible to determine whether salivary protein changes could be responsible for the increased prevalence of oral yeast colonisation in individuals with xerostomia. However, the study showed that factors other than low saliva flow may contribute to increased oral yeast colonisation in such individuals.

This project was supported by a University of Otago Faculty of Dentistry Fuller scholarship.
Acknowledgements

The completion of this thesis marked the end of an unforgettable journey. Thankfully, I have traveled this journey with the support and encouragement of numerous people especially my research supervisors, my family, friends and colleagues. It is a privilege to express my appreciation to all those who contributed in many ways to the success of this study and made it a valuable experience for me.

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It’s my fortune to gratefully acknowledge the support of some special individuals. I would like to pay deepest regards to my partner, my siblings and parents, for their support and constant care. They are always there for me during the happy and hard moments. A journey is different when you travel together.
Poster and Presentations

IADR New Zealand Section Poster competition

15th April 2012, School Of Dentistry, University of Otago, Dunedin, New Zealand (Appendix I)

Oral Yeast Carriage of Xerostomia Subjects and Age and Gender Matched Controls

Oral Presentations

(i) 12th July 2012 The Academy of Australian and New Zealand Prosthodontics conference, Cairns, Australia (Appendix II)

Oral yeast carriage and saliva protein profiles of xerostomia subjects and matched controls

(ii) 2nd August 2012 Research Day, School of Dentistry, University of Otago, Dunedin, New Zealand (Appendix III)

Oral yeast carriage and saliva protein profiles of xerostomia subjects and matched controls
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<td>AECG</td>
<td>American-European Consensus Group</td>
</tr>
<tr>
<td>AF</td>
<td>Antifungal</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immunodeficiency disease</td>
</tr>
<tr>
<td>ALS</td>
<td>Agglutinin-like sequence</td>
</tr>
<tr>
<td>ANA</td>
<td>Antinuclear antibody</td>
</tr>
<tr>
<td>anti-La (SS-B)</td>
<td>Anti-nuclear autoantibodies associated with Systematic Lupus Erythematosus, often present in Sjögren's syndrome</td>
</tr>
<tr>
<td>anti-Ro (SS-A)</td>
<td>Anti-nuclear autoantibodies associated with autoimmune diseases, often present in Sjögren's syndrome</td>
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<tr>
<td>bPRPs</td>
<td>Basic proline-rich proteins</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>C.</td>
<td>Candida</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming units</td>
</tr>
<tr>
<td>CBB</td>
<td>Coomassie Brilliant Blue</td>
</tr>
<tr>
<td>Cn</td>
<td>Control saliva wash sample code number n</td>
</tr>
<tr>
<td>1D</td>
<td>One-dimensional</td>
</tr>
<tr>
<td>2D</td>
<td>Two-dimensional</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DS</td>
<td>Denture stomatitis</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>e.g.</td>
<td>For example</td>
</tr>
<tr>
<td>et al.</td>
<td>et alii (and others)</td>
</tr>
<tr>
<td>EZBlue™</td>
<td>Coomassie Blue based commercial staining kit</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>Gel Doc™EZ</td>
<td>Analysis software of protein profiles: Gel Doc™. EZ system – Image Lab™</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycosylphosphatidylinositol</td>
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h  Hour
HPLC  High-performance liquid chromatography
HIV  Human immunodeficiency virus
HAART  Highly active antiretroviral therapy
HPV  Human papilloma virus
Hwp1p  Hyphal wall protein 1
IADR  International Association for Dental Research
IC  Invasive candidosis
IgA  Immunoglobulin A
kDa  Kilo Dalton
LC  Liquid chromatography
µg  Microgram
µl  Microliter
M  Marker lane
MALDI-TOF  Matrix-assisted laser desorption ionisation -Time-of-flight
min  Minute(s)
ml  Millilitre
mm  Millimetre
MS  Mass spectrometry
MW  Molecular weight
ng  Nanogram
NIH  National Institutes of Health
%  Percentage
PAGE  Polyacrylamide gel electrophoresis
PMMA  Polymethylmethacrylate
Pn  Patient saliva wash sample code number n
PRPs  Proline-rich-proteins
P-value  Estimated probability of rejecting the null hypothesis (H₀) of a study question when that hypothesis is true
r  Correlation coefficient
R²  Coefficient of determination
RDP  Removable dental prostheses
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<tbody>
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<td>SAP</td>
<td>Secreted aspartyl proteinases</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDA</td>
<td>Sabouraud dextrose agar</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>sec</td>
<td>Second</td>
</tr>
<tr>
<td>SFR</td>
<td>Salivary flow rate</td>
</tr>
<tr>
<td>SICCA</td>
<td>Sjögren's International Collaborative Clinical Alliance</td>
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<tr>
<td>sIgA</td>
<td>Secretory immunoglobulin A</td>
</tr>
<tr>
<td>SLR</td>
<td>Single lens reflex</td>
</tr>
<tr>
<td>spp.</td>
<td>Species</td>
</tr>
<tr>
<td>SS</td>
<td>Sjögren’s syndrome</td>
</tr>
<tr>
<td>SSA</td>
<td>Antibodies to Ro antigens</td>
</tr>
<tr>
<td>SSB</td>
<td>Antibodies to La antigens</td>
</tr>
<tr>
<td>St</td>
<td>Internal standard lane</td>
</tr>
<tr>
<td>SWS</td>
<td>Stimulated whole saliva</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethylethylenediamine</td>
</tr>
<tr>
<td>Tris</td>
<td>(Hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>UWS</td>
<td>Unstimulated whole saliva</td>
</tr>
<tr>
<td>V</td>
<td>Volt</td>
</tr>
<tr>
<td>w/v</td>
<td>Ratio of weight to volume</td>
</tr>
<tr>
<td>YEPD</td>
<td>Yeast extract peptone dextrose (medium)</td>
</tr>
</tbody>
</table>
Chapter 1: Introduction
1.1 Introduction

1.1.1 Background

1.1.1.1 Human saliva

The biochemical and physiochemical properties of saliva reflect the multifunctional roles of saliva in maintaining oral health. Saliva consists of water (99.5%), and proteins (0.3 %), as well as inorganic and trace components (0.2%) (Humphrey and Williamson, 2001; Van Nieuw Amerongen et al., 2004a). The proteins in saliva comprise of glycoprotein enzymes, immunoglobulin, proline-rich proteins and a range of peptides, including histatins, cystatins, and statherin (van Nieuw Amerongen et al., 2004b). Acquired salivary pellicles are present on many oral surfaces such as enamel, cementum, oral mucosa, and denture surfaces (Bennick and Cannon, 1978; Bradway et al., 1992; Edgerton et al., 1987). The salivary pellicles often consist of numerous salivary components, including mucins, glycoproteins, proline-rich proteins, histidine-rich proteins, enzymes and other host and microbial molecules (Whittaker et al., 1996). Saliva proteins contribute to salivary pellicle formation through specific adsorption onto the oral surface (Lendenmann et al., 2000), and pellicles have a different protein composition relative to whole saliva (Burgers et al., 2009; Hara and Zero, 2010).
1.1.1.2 Dry mouth (xerostomia)

Dry mouth (xerostomia) is defined as a subjective complaint of a dry mouth that usually, but not necessarily, results from a decrease in the production of saliva. Various local and systematic factors may contribute to the condition of dry mouth, including aging, certain illnesses including Sjögren’s syndrome, radiation therapy, medications and habits such as chronic mouth breathing. Dry mouth is also commonly seen in denture wearers. Denture wearers with xerostomia are believed to be more susceptible to oral candidosis. When xerostomia is associated with measurable salivary gland hypofunction, it can have severe effects on oral health. The amount and composition of secreted saliva depends on many factors, such as the flow rate, circadian rhythm effects (Dawes, 1972), the relative activities of different salivary glands (Gusman et al., 2004), and the ingestion of certain materials that can stimulate saliva flow. Saliva composition also varies with age, gender, and health status (Battino et al., 2002). Physiologically, in xerostomia subjects there is usually a marked decrease in the ability of the salivary glands to synthesize, transport and secrete saliva (Sreebny, 2004). Alternatively, xerostomia can occur because the rate of water absorption through the oral mucosa, together with evaporation of water from the oral mucosa, exceeds the salivary flow (Collins and Dawes, 1987). Despite the various causes of xerostomia, the saliva present in the mouth and the oral microenvironments of xerostomia patients are altered in comparison to that in normal healthy individuals (Calderone and Of, 2002; van den Berg et al., 2007).
1.1.1.3 C. albicans in the oral cavity

*C. albicans* is common oral commensal yeast that colonizes humans. Oral Candida carriage has been reported to be associated with a greater risk of both invasive candidosis and more frequent mucosal infections, such as oral candidosis. *C. albicans* is an opportunistic pathogen that can cause disseminated infections in immunocompromised individuals. Invasive candidosis (IC) is a leading cause of mycosis-associated mortality worldwide, and *C. albicans* remains the predominant cause of IC, accounting for over 50% of all cases (Leroy *et al.*, 2009; Lockhart *et al.*, 2009; Ruan and Hsueh, 2009). Although oral candidosis will occur in several immunocompromising conditions, such as in patients with lymphoma and in transplants recipients, it appears to be much more common in HIV-infected persons than under any other condition (Fidel, 2006). Clinically, signs and symptoms of oral candidosis can be indicative of underlying HIV infections, and in the 1980s it had a high incidence in this population, affecting 50-95% of all HIV patients at some stage of their infection (Rabeneck *et al.*, 1993). In recent years, the use of highly active antiretroviral therapy (HAART) has reduced the incidence of oral Candida infections in HIV positive patients (Kaufmann and Rosenberg, 2003). A statistically-significant link between poor oral hygiene, periodontal disease, chronic candidosis, human papilloma virus (HPV) and herpes virus infections with cancer has also been found, although the mechanisms involved are largely unknown (Meurman, 2010). Chronic Candida infection such as candida leukoplakia have been estimated to develop into carcinomas in 9–40% of cases (Hooper *et al.*, 2009). *C. albicans* cells form biofilms on the surfaces of mucosal tissues and prostheses. Infections
most often arise from the previous, asymptomatic yeast carriage rather than a \textit{de novo} infection. Thus, attachment to a surface is a critical step in fungal pathogenesis and, as all oral surfaces are bathed in saliva, saliva is an important factor in the attachment process.

There are few longitudinal studies of \textit{C. albicans} prevalence, but cross-sectional studies have shown that up to 60\% of healthy, non-hospitalized individuals may harbor \textit{C. albicans} in the oropharyngeal region (Arendorf and Walker, 1980; Brawner and Cutler, 1989). In a study of xerostomia patients, up to 92.9\% of patients with signs of dryness harbored oral yeasts (Shimizu \textit{et al.}, 2008). Adhesion is the first step in colonization, and both mucosal infections and IC usually originate from an endogenous source such as colonized oral or gut tissue (Cannon and Chaffin, 1999). \textit{C. albicans} has been found on various oral surfaces with different biochemical and biomechanical properties (Arendorf and Walker, 1980), including teeth, tongue, cheek, dental plaque and dental prostheses. In general, \textit{C. albicans} has a high potential to adhere to prosthetic materials (Blankenship and Mitchell, 2006). Once such a surface is exposed in the oral cavity, it is immediately coated with a salivary pellicle, which in turn significantly affects the process of yeast adhesion to the substratum.

In vitro studies have shown that \textit{C. albicans} adhesion to oral surfaces such as hydroxyapatite is promoted through interactions with salivary proteins that may be present in salivary pellicles (Cannon \textit{et al.}, 1995b; Samaranayake and MacFarlane, 1980). O'Sullivan \textit{et al.} (O'Sullivan \textit{et al.}, 1997) identified salivary basic proline-rich proteins as receptors for \textit{C. albicans} adhesion. Other factors in
whole saliva are reported to promote the adherence of *C. albicans* yeast cells to cultured epithelial cells (Holmes et al., 2002), and certain salivary proteins selectively absorbed to the silicone of voice prosthesis promote *C. albicans* adherence (Holmes et al., 2006b).

*C. albicans* can induce inflammatory disease of mucosal tissue (Budtz-Jorgensen, 1990). Numerous studies have demonstrated an association between *C. albicans* and denture stomatitis (Cahn, 1936; Davenport, 1970; Lehner et al., 1966), and the denture base can serve as a reservoir (Budtz-Jorgensen, 1972) of the yeast. To understand the pathogenesis of denture stomatitis, studies have evaluated mechanisms affecting *C. albicans* adhesion to surfaces (Quirynen et al., 1990), including cell surface hydrophobicity (Minagi et al., 1985) and the role of saliva (Cannon and Chaffin, 1999). Studies have investigated *C. albicans* adhesion to denture acrylic with different acrylic surface topography (Shakespeare and Verran, 1988), acrylic polymerizing methods (Moura et al., 2006), and methods of denture disinfection (Buergers et al., 2008; Samaranayake and MacFarlane, 1980). Various experimental methods have been employed to study the attachment of *C. albicans* to denture acrylic surfaces, including microscopy (McCourtie et al., 1986; Samaranayake and MacFarlane, 1980), image analysis (Busscher et al., 1997), bioluminescence cytotoxicity - a cell proliferation assay for viable eukaryotic cell quantification (Nikawa et al., 1997), and radioactive labeling of cells (Edgerton et al., 1993; Holmes et al., 1995).

While previous studies have focused on the role of reduced salivary flow in promoting yeast mucosal infection in xerostomia patients (Budtz-Jorgensen,
2000; Parvinen and Larmas, 1981; Tapper-Jones et al., 1980), the significance of altered saliva composition on *C. albicans* pathogenesis has not been investigated.

### 1.1.2 Aims

This research project consists of two cohort studies using saliva rinse samples collected from a group (20) of dry mouth patients attending the Oral Medicine Clinic and from age- and gender-matched control subjects recruited from other Departments in the School of Dentistry.

Aim 1: to compare yeast carriage in dry mouth patients and in age- and gender-matched control subjects.

Aim 2: to analyse salivary protein profiles, as measured by 1-dimensional polyacrylamide electrophoresis (PAGE), of saliva rinse samples from dry mouth patients and in age-and gender-matched control subjects.

### 1.1.3 Hypothesis

My hypothesis is that saliva composition plays a significant role in *C. albicans* colonisation and pathogenesis in dry mouth patients.

Research Hypotheses:

1. Prevalence of yeast carriage in subjects with xerostomia is significantly higher compared to that in healthy individuals.
2. Total numbers of yeast isolated from saliva rinse samples from subjects with xerostomia are significantly higher compared to those in healthy individuals

3. Saliva protein profiles of xerostomia subjects differ from those of age- and gender-matched healthy individuals

4. There are changes in saliva protein profiles that correlate to an increased oral yeast colonisation in xerostomia subjects

### 1.2 Project Outline

The project was conducted as a pilot study of a limited number of healthy subjects in order to establish methodology, followed by an extended study of 20 dry mouth subjects and 20 age- and gender-matched control subjects. During the extended stage, two linked cohort studies were carried out using standardised oral rinse samples obtained from dry mouth patients and from healthy individuals to identify and enumerate yeast present, and to analyse the component salivary proteins present (Figure 1.1). The salivary proteins were analysed with the Molecular Imager® Gel Doc Image lab system from Bio-Rad and with calibrated visual identification by three independent researchers.
Chapter 1. Introduction

Project Outline

Figure 1.1. Project outline.

1.2.1 Pilot study

In 2010 a pilot study using saliva from healthy individuals was conducted to validate the planned experimental approaches, under ethical approval (OTA/02/05/034). The following experimental procedures were tested:

- Saliva rinse sample collection (from 7 healthy volunteers)
- Yeast species identification and enumeration
- Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of saliva rinse samples
1.2.2 Extended study

1.2.2.1 Dry mouth patient sampling

An application was submitted to the Lower South Regional Ethics Committee for ethical approval of the study involving dry mouth patients, for sample collection beginning in November 2010. The ethical approval (LRS/10/09/034 - Appendix IV) and consent was obtained prior to the sample collection.

1.2.3 Experimental procedures

The experimental procedures developed in the pilot study were applied to the saliva rinse samples. The oral yeast carriage and saliva protein profiles of the dry mouth subjects and age- and gender-matched controls were determined.
Chapter 2: Literature Review

Oral Yeast Carriage and Saliva Protein Profiles of Xerostomia Subjects and Matched Controls
2.1 *C. albicans* and candidosis

2.1.1 *C. albicans* and *C. albicans* infection

*C. albicans* is a polymorphic fungus. Few fungi are true pathogens, capable of infecting healthy individuals. Several fungi, however, including *C. albicans*, are opportunist pathogens, that are normally well-tolerated by healthy individuals, only causing disease in hosts with compromised conditions (Figure 2.1)

![Figure 2.1](image)

Figure 2.1. *C. albicans* as commensal microorganism and pathogen, partially adapted from Holmes and Cannon (unpublished work).

*C. albicans* is the fungus most commonly detected in association with humans (Douglas, 2003), and it is found on the skin, gastrointestinal tract, and genitourinary tract (Budtz-Jorgensen, 1990). The pathogenic effects of *C. albicans* have been recognized since the nineteenth century (Barnett, 2008), and the association with prosthesis wearing has been reported since 1930’s (Cahn, 1936). *C. albicans* is often isolated from the oral cavity (Rossie and Guggenheimer, 1997) and is the species most frequently associated with oral diseases. Although it is rare, *C. albicans* is one of the leading causes (fourth most
common) of mortality due to systemic infections (Harriott and Noverr, 2009; Meiller et al., 2009).

Martin (Martin et al., 2003) conducted a longitudinal study by reviewing discharge data from approximately 750 million hospitalizations in the United States over the 22-year period 1979-2000. The author reported the number of cases of sepsis caused by fungi increased more than three times over this period. Epidemiological studies in different countries show a worldwide trend: that the main agent recovered in candidemia is \textit{C. albicans}. It has been reported that about 59% of the blood culture samples tested positive for yeast (Guery et al., 2009).

\textit{C. albicans} is the most common fungal species isolated from patients with device-related candidosis, and it has been shown to form biofilms on both superficial and invasive devices (Budtz-Jorgensen, 1972; Holmes et al., 2006a; Ramage et al., 2004). A large number of nosocomial infections caused by \textit{Candida} species derive from the use of different types of medical catheters and prostheses, including dentures via denture plaque as shown in Figure 2 (Budtz-Jorgensen, 1990; Fridkin and Jarvis, 1996; Odds, 1994). Furthermore, the organisms adhering to those surfaces may also be less susceptible to antifungal drugs (Akins, 2005; Chandra et al., 2001; Kalya and Ahearne, 1995) and the colonization can contribute to the deterioration of the prostheses (Busscher et al., 1997; Elving et al., 2000; van Weissenbruch et al., 1997). The release of microorganisms from biofilms is a factor contributing to the increased incidence
of acute disseminated nosocomial infections in the last decade (Ramage et al., 2009; von Fraunhofer and Loewy, 2009).

The mucosal infection, oral candidosis, is the most common fungal infection of the oral cavity (Davis, 2009). Many factors can increase susceptibility to oral candidosis. These factors include immunosuppression, endocrinopathies, nutritional deficiencies, medications, malignancies, dental prostheses, epithelial alterations, salivary changes (quantitative or qualitative), a high carbohydrate diet, age, poor oral hygiene, and a history of smoking. *C. albicans* characteristics, such as growth as mycelia, the production of proteinases, and the ability to adhere to surfaces or persist in the oral cavity, influence the host-pathogen relationship. In the years before the advent of highly active antiretroviral therapy (HAART), AIDS patients often suffered severe oral and pharyngeal candidosis (Fidel, 2006) and *C. albicans* oral infection remains a serious issue for AIDS patients.

Clinically, oral candidosis can be asymptomatic, but patients may complain of an altered taste sensation or dysphagia. Oral *Candida* infections can be classified as follows (Table 2.1)
Table 2.1. Description of types of oral candidosis (Cannon and Firth, 2006).

<table>
<thead>
<tr>
<th>Candida Infection</th>
<th>Clinical presentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute pseudomembranous</td>
<td>Multiple removable white plaques</td>
</tr>
<tr>
<td>Acute erythematous</td>
<td>Generalized redness of tissue</td>
</tr>
<tr>
<td>Chronic plaque like/nodular</td>
<td>Fixed white plaques on commissures</td>
</tr>
<tr>
<td>Chronic erythematous</td>
<td>Generalized redness of tissue on fitting surface of upper denture</td>
</tr>
<tr>
<td>Chronic pseudomembranous</td>
<td>Multiple removable white plaques</td>
</tr>
<tr>
<td>Candida-associated angular cheilitis</td>
<td>Bilateral cracks at angles of mouth</td>
</tr>
<tr>
<td>Median rhomboid glossitis</td>
<td>Fixed red/white lesion on dorsum of tongue</td>
</tr>
</tbody>
</table>

2.1.2 Factors contributing to infections by *C. albicans*

The ability of *C. albicans* to cause disease involves host recognition; binding of the organism to host cells, host cell defence proteins or microbial competitors that affect clearance by the host. Although the main factors that determine whether *C. albicans* causes an infection are host-related, several characteristics, so-called virulence factors, of *C. albicans* can also contribute to the development of an infection from an asymptomatic colonising strain. The morphological variations of *C. albicans* enable it to grow as hyphae and may enable the fungus to penetrate between epithelial cells. *C. albicans* cell-surface properties permit this microorganism to colonize various surfaces, interact with host tissues and with other microorganisms within biofilms. The ability of *C.
*C. albicans* to adhere to a wide range of surfaces and co-aggregate with other microorganisms increases its resistance to clearance by the host. Biofilms are often also resistant to the effects of antifungal drug therapy which poses clinical challenges in the management of *C. albicans* infections. The factors that contribute to the development of a *C. albicans* infection include adhesion, hyphal formation, phenotypic switching and extracellular hydrolytic enzyme production (Figure 2.3) (Cho, 2009; Pitarch *et al.*, 2006)

![Figure 2.3. *C. albicans* virulence factors. Schematic drawing based on (Cho, 2009).](image-url)
2.1.3 Polymorphism

Although *C. albicans* is often referred to as a dimorphic fungus, as it exists predominantly as yeast or hyphae, it is in fact polymorphic (Figure 2.4). As shown in Figure 2.4, *C. albicans* can undergo morphological transitions between ovoid, unicellular budding cells (yeast cells or blastopores) (A) and chains of filamentous, hyphal cells (B). Growth in a morphology known as pseudohyphae (C) can also occur. Although the degree of elongation of pseudohyphal cells can vary considerably, from relatively short to significant extended cells, they always display constrictions at their septa between individual cellular compartments. In contrast, true hyphae and their progenitors (germ tubes) show no constrictions, having parallel walls at their septa (Calderone and Of, 2002). Another morphology is a spore-like form known as chlamydomospores.

![Figure 2.4. C. albicans polymorphism (schematic drawing based on (Calderone and Of, 2002))](image)
These morphological transitions often reflect a response of *C. albicans* to environmental and microenvironmental changes. Under the conditions of physiological temperature (37°C), pH equal to or greater than 7.0, CO₂ concentration of 5.5% or the presence of serum, hyphal growth is stimulated. Growth in the yeast morphology is stimulated by lower temperatures, acidic pH, and absence of serum and high concentrations of glucose. Yeast cells are thought to be responsible for dissemination in the environment and finding new hosts, while the virulence of hyphae is suggested by their ability to penetrate deeper into tissues than the yeast form in superficial candidosis. The ability of *C. albicans* to undergo reversible morphological transitions between ovoid, unicellular budding cells and different degrees of filamentous growth could enhance survivability within the host and contribute to its virulence (Calderone et al., 2000).

### 2.1.4 Phenotypic switching of *C. albicans*

Phenotypic switching involves reversible and heritable switching between alternative cellular phenotypes, often distinguished by differences in microscopic and macroscopic morphology. In the white-opaque transition of the opportunistic pathogen *C. albicans*, growth as smooth, white colonies switches to growth as flat, grey (opaque) colonies. Several differences exist between the yeast from these two colony types, including cell shape (white cells are round or ovoid and opaque cells are elongated or bean-shaped), cell surface structures (pimples are found on opaque cells only) and production of germ tubes at 37°C, pH 6.7 by white cells but not by opaque cells, unless the opaque cells are grown on human
epithelial cells (Slutsky et al., 1987; Soll, 1992; 2009). These differences affect *C. albicans* pathogenicity, mating efficiency, and biofilm formation (Bennett et al., 2005; Lohse and Johnson, 2009). White and opaque cells have also been shown to differ in their interaction with immune cells, suggesting that white-opaque switching may be an adaptive mechanism to help *C. albicans* cells escape the attention of the host immune system (Lohse and Johnson, 2008).

### 2.1.5 *Candida* biofilms

One distinguishing feature of *C. albicans* biofilms is the variety of morphological forms present. Fully matured biofilms, consist of a dense network of yeast, hyphae and pesudohyphae (Hawser et al., 1998). A specific characteristic of a biofilm is the formation of a matrix of extracellular polymeric material, produced by microorganisms, and in which they exist. There are numerous factors affecting *Candida* biofilm formation, such as the *Candida* species and strains involved (Kuhn et al., 2002; Shin et al., 2002), characteristics of the colonized surface (Hannig and Hannig, 2009), salivary flow (Baillie and Douglas, 1999; Hawser et al., 1998), and presence of bacteria (Adam et al., 2002; Jenkinson and Douglas, 2002). It has been found that *C. albicans* isolates consistently produce more biofilm than other *Candida* species (Kuhn et al., 2002). Biofilm formation was slightly increased on latex or silicone elastomer, compared with polyvinylchloride (PVC), but decreased on polyurethane or 100% silicone (Hawser and Douglas, 1994). The conditioning of these surfaces with fibrinogen or collagen enhanced the formation of biofilms by *C. albicans*, and similarly
conditioning films of serum or saliva promoted biofilm formation on denture acrylic (Chandra et al., 2001; Nikawa et al., 1996).

The ability of *C. albicans* to form biofilms on various surfaces has been documented in several studies: (i) *Candida* biofilms were visualized using various methods, such as scanning electron microscopy (Ramage et al., 2004). The biofilms consisted of intricate networks of yeast cells and hyphae deeply embedded into imperfections, cracks, and crevices of the biomaterials of denture samples from patients with denture stomatitis (Ramage et al., 2004); (ii) in a mixed biofilm of bacteria and yeast from voice prosthesis, the predominant microorganism has been identified as *C. albicans* (Elving et al., 2003); (iii) the presence of *Candida* in a biofilm was detected in periodontal tissue (Jarvensivu et al., 2004), including apical periodontal tissue (Waltimo et al., 2003); and (iv) *C. albicans* co-aggregated with oral bacterial on saliva-coated surfaces (Holmes et al., 1995).

Bacteria are often found together with *Candida* species in polymicrobial biofilms *in vivo* suggesting that complex interspecies interactions take place within biofilms. Co-aggregation may play a significant role in the colonization of the mucosal and hard tissue surfaces in the oral cavity by yeast (Kolenbrander et al., 2010). A significant characteristic of microbial biofilms is their innate resistance to antifungal drugs. For example, when bacteria grow as a biofilm form they are 10-1000 times more resistant to antibiotics than are planktonic cells (Donlan, 2001). The mechanisms of antifungal resistance by *Candida* biofilms are not known, although several factors may contribute to the resistance: these
include metabolic activity of biofilm cells, extracellular polymeric matrix materials, expression of resistance genes, and the presence of a subtype of cells with a resistant phenotype (persister cells) (Douglas, 2003; Niimi et al., 2010) (Figure 2.5).

![Figure 2.5. Interactions between fungi and other microorganisms within biofilms leads to antifungal drug resistance (Niimi et al., 2010).](image)

Drug resistance of Candida biofilms was also found in in vitro experiments (Chandra et al., 2001; Hawser and Douglas, 1995). A study of a mixed species biofilm (Adam et al., 2002) indicated that the presence of C. albicans in a biofilm increased the resistance of slime-negative staphylococci to vancomycin, and that C. albicans resistance to fluconazole was enhanced in the presence of slime-producing staphylococci. A similar observation has been made with biofilms consisting of C. albicans and streptococci on denture acrylic (Jenkinson and Douglas, 2002). Other studies have demonstrated the drug resistance of Candida biofilms on a variety of surfaces including polyurethane,
silicone elastomeric and denture materials (Chandra et al., 2001; Mattos et al., 2009; von Fraunhofer and Loewy, 2009).

2.1.6 Cell surface

The cell wall is essential both to the biology of the microorganism and its interaction with the host tissue in health and disease. The *C. albicans* cell wall makes up approximately 30% of the dry weight of the cell (Sullivan et al., 1983). A number of studies have shown that the cell wall is composed of mannoproteins (20%-30%), β-1, 3-D-glucans (25%-35%), β-1,6-D-glucans (35%-45%) (Fukazawa and Kagaya, 1997), proteins (6%-25%) (Chaffin, 2008), and a small amount of chitin and lipids (Chaffin, 2008). The surface of the organism is the site of interaction between host and fungal proteins, and with the immune system. These interactions lead to either clearance, colonisation, or infection (Cannon and Chaffin, 1999). Several glycoproteins are located on the exterior of the cell wall, many of which are involved in these interactions with the immune system (Chaffin, 2008). In addition, a number of hydrolytic enzymes can be recovered from the *C. albicans* cell wall, and may also be secreted. These may damage host tissues and may also help provide nutrients (Calderone et al., 2000; Chaffin, 2008). Changes in the cell wall also ultimately determine the cell morphology, yeast, hyphal or pseudohyphal, which also contributes to the virulence of *C. albicans* as described previously.
2.1.7 *Candida* adhesion

The adhesion of *C. albicans* to surfaces appears to be a dynamic process, regulated by host environmental conditions and the phenotypic state of the organism (Cho, 2009; Kennedy *et al.*, 1989; McCourtie and Douglas, 1984). The *C. albicans* cell wall, cell morphology and the formation of biofilms, all contribute to the adhesion of *C. albicans* to various surfaces in the oral environment. Both mucosal and systemic candidosis usually originates from an endogenous source such as colonized oral or gut tissue (Cannon and Chaffin, 1999). Adhesion is the crucial first step for *Candida* survival and proliferation in the oral cavity (Cannon *et al.*, 1995a). Several surface proteins of *C. albicans* have been identified as adhesins. A major group of *C. albicans* adhesins is encoded by the *ALS* (agglutinin-like sequence) gene family (Figure 2.6). This family contains eight glycosylphosphatidylinositol (GPI)-linked cell surface proteins that mediate binding to diverse host substrates (Braun *et al.*, 2005; Zhao *et al.*, 2004). Each Als protein has an N-terminal domain that contains the substrate binding region, a C-terminal domain rich in serine and threonine, and a GPI anchoring sequence that is predicted to be cleaved as the protein is exported to the cell surface (Zhu and Filler, 2010). Hwp1p (hyphal wall protein 1) is a GPI protein that is expressed on the surface of *C. albicans* hyphae and mediates adherence to oral epithelial cells by a unique mechanism. The N-terminal region of Hwp1p functions as a substrate for epithelial cell-associated transglutaminases that covalently link it to other proteins on the surface of epithelial cells such as
human buccal epithelium cells (Sundstrom, 2002). The usual substrates for these transglutaminases are small proline-rich proteins. Thus, Hwp1 mediates epithelial cell adherence by functionally mimicking host cell proteins (Ponniah et al., 2007; Staab et al., 1999).

Figure 2.6. Adhesive factors in *C. albicans*. Schematic drawing based on (Cho, 2009).

The existence of several cell wall proteins with adhesin properties, such as Als proteins and Hwp1p, suggests that surface proteins with different specificities are necessary to adhere to different surfaces/tissues. More work is also needed in understanding the host factors that participate in *C. albicans* adhesion and the host responses to the presence of adherent organisms. The host protein(s) participating in cross-links to Hwp1p remain unknown (Sundstrom, 2002).
2.1.8 Secreted proteinases of *C. albicans*

Proteinases possess multiple functions ranging from regulation of cellular processes to non-specific degradation of proteins for the recycling of biomolecules. Extracellular proteolytic activity plays a central role in *Candida* pathogenicity and is carried out by a family of 10 secreted aspartyl proteinases (Sap proteins) (Naglik *et al.*, 2003). Saps have been shown to contribute to the *C. albicans* infection process, including mucosal and systemic infections (Figure 2.7) (Albrecht *et al.*, 2006).

![Figure 2.7. Possible roles of Saps in *C. albicans* pathogenicity (schematic drawing based on (Naglik *et al.*, 2004))](image)

Although the consequences of proteinase secretion during human infections is not known precisely, both animal and human studies have implicated the proteinases in *C. albicans* virulence in one of the following seven ways (Naglik *et al.*, 2004), by showing: (i) a correlation between Sap production *in vitro*
and *Candida* virulence, (ii) Sap substrate specificity includes human proteins, (iii) an association of Sap production with other virulence processes of *C. albicans*, (iv) *SAP* gene expression during *Candida* infections, (v) Sap protein production and Sap immune responses in animal and human infections, (vi) modulation of *C. albicans* virulence by aspartyl proteinase inhibitors, and (vii) *SAP*-disrupted *C. albicans* mutants have reduced virulence. The function of Saps in digesting or disrupting host cells during the infective process may provide a way to obtain nutrients, to facilitate adhesion and tissue invasion, and to avoid or resist antimicrobial attack by the host (Naglik *et al.*, 2003).

### 2.1.9 *C. albicans* as a major cause of denture stomatitis.

The etiology of denture stomatitis is multifactorial, involving both local and systemic predisposing factors. It was observed more than 100 years ago, as “sore mouth under plates” (Black, 1885). Black detected acidogenic microorganisms from the fitting surface of dentures and corresponding mucosa as a causative factor of this pathology (Black, 1886). Subsequently, in 1936, Cahn suggested *C. albicans* as a cause of denture-induced stomatitis (Cahn, 1936).

The prevalence of denture stomatitis has been shown to vary between 22.5% and 65% among denture wearers (Budtz-Jorgensen *et al.*, 1975; Moskona and Kaplan, 1992). The populations sampled, diagnostic criteria, and methodology used in these different studies are probably responsible for the wide range in prevalence rates. Plaque accumulation on the fitting surface of
removable dentures plays a critical role in promoting a switch from a commensal to a pathogenic oral flora (Budtz-Jorgensen, 1972).

2.2 Salivary factors

2.2.1 Saliva and salivary glands

Saliva is critical to the preservation and maintenance of oral and general health. Whole saliva is a complex mixture of fluids from major and minor salivary glands and from gingival crevicular fluid, and also contains oral microbes and food debris. Many oral and systemic conditions can manifest themselves as changes in the flow and composition of saliva (Kaufman and Lamster, 2002).

The major salivary glands include the paired parotid glands, which are situated in the parotid space posterior to the mandibular ramus and anterior inferior to the ear; the submandibular and sublingual glands, which are found in the floor of the mouth, inferior and posterior to the body of mandible. Minor salivary glands are found in the lower lip, tongue, palate, cheeks, and pharynx. Salivary glands consist of highly specialised epithelial-derived cells with two fairly well-defined morphological and functional segments, the acinar cells at the secretory end of the gland and a system of duct glandular cells of varying complexity. Essentially, there are two stages in salivary fluid secretion. Initial secretion of an aqueous plasma-like primary fluid by the acinar cells and its subsequent modification during passage through the water-impermeable ductal cell system. The acinar cells produce and secrete 85-90% of salivary proteins (Baum, 1989). The second stage of saliva secretion is fluid modification within
the ductal region. In this segment of the gland there is considerable electrolyte reabsorption, rendering the saliva hypotonic, as well as the secretion of additional exocrine proteins.

### 2.2.2 Salivary flow rate

At rest, without exogenous or pharmacological stimulation, there is a small, continuous salivary flow, termed basal unstimulated secretion, which forms a film that covers, moisturizes, and lubricates the oral tissues. In contrast, stimulated saliva is produced at a greater rate as a result of mechanical, gustatory, olfactory, or pharmacological stimulus, and contributes around 80-90% of daily saliva production. Other factors that have an impact on secretion include pain, certain medications and several local and systemic diseases affecting the glands (Shannon, 1972).

The salivary flow rate and saliva composition vary between different salivary glands. The percentage contributions of the different salivary glands to whole unstimulated saliva are as follows: 20% from parotid, 60% from submandibular, 7-8% from sublingual, and minor mucous glands contribute less than 10%. When the flow is stimulated, the parotid glands contribute more than 50% of total salivary volume (Dawes and Ong, 1973; Edgar, 1998; Schneyer, 1955; Schneyer and Levin, 1955), and the submandibular contribution accounts for about 30% (Ferguson, 1975; Schneyer, 1955)(Figure 2.8).
Chapter 2. Literature Review

Figure 2.8. The major salivary glands.

The positions of the major salivary glands (Lee et al., 2009) are superimposed on a photograph of one of my patients seen in a prosthodontic clinic at University Of Otago, School of Dentistry. Variations in salivary flow can be affected, reversibly or irreversibly, by numerous physiological and pathological factors, including the degree of hydration, body position, exposure to light, previous stimulation, circadian (daily) and circannual (annual) rhythms, gland size and drug use (Dawes, 1987). Total saliva flow rate ranges from 0.03 ml/min to 7 ml/min (Edgar, 1998) with an average daily flow of whole saliva about 500 ml - 1.5 L in healthy people (Humphrey and Williamson, 2001). An unstimulated flow rate $\leq 0.1$ ml/min (Sreebny and Valdini, 1987) or a reduction of 50% base flow rate in an individual should be considered hypofunction (Edgar, 1990; Ship et al., 1991) (Table 2.2). It must be emphasized that there is great individual variability in response to salivary stimulation, and thus the secretion of saliva. The salivary flow rate can vary on a daily and circannual basis. The circadian flow rate is low during sleep.
Chapter 2. Literature Review

(Dawes, 1972); the circannual flow is low during the summer and reaches the highest values in winter (Edgar, 1990; Shannon, 1972).

Table 2.2. Salivary flow rates (Edgar, 1990).

<table>
<thead>
<tr>
<th></th>
<th>Normal Saliva Flow Rate (ml/min)</th>
<th>Abnormal Saliva Flow Rate (ml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstimulated Whole Saliva</td>
<td>0.3-0.4</td>
<td>≤0.1</td>
</tr>
<tr>
<td>Stimulated Whole Saliva</td>
<td>1-3</td>
<td>≤0.5</td>
</tr>
</tbody>
</table>

Decreased salivary flow results in clinically significant oral discomfort, and may result in increased caries, susceptibility to oral candidosis, or altered taste sensation (Mese and Matsuo, 2007).

2.2.3 Salivary components and function

Whole saliva contains the combined fluids and solids present in the oral cavity, which is formed primarily from salivary gland secretions, fluids from gingival cevices, food remnants, exfoliated oral tissue cells and large numbers of microorganisms, particularly bacteria (Vitorino et al., 2004). A variety of electrolytes, including sodium, potassium, calcium, magnesium, bicarbonate, and phosphates ions can be found in saliva, as well as immunoglobulins, proteins, enzymes, mucins, and nitrogenous products, such as urea and ammonia.

Saliva has many essential functions. As the first digestive fluid in the alimentary canal, saliva is secreted in response to food, assisting intake and initiating the digestion of starch and lipids. During this process, saliva acts as a solvent of taste substances and affects taste sensitivity. Clinically, a more
important role is in the maintenance of oral health, including the protection of
teeth and mucosa from infections, maintenance of the milieu of taste receptors,
and communication ability through speech. The ways salivary components affect
salivary functions are summarised as follows (and see Figure 2.9).

(1) Antibacterial, antifungal, antiviral functions are provided by
immunoglobulins, cystatins, mucins, histatins, and enzymes. Proteins and mucins
serve to aggregate, and/or attach to oral microorganisms and help clear
microorganisms from the mouth in combination with saliva flow, providing
mucosal protection (Amerongen and Veerman, 2002; Kaplan and Baum, 1993).

(2) Buffering action and lubrication: bicarbonates, phosphates, and proteins act to
modulate the pH and the buffering capacity of saliva; proline-rich glycoproteins
and mucins together with water act to lubricate oral surfaces;

(3) Protection of teeth: calcium, phosphate, and proteins work together to
modulate demineralization and remineralization;

(4) Food digestion, taste: enzymes help digest food, water solubilizes chemicals
that can be detected by taste buds, and mucins help form food boluses.
2.2.4 Salivary proteins and \textit{C. albicans} colonization of the oral cavity

Saliva has a defensive role in the oral cavity that is both non-specific and targeted. Saliva contributes to physical removal of potential pathogens and also has antimicrobial properties through the presence of innate defense molecules, such as lysozyme, histatin, lactoferrin, calprotectin and IgA (Dodds \textit{et al.}, 2005) some of which can kill \textit{C. albicans} cells. Saliva also provides water, nutrients and adherence factors that permit microbial growth (Marcotte and Lavoie, 1998). Thus the multifunctional components of saliva could contribute to the dual effects, both countering and supporting adhesion of \textit{C. albicans} to oral surfaces.

Selective adsorption to oral surfaces of proteins from saliva has been demonstrated \textit{in vivo}; (Edgerton and Levine, 1992; Holmes \textit{et al.}, 2006b) for tooth surfaces (Edgerton and Levine, 1992) oral epithelial tissue (Holmes \textit{et al.}, 2002),
denture base materials (Edgerton et al., 1987), titanium (Kohavi et al., 1995) and for nickel chromium alloy (Ozden et al., 2002). Different salivary components were detected in vivo on different restorative materials (Edgerton and Levine, 1992). In one study, cystatin, proline-rich proteins (PRPs) and low-molecular weight mucin were not detected in denture pellicles by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), whereas, amylase, lysozyme, sIgA, albumin and high molecular weight mucin were present (Edgerton and Levine, 1992). In contrast, fractionation of saliva revealed that basic proline-rich proteins (bPRPs) and statherin promote C. albicans binding to hydroxyapatite beads (O'Sullivan et al., 1997) and parotid saliva that is rich in bPRPs significantly increased the binding of C. albicans cells to denture acrylic compared to submandibular/sublingual saliva (Vasilas et al., 1992). A number of PRPs, mostly acidic PRPs, have been described and implicated in the binding of some bacteria to pellicles (Douglas, 1994). Binding of radiolabelled yeast cells to membranes containing electrophoretically separated saliva proteins, identified four major reactive species (O'Sullivan et al., 1997). Purification and partial-sequence analysis of these proteins indicated that they were bPRPs. Histatin-5 exhibits antifungal activities against biofilms of C. albicans and C. glabrata developed on denture acrylic (Konopka et al., 2010), and histatin-5 has been proposed to play a protective roles in oral cavity against C. albicans (Peters et al., 2010). Mucins in human submandibular and sublingual saliva bind to both yeast cells and to germ tubes, as detected by their presence in an extract of saliva-incubated Candida cells (Edgerton et al., 1993). Mucins may enhance C.
*albicans* accumulation (Arendorf and Walker, 1987), while albumin is thought not to be involved in the adhesion process (Burgers *et al*., 2010).

### 2.3 Dry mouth (xerostomia) and hyposalivation

Dry mouth is characterized by a subjective complaint of having a dry mouth. This symptom may or may not be associated with salivary hypofunction - a measurable decrease in salivary flow. Therefore the degree of the dry mouth and salivary hyposalivation do not always correlate (Fox *et al*., 1987). The term “xerostomia” and “dry mouth” are used interchangeably in the literature. Xerostomia is a medical term; dry mouth is often used both in the literature and by lay-people as a descriptive term of the associated complaint or discomfort. For that reason, both the term “dry mouth” and “xerostomia” will be used in the following sections.

#### 2.3.1 Etiology of dry mouth

Dry mouth can be associated with systemic diseases, induced by salivary gland dysfunction, as well as being a side-effect of radiation or frequent drug use (Table 2.3). The most common cause is the use of certain systemic medications, which put the elderly at greater risk because they are usually more medicated. Other causes include high doses of radiation and diseases such as Sjögren's syndrome (Amerongen and Veerman, 2002; Mese and Matsuo, 2007).
Table 2.3. Causes of dry mouth. Based on (Napeóas et al., 2009).

<table>
<thead>
<tr>
<th>Causes of complaints of dry mouth</th>
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</thead>
<tbody>
<tr>
<td><strong>Salivary diseases</strong></td>
</tr>
<tr>
<td>Sjögren’s syndrome</td>
</tr>
<tr>
<td>Autoimmune disorder (e.g. rheumatoid arthritis)</td>
</tr>
<tr>
<td>Diabetics</td>
</tr>
<tr>
<td>HIV</td>
</tr>
<tr>
<td>Hepatitis</td>
</tr>
<tr>
<td>End stage renal dysfunction</td>
</tr>
<tr>
<td>Salivary gland tumor</td>
</tr>
<tr>
<td>Salivary trauma</td>
</tr>
<tr>
<td><strong>Side effects of therapeutic intervention</strong></td>
</tr>
<tr>
<td>Radiotherapy</td>
</tr>
<tr>
<td>Medications</td>
</tr>
<tr>
<td>Antihypertensive</td>
</tr>
<tr>
<td>Antidepressant (tricyclic)</td>
</tr>
<tr>
<td>Antispasmodic</td>
</tr>
<tr>
<td><strong>Non-salivary</strong></td>
</tr>
<tr>
<td>Dehydration</td>
</tr>
<tr>
<td>Cognitive alteration and psychological</td>
</tr>
<tr>
<td>Oral sensory dysfunction</td>
</tr>
<tr>
<td>Mouth breathing</td>
</tr>
<tr>
<td>Nutritional deficiencies</td>
</tr>
</tbody>
</table>

The effects of dry mouth vary, depending on the cause. The lower the secretion rate of whole saliva, the thinner the film of residual saliva covering the oral mucosa surfaces (Wolff and Kleinberg, 1998). It has been reported that the protein concentration in unstimulated whole saliva and residual saliva in hyposalivators is higher than those in normal salivators, a difference that was more apparent in patients with severe dry mouth symptoms (Lee et al., 2002).
2.3.2 Dry mouth and medication

The most common cause of dry mouth is the use of medications. Many medications can lead to dry mouth, however only a few have been tested for actual effects on salivary flow (Napeòas et al., 2009). Among those medications, tricyclic antidepressants, antihypertensive medications, medications with anticholinergic activities are certainly implicated in both xerostomia and hyposalivation (Amerongen and Veerman, 2002; Atkinson et al., 2005). A study by Osterberg and colleagues showed that the complaint of dry mouth was positively correlated with the intake of anticholinergic drugs, antihistamines, sedatives and hypnotics (Osterberg et al., 1984). Moreover, the feeling of dryness increased with the number of drugs taken in combination. The condition of dry mouth may also be caused by other factors, including hyposalivation (Sreebny and Valdini, 1988), sialochemistry alterations (Fox et al., 1985) and changes in oral moisture perception (Fox et al., 1987). A distinction should be made between medications that cause dry mouth and those that cause hyposalivation, as a subjective complaint of dry mouth does not always correlate to a reduced salivary flow. The variety of medications that cause dry mouth and the variety of medical conditions in these patients necessitating the medication complicates analysis of dry mouth in these patients.
2.3.3 Dry mouth and head/neck radiation therapy

Radiotherapy plays an important role in the management of patients with head and neck cancer. The majority of invasive head and neck cancers will need radiotherapy as a primary treatment, as an adjunct to surgery, or in combination with chemotherapy (Dobbs et al., 2009). The fundamental aim of the radiotherapy is the local control of the tumor. Although thorough protocols have been developed to minimize the oral side-effects of radiotherapy of the head and neck region (Schiodt and Hermund, 2002), the consequences of radiation-induced salivary gland injury and the other oral side-effects of head and neck radiotherapy are still difficult to manage. Salivary glands are radiosensitive - the radiation can cause acinar cell atrophy and chronic inflammation of the salivary glands, which results in varying degrees of temporary or permanent dry mouth (Vissink et al., 2003). The extent of radiation-induced salivary dysfunction primarily depends on the radiation dose and field. In patients receiving radiotherapy, both resting and stimulated flow rates can be significantly impaired (Valdez et al., 1993). A significant correlation between the salivary flow rate and Candida colonization and clinical presentation was detected (Mann–Whitney test, p=0.031) in a recent study (Karbach et al., 2012). The most severe form of dry mouth is that in which salivary glands are damaged by radiation therapy used to treat malignancies, and where salivary glands can not be excluded from the field of radiation (Sennhenn-Kirchner et al., 2009).
2.3.4 Dry mouth and systemic diseases

A number of systemic conditions predispose individuals to dry mouth; among these Sjögren’s syndrome is one of the most commonly reported (Boutsi, 2000). Sjögren’s syndrome is a chronic autoimmune disease characterized by lymphocytic infiltration of the exocrine glands, especially the lacrimal and salivary glands. In patients with Sjögren’s syndrome, in addition to dry mouth, they may also have symptoms of dry eyes. This syndrome is classified into primary Sjögren’s syndrome, with symptoms primarily including dry mouth and dry eyes, and secondary Sjögren’s syndrome, when dry mouth is associated with other connective tissue diseases such as rheumatoid arthritis (Fox, 2003). One of the main diagnostic criteria for Sjögren’s syndrome is acinar cell atrophy observed in salivary gland biopsies.

Diabetes mellitus is another risk factor for developing dry mouth. Diabetes mellitus consists of a group of disorders, which are characterized by a lack of insulin production or insulin resistance. Patients suffering from diabetes, especially those who have poor glycemic control, are more susceptible to a decreased normal salivary flow rate, and to complain of dry mouth (Mattson and Cerutis, 2001).

Other diseases that cause a dysfunction of the immune system, such as HIV/AIDS, as well as hepatitis, uncontrolled hypertension, and severe renal disease all can contribute to dry mouth through the disease itself or in combination with the side effects of medications (NapeÔas et al., 2009).
2.3.5 Sjögren's syndrome overview

Sjögren's syndrome is a common autoimmune rheumatic disease. Prevalence of the disease is second to rheumatoid arthritis, with about 1% (1:100) of the population affected. Sjögren's syndrome is more commonly found in middle-aged women than in men, with a ratio of 9:1 (Hammi et al., 2005). The most common symptoms of Sjögren's syndrome are extreme tiredness, dry eyes, and dry mouth. These latter two symptoms occur due to the reduction of flow in the salivary and lacrimal glands. Other symptoms associated with the disease include swelling of the salivary glands, sialorrhea, and tooth loss (Mignogna et al., 2005). Sjögren's syndrome also has an impact on the peripheral and central nervous system, as well as affecting critical areas such as muscles, bone marrow, joints, kidneys, pancreas, and other organs (Tzioufas and Vlachoyiannopoulos, 2012). When Sjögren's syndrome appears alone, it is diagnosed as primary Sjögren's syndrome. When it occurs alongside another autoimmune disease, such as rheumatoid arthritis or systemic lupus erythematosus, it is then diagnosed as secondary Sjögren's syndrome. Along with Sjögren's syndrome, individuals may experience other diseases such as fibromyalgia, migraines, Raynaud's phenomenon, and hypothyroidism (Hammi et al., 2005). Research also shows that systemic extra glandular manifestations, including lymphoma, appear in one-third of individuals with primary Sjögren's syndrome (Ryu et al., 2006). The classification criteria introduced in 1985 by Breiman et al. and the European preliminary criteria (Vitali et al., 1993) were used commonly for research. The European criteria for diagnosis rely upon combination of ocular symptoms, oral symptoms, ocular signs, and salivary glands involvement (Vitali et al., 2002). The
American-European Consensus Group classification criteria (AECG) have been used since 2002 as a new "gold standard" (Vitali et al., 2002). The American-European criteria are based on the presence of 4/6 criteria (including the objective criteria, oral and ocular symptoms) when either anti-60 kD Ro antibodies or histopathology are positive (Table 2. 4) (Langegger et al., 2007). If Sjögren's syndrome is suspected, procedures such as salivary gland imaging, sialography, magnetic resonance imaging, or technetium-99m pertechnetate scintigraphy can be carried out, depending upon the type of information that is needed for diagnosis (Rabinov and Weber, 1985; Luyk et al., 1991; Baum et al., 1996; PC Fox et al., 1998). In suspected cases of Sjögren's syndrome, serologic evaluation is important, since non-specific autoimmune markers - such as rheumatoid factor, antinuclear antibodies, elevated serum immunoglobulin, total protein levels, and increased erythrocyte sedimentation rate, or the more specific test for anti-Ro (SS-A) and anti-La (SS-B) autoantibodies help in the definitive diagnosis of Sjögren's syndrome. However, no single serologic marker is found in all cases of Sjögren's syndrome (Fox and Fox, 2008).
### Table 2.4. Revised international classification criteria for Sjögren's syndrome (Vitali et al., 2002).

#### I. Ocular symptoms: a positive response to at least one of the following questions:

1. Have you had daily, persistent, troublesome dry eyes for more than 3 months?
2. Do you have a recurrent sensation of sand or gravel in the eyes?
3. Do you use tear substitutes more than 3 times a day?

#### II. Oral symptoms: a positive response to at least one of the following questions:

1. Have you had a daily feeling of dry mouth for more than 3 months?
2. Have you had recurrently or persistently swollen salivary glands as an adult?
3. Do you frequently drink liquids to aid in swallowing dry food?

#### III. Ocular signs—that is, objective evidence of ocular involvement defined as a positive result for at least one of the following two tests:

1. Schirmer's I test, performed without anaesthesia (≤5 mm in 5 minutes)
2. Rose bengal score or other ocular dye score (≥4 according to van Bijsterveld's scoring system)

#### IV. Histopathology: In minor salivary glands (obtained through normal-appearing mucosa) focal lymphocytic sialoadenitis, evaluated by an expert histopathologist, with a focus score ≥1, defined as a number of lymphocytic foci (which are adjacent to normal-appearing mucous acini and contain more than 50 lymphocytes) per 4 mm² of glandular tissue

#### V. Salivary gland involvement: objective evidence of salivary gland involvement defined by a positive result for at least one of the following diagnostic tests:

1. Unstimulated whole salivary flow (≤1.5 ml in 15 minutes)
2. Parotid sialography showing the presence of diffuse sialectasias (punctate, cavitary or destructive pattern), without evidence of obstruction in the major ducts
3. Salivary scintigraphy showing delayed uptake, reduced concentration and/or delayed excretion of tracer

#### VI. Autoantibodies: presence in the serum of the following autoantibodies:

1. Antibodies to Ro(SSA) or La(SSB) antigens, or both

Revised Rules for Classification:

For primary Sjögren’s syndrome:

In patients without any associated disease, primary Sjögren’s syndrome may be defined as follows:
a. The presence of any 4 of the 6 items (Table 2.4) is indicative of primary Sjögren’s syndrome, as long as either item IV (Histopathology) or VI (Serology) is positive, or

b. The presence of any 3 of the 4 objective criteria items (that is, items III, IV, V, VI), or

c. The classification tree procedure represents a valid alternative method for classification, although it should be more properly used in clinical-epidemiological survey

For secondary Sjögren’s syndrome:

In patients with a potentially associated disease (for instance, another well defined connective tissue disease), the presence of item I or item II plus any 2 from among items III, IV, and V may be considered as indicative of secondary Sjögren’s syndrome.

Exclusion criteria:

- Past head and neck radiation treatment
- Hepatitis C infection
- Acquired immunodeficiency disease (AIDS)
- Pre-existing lymphoma
- Sarcoidosis
- Graft versus host disease
- Use of anticholinergic drugs (within a period less than 4 x the half life of the drug)
Recently, a new set of classification criteria for Sjögren’s syndrome has been released by an international team of researchers (Shiboski, 2012). The classification criteria are the first for Sjögren’s syndrome to be based solely on objective clinical tests. The new criteria come from the Sjögren's International Collaborative Clinical Alliance (SICCA). In the SICCA study, an international expert panel of ophthalmologists, rheumatologists, and oral medicine/pathology specialists compiled a consensus list of existing diagnostic tests to assess the various components of Sjögren’s syndrome (oral, ocular, and systemic), by combining the expert consensus and data collected from a wide range of study participants, the SICCA scientists developed a set of preliminary classification criteria for Sjögren’s syndrome. These criteria were validated using novel statistical methods and comparisons with existing classification approaches, mostly the AECG criteria as previously discussed. Based on this intensive process, they stipulate that to be classified with Sjögren’s syndrome, research participants must be positive for at least two of three objective diagnostic tests:

- Anti-SS-A/B blood test: There are two scenarios: 1) Positive serum levels of either the SSA and/or SSB antibody and/or 2) positive serum levels of the rheumatoid factor antibody (RA) and elevated antinuclear antibody (ANA) titers. All are associated with the syndrome.

- Ocular surface staining: Measures the dissipation rate of a specialized dye that is applied to the tear film that bathes the surface of the eye. A score of three or more is considered to be positive.
• Salivary gland biopsy: A pathologist examines the biopsy for sites of inflammation. One or more sites of inflammation per 4 mm² area is considered positive.

Stephen Shiboski (Shiboski, 2012) noted that because the AECG criteria can be applied in multiple ways, their application is more subjective and may result in a higher risk of misclassification. The National Institutes of Health (NIH), the American medical research agency supports the new classification criteria for Sjögren’s syndrome.

2.3.5.1 Sjögren's syndrome and saliva proteins

As salivary gland dysfunction is a critical component of in the Sjögren’s syndrome sequelae, several studies have searched for saliva markers that could be used in early diagnosis methodologies (Kalk et al., 2002; Peluso et al., 2007). In the rapidly progressing field of disease diagnostics based on salivary biomarkers, it is becoming clear that a combination of several biomarkers, rather than a single one may define a specific disease (Wong, 2006). Having a biomarker set specifically for Sjögren’s syndrome will assist in the clinical diagnosis and in measuring the severity status of Sjögren’s syndrome and furthermore may contribute to the understanding of the molecular mechanism of this syndrome. Previous studies examining saliva from Sjögren’s syndrome patients have shown that protein expression differences exist in comparison with healthy individuals (Giusti et al., 2007; Peluso et al., 2007; Ryu et al., 2006). Studies reported the
discovery of alteration of salivary proteins in saliva samples of Sjögren’s syndrome patients (Hu et al., 2005; Yaltirik et al., 2005).

### 2.3.6 Diabetes and other conditions

Diabetes mellitus is another risk factor for developing dry mouth. Diabetes mellitus consists of a group of disorders, which are characterized by a lack of insulin production or insulin resistance. Patients suffering from diabetes, especially those who have poor glycaemic control, are more susceptible to a decreased normal salivary flow rate, and to complain of dry mouth (Mattson and Cerutis, 2001).

Other diseases that cause a dysfunction of the immune system, such as HIV/AIDS, as well as hepatitis, uncontrolled hypertension, and severe renal disease all can contribute to dry mouth through the disease itself or in combination with the side-effects of medications (Napeòas et al., 2009).

### 2.3.7 Dry mouth diagnosis and management

A systematic approach should be employed to determine the aetiology of dry mouths, with distinctions made between patients with subjective complaints of dry mouth alone and those with measurable salivary gland dysfunction. In general, salivary dysfunction is difficult to assess, because of the wide range of variable properties. Also, the diagnosis or assessment of dry mouth can be relatively subjective unless an individual’s base saliva flow rate has been
established. Clinically, symptoms such as dry lips, buccal mucosa dryness, lack of salivation on palpation, and high caries rates should lead to further investigation such as saliva flow rate measurements, minor salivary gland biopsy, and other diagnostic evaluations.

The management of dry mouth varies, depending on the causes. Usually it consists of alleviating symptoms, treating oral conditions, and treating any underlying systemic conditions. The suggested approach has been summarized below (Table 2.5).

### Table 2.5. Dry mouth management. Adapted from (Porter et al., 2004).

<table>
<thead>
<tr>
<th>Alleviating symptoms</th>
<th>Oral hygiene intervention</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saliva substitutes: moisturing gels</td>
<td>Plaque control: Good personal hygiene care; alcohol - free mouth wash</td>
</tr>
<tr>
<td>Salivary stimulants: Pilocarpine, and others</td>
<td>Dietary advice: avoidance of dry food, sweets, alcohol, smoking</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Oral hygiene intervention</td>
<td></td>
</tr>
<tr>
<td>Plaque control: Good personal hygiene care; alcohol - free mouth wash</td>
<td></td>
</tr>
<tr>
<td>Dental advice: avoidance of dry food, sweets, alcohol, smoking</td>
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<td></td>
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<tr>
<td>Dentures care</td>
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<td>Denture hygiene</td>
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<tr>
<td>Denture to be fitted well</td>
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<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Treating underline systemic condition</td>
<td></td>
</tr>
<tr>
<td>Systemic therapies</td>
<td></td>
</tr>
</tbody>
</table>

#### 2.3.8 Dry mouth as a predisposing factor for oral candidosis

A continuous saliva flow is important for oral health. Saliva not only contains antimicrobial peptides such as lactoferrin, lysozyme, and immunoglobulin A, but also removes organisms from the oral cavity through its flushing effect. It has been shown that low salivary flow rates are associated with
higher *Candida* counts, and predisposing factors such as dry mouth, are associated with a greater diversity of *Candida* species colonizing the oral cavity (Table 2.6) (Torres et al., 2002; Torres et al., 2007). The reported prevalence of yeast carriage in dry mouth subjects ranges from 25% to 75%, depending on the population studied (Stenderup, 1990).

<table>
<thead>
<tr>
<th>Species</th>
<th>Median salivary flow rates (ml/min)</th>
<th>Salivary flow rate range (ml/min)</th>
<th>Median Candida count (Cfu)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. albicans</em></td>
<td>0.6</td>
<td>0.01-2.04</td>
<td>2000</td>
<td>0.0002</td>
</tr>
<tr>
<td><em>C. parapsilosis</em></td>
<td>0.47</td>
<td>0.01-1.4</td>
<td>420</td>
<td>0.01</td>
</tr>
<tr>
<td><em>C. tropicalis</em></td>
<td>0.7</td>
<td>0.2-1.96</td>
<td>3200</td>
<td>0.87</td>
</tr>
</tbody>
</table>

P values are given for the relationship between the flow rate and *Candida* count (Torres et al., 2002).

In addition to effects on the salivary flow rate, dry mouth may also affect salivary components, such as the antimicrobial proteins in saliva, including lactoferrin, sialoperoxidase, lysozyme, histidine-rich polypeptides and specific anti-*Candida* antibodies, which can help prevent candidosis.

### 2.4 Prosthodontic considerations – prosthesis surfaces

#### 2.4.1 Denture plaque

The concept of plaque was first introduced by Williams in 1897 (Nikawa *et al.*, 1998), and was defined as a structure consisting of a dense microbial layer on teeth. Denture plaque is very similar, it is a biofilm comprising
microorganisms and their metabolites embedded in a polymeric matrix of microbial origin located on the denture surface (Figure 2.2). The microbial composition of denture plaque resembles that of plaque found in other parts of the mouth, except for an increased number of Candida cells (Arendorf and Walker, 1980; Davenport, 1970). These cells attach to the denture and often cause denture-induced stomatitis (Pereira-Cenci et al., 2008). Denture plaque develops from adherence, aggregation and growth of microbes from saliva and the oral mucosa, in the absence of adequate denture hygiene, and derives nutrients from saliva, the oral mucosa and the diet (Radford et al., 1999; Theilade et al., 1983).

Although the composition of denture plaque differs between individuals, and at different locations on the denture, the predominant microflora in denture plaque includes streptococcal species, gram-positive and gram-negative rods and yeast. In relatively stagnant sites, where the protection from saliva flow and mechanical removal forces in the mouth is provided, plaque tends to be more acidogenic, favoring streptococci and Candida. In vitro studies have also shown the high acidogenicity of C. albicans (Nikawa et al., 1993; Samaranayake et al., 1983; Samaranayake et al., 1986a). These studies reported that the pH of the medium or saliva, supplemented with glucose or sucrose, decreased from neutral or weak acid to around 3.0 within 72h of incubation, and was accompanied by C. albicans growth. The reduction in pH caused by Candida acid production is known to promote tissue damage and subsequent Candida invasion, and also to facilitate adherence (Odds, 1994).
2.4.2 Denture acrylic as a denture base material

In the UK in 2000, it was estimated that over 15 million people were wearing dentures (Kelly *et al.*, 1998) - representing a special concern for oral health care and health care in general. Over the years, a variety of materials has been used for the fabrication of dentures, currently, the most commonly used materials are polymers such as polymethylmethacrylate (PMMA) or acrylic resin (Zarb *et al.*, 2003). The denture base can be formed through a polymerizing process by mixing liquid monomer methylmethacrylate and polymer powder. There are several methods to activate the polymerization process, such as heat, chemical reaction, visible light initiation, or through electromagnetic radiation in the case of microwave-activated resin. The most commonly used method in dentistry is chemical polymerization by using an initiator to activate the process.

Although dental acrylic is a biocompatible material, it has inherent limitations as a material used in oral cavities. The main problem of denture acrylic is the porosity of the surface formed during the processing. During use, both the thermal and mechanical cycling can produce cracks and crazes in dentures, which increase plaque retention. Microbial biofilm formation on dental acrylic surfaces has been well documented, and directly contributes to oral microbial infections. Denture acrylics are sometimes used in combination with soft liners and tissue conditioners to improve comfort and fit, and to reduce irritation of the mucosa. Denture soft lining materials such as silicone are flexible in order to provide cushioning of the hard denture acrylic base and hence are more permeable with more porous structures and more surface irregularities in which
microbial cells may be retained (Allison and Douglas, 1973). Therefore, these silicone materials can enhance microbial colonization and penetration, resulting in deterioration, loss of the functional properties of dentures and increased microbial infection.

2.4.2.1 Denture wearing as a predisposing factor for oral candidosis

Wearing dentures produces a microenvironment conducive to the growth of *Candida* with low oxygen tension and low pH (von Fraunhofer and Loewy, 2009), which may be due to enhanced adherence of *Candida* to acrylic, reduced saliva flow under the surfaces of the denture fittings, improperly fitted dentures, and poor oral hygiene. The denture-mucosal interface offers a unique ecological niche for microorganism colonization because of the relatively anaerobic and acidic environment favoring yeast proliferation (Budtz-Jorgensen, 1972; 2000). It has been demonstrated that *Candida* cells have an affinity for the tissue side of a denture (Muzyka and Glick, 1995; Samaranayake and MacFarlane, 1980), which may reflect the lack of saliva flow and other environmental factors. Additionally, mechanical trauma from a poorly fitting prosthesis or failure to provide adequate access for cleaning of the denture increases the risk of plaque accumulation and subsequent tissue colonization and penetration (Figure 2. 10). The denture may also act as a reservoir of infection for respiratory and systemic opportunistic pathogens (Sumi *et al.*, 2003), and presents a niche for antibiotic-resistant biofilms.
2.5 Conclusions

The multifaceted spectrum of *Candida* infections, in particular related to prosthodontic treatment, continues to represent an important area of dental research. *C. albicans*-related infection can arise from *C. albicans* cells forming biofilms on the surfaces of mucosal tissues and prostheses, and infections most often occur from the previous, asymptomatic yeast carriage rather than as *de novo* infections. Recognition of the importance of saliva in modulating oral infection undoubtedly will continue to increase because of its many functions. Saliva quantity and functionality is affected by the health status of the host. Dry mouth is common in denture wearers, and patients with such a condition are reported to be more susceptible to oral candidosis. Prosthesis surfaces can serve as a reservoir to harbor biofilms, and co-aggregation of microorganisms within the biofilm increases the drug resistance of the microorganisms. The combination of dry mouth, denture wearing and oral *Candida* infection can present a challenge for clinical prosthodontic management. With an increasingly expanding variety of biomaterials applied in prosthodontics practice, it is essential to have an
understanding of *Candida* infections in relation to dry mouth and denture wearing. Salivary diagnostics has an important future. There are rapidly evolving innovations for the use of salivary diagnostics in human disease (Baum *et al.*, 2011). Systems biology tells us the biological associations of different systems in our body. The salivary glands, saliva, and its constituents, overlaid with a wealth of knowledge for potential clinical applications, present opportunities for oral and systemic disease detection and may affect the management of such conditions. The innovative application of salivary diagnostics in the dentist’s office is being explored (Giannobile and Wong, 2011). It should be noted that while the scientific rationale for salivary biomarkers reflecting oral diseases is in place, studies are ongoing to delineate the mechanisms of how systemic diseases can be reflected in saliva composition.
Chapter 3: Materials and Methods
3.1 Equipment

A list of the equipment used in this study is provided in Table 3.1.

Table 3.1. Equipment used.

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Manufacturer and Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Balance</td>
<td>Sartorius AG, Goettingen, Germany</td>
</tr>
<tr>
<td>Canon G10 camera</td>
<td>Canon, Lake Success, New York, USA</td>
</tr>
<tr>
<td>Centrifuge</td>
<td>Heraeus Labofuge 400, DJB Labcare Ltd, Newport Pagnell, Buckinghamshire, England</td>
</tr>
<tr>
<td>Film processor</td>
<td>Curix 60, Agfa-Gevaert, NV, Mortsel, Belgium</td>
</tr>
<tr>
<td>Epson Scanner</td>
<td>Epson, Auckland, New Zealand</td>
</tr>
<tr>
<td>Freezer, -20°C</td>
<td>Forma, ThermoQuest Analytische Systeme GmbH, Egelsbach, Germany</td>
</tr>
<tr>
<td>Freezer, -80°C</td>
<td>New Brunswick Scientific, John Morris Scientific Ltd, Christchurch, New Zealand</td>
</tr>
<tr>
<td>Heat sealer</td>
<td>Steriking RS1100, Van Dijk Medizin Technik GmbH, Straelen, Germany</td>
</tr>
<tr>
<td>Gel documentation system for SDS-PAGE gels</td>
<td>Bio-Rad Chemidoc, Bio-Rad Laboratories GmbH, Muenchen, Germany</td>
</tr>
<tr>
<td>Ice machine</td>
<td>Hoshizaki F-120B, Steelfort, Palmerston North, New Zealand</td>
</tr>
<tr>
<td>Shaking incubator</td>
<td>Bio-Line, Edwards Instrument Company, Narellan, Australia</td>
</tr>
<tr>
<td>Magnetic stirrer/hotplate</td>
<td>Chiltern MM31, Chiltern Scientific, Chicago, USA</td>
</tr>
<tr>
<td>Microplate reader</td>
<td>BioTek® Synergy 2 SL Luminescence, Wynoski, USA</td>
</tr>
<tr>
<td>pH-Meter</td>
<td>Eutech CyberScan pH 510 Bench pH/mV Meter, Thermo Fisher, Singapore</td>
</tr>
<tr>
<td>Refrigerators</td>
<td>Cyclomatic Frigidaire 370, Piraeus International, Baltimore, USA</td>
</tr>
<tr>
<td></td>
<td>Prestcold, McAlpine Prestcold Limited, Biolab Scientific, Auckland, New Zealand</td>
</tr>
<tr>
<td>Shaking platform</td>
<td>Ika Schüttler MTS 4, Ika® Works, Selangor, Malaysia</td>
</tr>
<tr>
<td>Vertical gel electrophoresis system</td>
<td>Bio-Rad Mini-Protean® II, Bio-Rad Laboratories GmbH</td>
</tr>
<tr>
<td>Water bath</td>
<td>Julabo TW12, Sigma-Aldrich New Zealand Ltd, Auckland, New Zealand</td>
</tr>
</tbody>
</table>
3.2 Chemicals and buffers

General chemicals were of analytical grade, unless otherwise specified, and were obtained from Ajax Chemicals (Auburn, NSW, Australia); BDH (Poole, England); Gibco BRL, Life Technologies, Inc. (Gaithersburg, MD, USA); Riedel-de Haen AgGT, (Germany); Sigma Chemical Co. (St. Louis, MO, USA) or Scientific Supplies Ltd (Auckland, New Zealand).

Ultrapure agarose, ultrapure Tris (Tris [hydroxymethyl]methylamine), ammonium persulphate and prestained molecular weight markers were obtained from Gibco-BRL, Life Technologies, Inc. (Gaithersburg, MD, USA). TEMED (N,N,N',N'-Tetramethylethylenediamine) was purchased from BDH Chemicals Ltd. and 40% Acrylamide/Bis-acrylarnide - solution (37.5:1) from Bio-Rad Laboratories. glycine was obtained from Ajax Chemicals, Bacto yeast extract from Difco (Becton Dickinson, (BD) Sparks, MD; USA), formaldehyde and bromophenol blue from Sigma Chemical Co, dithiothreitol (DTT) from Life Technology, New Zealand, glucose and glycerol from Merck (Manukau City, Auckland)

3.2.1 Buffers

3.2.1.1 KCl buffer

KCl buffer contained 2 mM KH$_2$PO$_4$, 2 mM K$_2$HPO$_4$.3H$_2$O, 5 mM KCl, 1 mM CaCl$_2$, pH 6.5. The KH$_2$PO$_4$, K$_2$HPO$_4$.3H$_2$O and KCl were dissolved in 800 ml H$_2$O and the pH adjusted to 6.5; the CaCl$_2$, was dissolved in 200 ml H$_2$O. These
two solutions were autoclaved separately and mixed together after the solutions had cooled, to prevent CaPO₄ precipitation.

### 3.2.1.2 Separating buffer

Separating buffer contained 18.2 g Tris (final concentration 1.5 M), 0.4 g SDS (final concentration 14 mM) per 100 ml water, adjusted to pH 8.8 with HCl. The buffer was then filtered through Whatman No. 1 paper and stored at 4°C.

### 3.2.1.3 Stacking buffer

Stacking buffer contained 6.1 g Tris (final concentration 0.5 M), 0.4 g SDS (final concentration 14 mM) in 100 ml water, adjusted to pH 6.8 with HCl. The buffer was then filtered through Whatman No. 1 paper and stored at 4°C.

### 3.2.1.4 Sample buffer

Sample buffer (10 ml stock) contained 2.5 ml stacking gel buffer, 0.2 ml 2-mercaptoethanol, 1 ml 20% (w/v) SDS, and 6.3 ml water and was stored as frozen aliquots.

### 3.2.1.5 Electrophoresis running buffer

Electrophoresis running buffer contained, per litre, 14.4 g glycine (192 mM), 3.0 g Tris (25 mM); 1 g SDS (3.5 mM).

### 3.2.1.6 Protein stains

Coomassie blue R250 protein stain contained, per litre, 0.2 g Coomassie blue R250 (Invitrogen, Life Technologies, Auckland, New Zealand), 400 ml methanol,
500 ml water, and 100 ml glacial acetic acid. Coomassie destain solution contained, per litre, 200 ml methanol, 700 ml water, 100 ml glacial acetic acid.

3.2.1.7 Protein assay and analysis kits

Protein assay kits were purchased from Bio-Rad Laboratories. The high molecular weight gel filtration calibration kit was obtained from Pharmacia Fine Chemicals AB (Uppsala, Sweden).

3.3 Study participants

3.3.1 Ethical approval

Ethical approval for the study was applied for and obtained (Ref OTA/02/05/034) prior to the collection of saliva-rinse samples from healthy individuals recruited for the pilot study. For patient and control samples, ethical approval for the collection and use of the saliva rinses for the research project was gained from the Lower South Regional Ethics Committee, Health and Disability Ethics Committees, Ministry of Health (Ref LRS/10/09/034). Copy of ethical approval letter is appended (Appendix IV).

3.3.2 Study participant selection criteria

Dry mouth subjects were recruited from Oral Medicine clinics and age- and gender-matched control subjects were recruited from patients attending other clinics in the School of Dentistry (Table 3.2). An information sheet (Appendix V
and VI) and a consent form (Appendix IV were provided and explained to each participant. Consenting participants completed a confidential questionnaire (Appendix VIII) to record their age, gender, ethnicity, general medical condition, oral health condition and current medication status. Twenty dry mouth subjects were recruited and these were matched with twenty healthy controls. Each participant was assigned a unique identifier.
Table 3.2. Subject selection criteria

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Inclusion criteria for patients</th>
<th>Inclusion criteria for controls</th>
<th>Exclusion criteria for patient samples</th>
<th>Exclusion criteria for control subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inclusion criteria for patients</td>
<td>1. Individuals attending the Oral Medicine clinic with complaints of a dry mouth and/or suspected Sjögren’s syndrome</td>
<td>1. Any healthy individual attending the School of Dentistry with no complaint of dry mouth or signs of Sjögren’s syndrome or any other systemic condition</td>
<td>1. Insufficient medical information obtained</td>
<td>1. Individuals complaining of a dry mouth</td>
</tr>
<tr>
<td></td>
<td>2. Any age or gender</td>
<td>2. Age and gender matching potential to the patient subjects</td>
<td>2. Saliva samples were not stored in a glass container</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3. Saliva rinse could be collected in a glass container</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4. Adequate medical information could be obtained</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Surfaces play a vital role in biology and medicine with most biological reactions occurring at surfaces and interfaces. Samples collected into a plastic container were eliminated due to the consideration that proteins may be absorbed to the plastic surfaces (Castner and Ratner, 2002); Holmes, A.R, unpublished observation.
3.3.3 Participants data collection

General data regarding the age, gender, ethnicity, general medical condition, oral health condition, denture wearing status and current medication intake of the subjects were obtained from the confidential questionnaire (Appendix VIII) and clinical notes. The data were checked against the Sjögren’s syndrome diagnostic criteria (Vitali et al., 2002). In some cases a lip labial biopsy was performed as part of other ongoing treatment, and a histology report was obtained from the Department of Oral Diagnostic and Surgical Sciences at the University of Otago School of Dentistry. For some patients, previous antinuclear antibodies test results from the Southern Community Laboratories, Dunedin, were obtained from patient files.

3.4 Collection of saliva rinses

With informed consent, saliva rinse samples were collected from twenty xerostomia subjects, or from the healthy volunteer controls, with informed consent at least one hour after eating. The same saliva samples collected from the subjects were used throughout the course of this study. Subjects were asked to rinse their mouths with 10 ml food-grade water from a commercially bottled source for 30 s without swallowing before collecting it into a sterile glass container, from which the saliva rinse was then transferred into a universal bottle and stored on ice. The total volume of the oral rinse was measured following the
sample collection. Individual saliva rinse samples were split in two portions and kept separately at -20°C.

3.5 Yeast media and growth conditions

3.5.1 Yeast media

YEPD medium contained, per litre, 10 g yeast extract (Becton Dickinson (BD)), 20 g Bacto® peptone (BD), 20 g glucose, (pH 5.7). YEPD agar was made by the addition of 20 g (2 % (w/v)) agar (Danisco NZ Ltd, East Tamaki, NZ) to YEPD prior to sterilization. Glucose salts biotin (GSB; (Holmes and Shepherd, 1988)) medium contained, per litre, 1 g (NH₄)₂SO₄ (7.57 mM), 2 g KH₂PO₄ (14.7 mM), 50 mg MgSO₄·7H₂O (0.2 mM), 50 mg CaCl₂·2H₂O (0.34 mM), 0.05 mg biotin (Sigma Chemical Co), and 20 g glucose (111 mM), pH 4.5.

3.5.2 Growth of yeast and yeast identification

Portions (100 µl and 10 µl) of the oral rinse were spread on CHROMagar Candida (Chromagar Co, Paris, France) agar plates. The plates were incubated for 48 to 72 h at 30°C before any colonies present were presumptively identified by their colour according to the manufacturer’s instructions, and counted. CHROMagar Candida presumptively identifies C. albicans (green), C. glabrata (purple) and C. krusei (pale rose) based on colony colour formed from a
chromogenic substrate by yeast hexosaminidase activity (Odds and Bernaerts, 1994; Beighton et al., 1995; Williams and Lewis, 2000).

Yeast strains were subcultured onto YEPD agar plates and samples of the yeast strains were stored in YEPD broth + 15 % (w/v) glycerol at -80 °C.

### 3.6 Protein band separation

#### 3.6.1 SDS-PAGE gel preparation

SDS-PAGE was performed according to the method described by Laemmli (Laemmli, 1970). A uniform concentration of acrylamide in the separating gel 10% (total concentration of acrylamide plus bisacrylamide), was achieved by using 1.5 ml of an acrylamide stock (40% acrylamide/bis solution, BioRad) with a constant amount separating gel buffer (Section 3.2.1.2), and a compensating amount of water (3ml) to a total volume of 6 ml. The polymerization of the acrylamide was catalysed by adding TEMED (4 µl) and 10% (w/v) ammonium persulphate (30 µl). The gel mix was poured into the gel mould and overlayed with 0.1% (w/v) SDS until the gel was set and then the SDS solution was removed. A stacking gel mix comprising: 40% acrylamide stock 0.25 ml, stacking gel buffer (Section 3.2.1.3) 0.5 ml, water 1.25 ml, TEMED 1µl and 10% (w/v) ammonium persulphate 15 µl was poured on to the stacking gel and a
10-well comb placed on top. When the stacking gel had set, the comb was removed and the wells were rinsed with distilled water (Section 3.2.1.5).

### 3.6.1.1 SDS – polyacrylamide gel electrophoresis (SDS – PAGE)

The polymerised gels were mounted on the gel apparatus and loaded with running buffer. Saliva rinse samples (25 µl) were mixed with 5 µl 6 x sample buffer (Section 3.2.1.4), vortexed, and heated (10 min, 70 °C) prior to loading. The samples were loaded into individual wells on the polyacrylamde gel and the PageRuler™ Plus Prestained Protein Ladder, (Fermentas International Inc, Thermo Scientific, Maryland, USA, batch number 1811) were used as markers. The gels were electrophoresed at 100 V (Solstat® electrophoresis power pack Model ES 300, Solstat Ltd., Christchurch, NZ) at room temperature for 45 min to 1.5 h.

All gels were run in pairs; one was subsequently stained with EZBlue™ (Sigma-Aldrich Biotechnology LP and Sigma-Aldrich Co) and the other with Silver stain (Morrissey, 1981). The salivary protein profile of saliva rinses from dry mouth subjects and those from control subjects were recorded by digital photography using a Nikon SLR digital camera and also analysed with Gel Doc™ EZ system (Life Science, Bio-Rad laboratories, Inc).
3.7 Protein visualization methods

3.7.1 Coomassie blue staining - EZBlue™

The EZBlue™ (Sigma-Aldrich Co) staining protocol was described below. The SDS-PAGE gels were rinsed with water for 5 min, 3 times. The gels were then left in 20-40 ml of EZBlue™ Gel staining reagent and gently shaken on a reciprocal shaker for 45 min to 1 h (or overnight) and the protein band development was checked periodically. The gels were then washed with distilled water to remove excess staining, with frequent changing of the water, until the background became clear.

3.7.2 Silver staining

Electrophoretically separated proteins in SDS-polyacrylamide gels were also visualised by staining with 0.1% (w/v) silver nitrate (Morrissey, 1981). The gel was incubated in Fix 1 (containing 50% methanol and 10% acetic acid) for 30 min at 20°C on a reciprocal shaker (50 – 60/min), then agitated on a reciprocal shaker for 30 min in Fix 2 (containing 5% methanol and 7% acetic acid), prior to rinsing several times with DW before incubating in a solution of DTT (5 µg/ml) for 30 min on a reciprocal shaker and then in a 0.1% (w/v) solution of silver nitrate for 30 min on a reciprocal shaker. The gel was then washed in 2 x 50 ml developer (containing per litre, 30 g (3%) anhydrous Na₂Ca₃ in distilled water and 500 µl 37% formaldehyde) followed by incubation in
developer (100 ml). Colour development was stopped with the addition of 2 ml citric acid (2.3 M).

### 3.7.3 Bradford protein assay

The Bio-Rad Protein assay kit (Bio-Rad Laboratories) was used to estimate the protein concentrations present in saliva rinse samples, based on the method of Bradford (1976). Briefly, portions (180 µl) of Bradford reagent diluted (1:4 in distilled water) were added to the samples (20 µl) in a 96 well microtitre plate, and incubated at 20°C for 5 min. Samples were analyzed in duplicate. The absorbance at 590 nm was measured using a microplate reader (Table 3.1). Protein concentrations were determined by comparison to a standard curve prepared for bovine γ-globulin (concentration range 0.1 to 0.5 µg/µl) and using Gen 5 data analysis software (BIO-TEK™ Instruments Inc.).

### 3.7.4 Gel Doc™ EZ analysis of proteins in patient and control samples

Patient and control saliva samples were run on commercially purchased Ready Gel Precast Polyacrylamide Gels, (Bio-Rad Laboratories, Inc). SDS-PAGE was conducted and the protein bands were visualised with both EZblue™ and silver staining methods as described in sections 3.6.1.1, 3.7.1 and 3.7.2.
The protein bands were identified using visual inspection and the Gel Doc™ EZ system (Bio-Rad laboratories, Inc). Visual inspections of digital images were done by three independent researchers evaluating the visible bands and agreement was reached on the number and location of the bands detected. The process of analysing SDS-PAGE gels using the Gel Doc™ EZ system is illustrated in Figure 3.1 and Figure 3.2.

![Figure 3.1. Gel Doc™ EZ system analysis process.](image)

![Figure 3.2. Example of the Gel Doc™ EZ system, protocol setup screen](image)

The automated protein studies were conducted by selecting “Analyze molecular weight” protocol as shown in the Figure 3.2.
3.8 Statistical analysis

Comparison between xerostomia subjects and matched controls using the Mann-Whitney test.
Chapter 4: Study Participants
4.1 Introduction

It has been reported that 16% - 29% of the population experience a dry mouth sensation at some point of time (Thomson et al., 2006). The subjective sensation of oral dryness generally results from a decrease in the production of saliva, which may alter the oral microenvironment, and result in yeast colonization. This colonization is a prerequisite for candidosis (Odds and Bernaerts, 1994), and C. albicans remains the dominant cause of both opportunistic yeast infections and life-threatening systemic yeast infections in immunocompromised individuals.

In order to investigate the clinical factors that might influence the diversity and the degree of Candida species carriage in saliva, we conducted a cross-sectional study of saliva samples from 20 patients with self-reported dry mouth and 20 age- and gender-matched controls. Relevant clinical data was collected by a confidential questionnaire (Appendix VIII) at the same time as collection of unstimulated saliva rinse samples as described in section 3.4. The project was approved by the Lower South Regional Ethics Committee (approval number LRS/10/09/034).
4.2 Overview of study participants’ general data

Study participants were asked to complete a confidential questionnaire, to obtain information concerning their sensation of dry mouth, other medical conditions, their medications and denture-wearing status (Table 4.1). Patients that had been formally diagnosed with, or showed strong indication of, Sjögren’s syndrome, were referred to the study by the Department of Oral Diagnostic and Surgical Sciences. Once 20 dry mouth patients had been enrolled, 20 age- and gender-matched control patients with no dry mouth symptoms or sensations were enrolled from the wider patient base of the School of Dentistry.
Table 4.1. General data collected for study participants.

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>Age</th>
<th>Gender</th>
<th>Dentures</th>
<th>Diabetics</th>
<th>Other Medical condition recorded on questionnaire</th>
<th>Medications</th>
</tr>
</thead>
<tbody>
<tr>
<td>P3</td>
<td>65</td>
<td>F</td>
<td>No</td>
<td>No</td>
<td>Hypertension,</td>
<td>Bendrofluazide, Lipex, Hydroxychloroquine</td>
</tr>
<tr>
<td>P4</td>
<td>86</td>
<td>F</td>
<td>No</td>
<td>No</td>
<td>Pernicious anemia</td>
<td>Felozipine, Celiropol, B11 Injections</td>
</tr>
<tr>
<td>P5</td>
<td>71</td>
<td>F</td>
<td>No</td>
<td>No</td>
<td>Undiagnosed connective tissue disorder, Swelling of submandibular glands</td>
<td>Venlafaxine, Cartex, Ayclovir</td>
</tr>
<tr>
<td>P6</td>
<td>73</td>
<td>F</td>
<td>Yes</td>
<td>No</td>
<td>Asthma and hypertension</td>
<td>Accupril, M-Edon, Musilax, Combivent Inhaler, Severeol, Cholecalciferol, Lansoprazole, Laxaid, Loratidine, Celebrex, Fexa Tablet, Clarithromycin, Frusinide, Apo-Amiodipine, Ami-triptiline Hydrochloride, Livostin, Nitrazepam, Hydroxyethylamin, Reclazone</td>
</tr>
<tr>
<td>P8</td>
<td>56</td>
<td>M</td>
<td>No</td>
<td>No</td>
<td>Glaucoma</td>
<td>Loxamine, Losec, Acupril, Atensol</td>
</tr>
<tr>
<td>P9</td>
<td>56</td>
<td>M</td>
<td>No</td>
<td>No</td>
<td>Sjögren’s syndrome</td>
<td>Naprosyn, Losec, Hydroxychloroquine</td>
</tr>
<tr>
<td>P10</td>
<td>73</td>
<td>F</td>
<td>Yes</td>
<td>No</td>
<td>Asthma</td>
<td>Losec, Lipex, Drexpin, Verapamil</td>
</tr>
<tr>
<td>P11</td>
<td>35</td>
<td>F</td>
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<td>No</td>
<td>Suspected Lupus, Sjögren’s syndrome , Connective tissue disorder, Thrombosis, chronic sialadenitis</td>
<td>Naprosyn, Inhibucle (=Cilapril)</td>
</tr>
<tr>
<td>P12</td>
<td>56</td>
<td>F</td>
<td>No</td>
<td>No</td>
<td>Back and neck pain</td>
<td>Diclofenac, Omeprazole, Ami-triptiline</td>
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<tr>
<td>P13</td>
<td>24</td>
<td>F</td>
<td>No</td>
<td>No</td>
<td>Sjögren’s syndrome , migraine</td>
<td>Gabapentin, Brafen, Ami-triptylinae,</td>
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<td>34</td>
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<td>No</td>
<td>Lupus</td>
<td>Brafen, Symbicort, Fluomazol</td>
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<td>Heart problems, Crohn’s, Sjögren’s syndrome</td>
<td>Loperamide, Verapamil, Calcitriol, Pantoprozone</td>
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<tr>
<td>P16</td>
<td>64</td>
<td>F</td>
<td>No</td>
<td>No</td>
<td>Rheumatoid arthritis</td>
<td>Fosamex, B12, Anti-reflex</td>
</tr>
<tr>
<td>P17</td>
<td>67</td>
<td>F</td>
<td>Yes</td>
<td>No</td>
<td>Sjögren’s syndrome, hypertensive disease</td>
<td>Fosamex, Plaquenil, Bendrofluazide, Lacrilube, Lapiuffin, Climbased Propionate, Simvastatin</td>
</tr>
<tr>
<td>P18</td>
<td>51</td>
<td>F</td>
<td>No</td>
<td>No</td>
<td>Lupus</td>
<td>Omeprazole, Cholecalciferol, Ibuprofen, Hydroxychloroquine, Piroxicam</td>
</tr>
<tr>
<td>P19</td>
<td>71</td>
<td>F</td>
<td>No</td>
<td>No</td>
<td>Lupus</td>
<td>Ami-triptiline</td>
</tr>
<tr>
<td>P20</td>
<td>69</td>
<td>F</td>
<td>No</td>
<td>No</td>
<td>Hypertension</td>
<td>Cilaipril, Lipitor</td>
</tr>
<tr>
<td>P21</td>
<td>53</td>
<td>F</td>
<td>Yes</td>
<td>No</td>
<td>Sarcoïdosis</td>
<td>No medication reported</td>
</tr>
<tr>
<td>P22</td>
<td>45</td>
<td>F</td>
<td>No</td>
<td>No</td>
<td>Lupus, Raynauld’s, Bi- cuspid Valve Defect</td>
<td>Zapolione, Aspirin, Hydroxychloroquine Sulphate, Quinipine Fumarate, Chonzebran, Paracetamol, Microfax Enema, Naprexen</td>
</tr>
<tr>
<td>P23</td>
<td>42</td>
<td>F</td>
<td>Yes</td>
<td>No</td>
<td>Rheumatoid Thyroid disease</td>
<td>Prednisone, Thryoxine, Salazopyrin</td>
</tr>
</tbody>
</table>
### 4.3 Age and gender distribution

The median age of the patients was 59.5 years of age, and ranged from 24 to 86 years old. The median age was the same for the controls, which were selected to match the age and gender of the patient group. Eighteen subjects (90%) of each group were females and two (10%) were males.

![Age distribution of xerostomia subjects and controls](image)

**Figure 4.1.** Age distribution of xerostomia and control participants
Figures 4.1 and 4.2 show the age distribution of the patient subjects. The xerostomia age distribution showed a high proportion of elderly subjects, which is in line with findings of an earlier study (Nederfors, 2000) of a large Swedish adult population, in which the frequency of reporting xerostomia was significantly greater in older people and in women (Nederfors, 2000).

**4.4 General medical conditions**

All xerostomia subjects participants claimed to suffer from one or more medical conditions other than dry mouth (Table 4.2) and 85% of those were taking a wide variety of prescribed and non-prescribed (over-the-counter) drugs, unrelated to their dry mouth condition. In the control group, fourteen subjects were healthy, whilst the remaining six participants reported other illnesses.
### Table 4.2. General medical conditions of study participants.

<table>
<thead>
<tr>
<th>Underlying diseases</th>
<th>Number of xerostomia subjects</th>
<th>% of xerostomia subjects</th>
<th>Number of control subjects</th>
<th>% of control subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>0</td>
<td>14</td>
<td>70</td>
</tr>
<tr>
<td>Diabetes</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Cardiovascular diseases</td>
<td>8</td>
<td>40</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>Endocrine and metabolic diseases</td>
<td>3</td>
<td>15</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Respiratory diseases</td>
<td>3</td>
<td>15</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>Autoimmune connective tissue disorders</td>
<td>12</td>
<td>60</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>Gastrointestinal diseases</td>
<td>2</td>
<td>15</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Neurological diseases</td>
<td>1</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Others</td>
<td>2</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

The table above clearly shows that the xerostomia subjects suffer from more, and a wider variety of, illnesses than their matched controls. All xerostomia subjects reported at least one other medical condition in addition to their dry mouth complaint. One xerostomia subject reported as many as five additional disorders. Of the xerostomia subjects with heart-related problems, hypertension was reported by six times more than the controls. One xerostomia subject had a structural heart valve problem and the others had non-specified heart problems. Of
the two subjects in the control group who reported cardiovascular problems, both mentioned hypertension.

### 4.5 Medication

Table 4.3 categorizes the intake of medications by the members of both groups at the time when the information was collected. All of the subjects in the xerostomia group are on (mainly prescribed) drugs, compared to half of the control group subjects. The xerostomia group, on average, used greater than triple the number of pharmaceuticals than the control group, however there might be a discrepancy between the reported and actual consumption of medications.

<table>
<thead>
<tr>
<th>Medication</th>
<th>Numbers of xerostomia subjects taking medication</th>
<th>% of xerostomia subjects taking medication</th>
<th>Numbers of control subjects taking medication</th>
<th>% of control subjects taking medication</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tricyclic antidepressant</td>
<td>6</td>
<td>30</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Antihypertensive</td>
<td>11</td>
<td>55</td>
<td>5</td>
<td>25</td>
</tr>
<tr>
<td>Anticholinergic</td>
<td>17</td>
<td>85</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>Osteoporosis/arthritis medication</td>
<td>14</td>
<td>70</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Cholesterol-lowering medication</td>
<td>4</td>
<td>20</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>Cardio medication</td>
<td>1</td>
<td>5</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>Diuretics, gastro/bowel medication</td>
<td>7</td>
<td>35</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>Antihistaines asthma medication</td>
<td>2</td>
<td>10</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>Other</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>50</td>
</tr>
</tbody>
</table>
The numbers of xerostomia and control subjects taking different types of medication are related to their underlying medical conditions (Table 4.1). The most notable difference between the subject groups was the taking of osteoporosis and anti-arthritic drugs (Table 4.3). The proportion taking blood pressure-lowering medications and the proportion taking antihistamines are the same for both groups, but for all other medications a higher proportion of the xerostomia group was taking them than the control group. Nine xerostomia subjects who mentioned the use of medications such as vitamin injections or paracetamol, which has been categorized as ‘other’.

Table 4.4. Medications taken by participants that are associated with dry mouth, classification based on (Scully and Felix, 2005).

<table>
<thead>
<tr>
<th>Medications</th>
<th>Numbers of xerostomia subjects samples</th>
<th>Numbers of control samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Directly damaging salivary glands</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>With anticholinergic activity a</td>
<td>17</td>
<td>2</td>
</tr>
<tr>
<td>Acting on sympathetic system b</td>
<td>12</td>
<td>4</td>
</tr>
<tr>
<td>Diuretic medication</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>

a. An anticholinergic drug blocks the passage of impulses through the parasympathetic nerve. Examples of such drugs are anti-depressants, antihistamines or antireflux agents like omeprazole (Scully, 2003).

b. Drugs acting on the sympathetic system are agents affecting the function of, or mimicking the actions of, the autonomic nervous system and thereby having an effect on such processes as respiration, circulation, digestion, body temperature regulation, certain endocrine gland secretions, etc. (Scully, 2003).

The drug groups in Table 4.4 are those which, according to Scully et al. (2003) are associated with dry mouth (Scully, 2003). The total number of xerogenic pharmaceuticals used by the xerostomia group is about five times that used by the control subjects.
Chapter 4. Study Participants

Oral Yeast Carriage and Saliva Protein Profiles of Xerostomia Subjects and Matched Controls

Table 4.5. Number of study participants taking drugs associated with dry mouth

<table>
<thead>
<tr>
<th>Number of drugs associated with dry mouth</th>
<th>Number of xerostomia subjects</th>
<th>Number of control subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2</td>
<td>14</td>
</tr>
<tr>
<td>1</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>≥ 2</td>
<td>11</td>
<td>2</td>
</tr>
</tbody>
</table>

Ninety percent of xerostomia subjects were taking at least one xerogenic medication compared to only 30% of control participants (Table 4.5). Furthermore, over half of the xerostomia subjects was using two or more of such drugs, in contrast to only 10% (2) of the control subjects.

4.6 Sjögren’s syndrome criteria assessment of xerostomia subjects

According to the revised international classification criteria for Sjögren’s Syndrome (Vitali et al., 2002), primary Sjögren’s syndrome requires the presence of four out of six criteria, which include both subjective and objective criteria (Table 2.4). The objective evidence of salivary gland involvement includes low unstimulated salivary flow (≤ 0.1 ml/min, criterion V). Other objective criteria include ocular signs (criterion III), either a positive serum anti - Ro/SSA and/or anti - La/SSB antibody test (criterion VI) or positive histopathology for focal lymphocytic sialadenitis from a labial salivary gland biopsy (criterion IV). Secondary Sjögren’s syndrome is diagnosed when, in the presence of a connective-tissue disease, symptoms of oral or ocular dryness exist in addition to
the positive finding for ocular signs, oral signs and positive minor salivary gland biopsy findings. It should be explained the reason why the revised international classification criteria for Sjögren’s Syndrome of 2002 (Vitali et al., 2002) were used to assess the condition of xerostomia subjects is due to the reason that the most recent diagnostic criteria for Sjögren’s Syndrome were not available at the time when the saliva samples collected.

The assessment revealed that all 20 xerostomia subjects displayed oral symptoms by having a feeling of dry mouth, 16 out of 20 xerostomia subjects had reduced SFR (meeting AECG criterion V), and five xerostomia subjects tested positive for serum anti - Ro/SSA and/or anti - La/SS-B autoantibodies (Table 4.6 and Table 4.7).

Five xerostomia subjects (P9, P13, P16, P17, P19) could be classified Sjögren’s Syndrome because they met four diagnostic criteria for Sjögren’s syndrome (Table 4.6 in Chapter 4 section 4.6 below); all five subjects had positive results for antibodies to Ro/SSA or La/SSB antigens or both, although only two had Sjögren’s syndrome confirmed by biopsy.
### Table 4.6. Simplified Sjögren’s syndrome diagnostic criteria used in this study.

<table>
<thead>
<tr>
<th>Patient Subjects</th>
<th>Dry Mouth (II)</th>
<th>Ocular Signs and Symptoms* (I and III)</th>
<th>Biopsy (IV)</th>
<th>SFR (ml/min) (V)</th>
<th>Antibodies (α-Ro/Lo) (VI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P3</td>
<td>Y</td>
<td>Not reported (NR)</td>
<td></td>
<td>0.02</td>
<td>Negative</td>
</tr>
<tr>
<td>P4</td>
<td>Y</td>
<td>NR</td>
<td>Negative</td>
<td>0</td>
<td>Negative</td>
</tr>
<tr>
<td>P5</td>
<td>Y</td>
<td>NR</td>
<td>Negative</td>
<td>0.1</td>
<td>Negative</td>
</tr>
<tr>
<td>P6</td>
<td>Y</td>
<td>NR</td>
<td>Negative</td>
<td>0.1</td>
<td>Negative</td>
</tr>
<tr>
<td>P8</td>
<td>Y</td>
<td>NR</td>
<td>NA</td>
<td>0.1</td>
<td>Negative</td>
</tr>
<tr>
<td>P9</td>
<td>Y</td>
<td>NR</td>
<td>Positive</td>
<td>0.07</td>
<td>Positive</td>
</tr>
<tr>
<td>P10</td>
<td>Y</td>
<td>NR</td>
<td>NA</td>
<td>0.08</td>
<td>Negative</td>
</tr>
<tr>
<td>P11</td>
<td>Y</td>
<td>NR</td>
<td>Negative</td>
<td>0.3</td>
<td>Negative</td>
</tr>
<tr>
<td>P12</td>
<td>Y</td>
<td>NR</td>
<td>NA</td>
<td>0.08</td>
<td>Negative</td>
</tr>
<tr>
<td>P13</td>
<td>Y</td>
<td>NR</td>
<td>Positive</td>
<td>0.1</td>
<td>Positive</td>
</tr>
<tr>
<td>P14</td>
<td>Y</td>
<td>NR</td>
<td>NA</td>
<td>0.1</td>
<td>Negative</td>
</tr>
<tr>
<td>P15</td>
<td>Y</td>
<td>NR</td>
<td>NA</td>
<td>0</td>
<td>Negative</td>
</tr>
<tr>
<td>P16</td>
<td>Y</td>
<td>Y</td>
<td>Negative</td>
<td>0</td>
<td>Positive</td>
</tr>
<tr>
<td>P17</td>
<td>Y</td>
<td>Y</td>
<td>Negative</td>
<td>0</td>
<td>Positive</td>
</tr>
<tr>
<td>P18</td>
<td>Y</td>
<td>Y</td>
<td>Negative</td>
<td>0.22</td>
<td>Negative</td>
</tr>
<tr>
<td>P19</td>
<td>Y</td>
<td>Y</td>
<td>NA</td>
<td>0.04</td>
<td>Positive</td>
</tr>
<tr>
<td>P20</td>
<td>Y</td>
<td>Y</td>
<td>NA</td>
<td>0.04</td>
<td>Negative</td>
</tr>
<tr>
<td>P21</td>
<td>Y</td>
<td>NR</td>
<td>NA</td>
<td>0.25</td>
<td>Negative</td>
</tr>
<tr>
<td>P22</td>
<td>Y</td>
<td>Y</td>
<td>NA</td>
<td>0.1</td>
<td>NA</td>
</tr>
<tr>
<td>P23</td>
<td>Y</td>
<td>Y</td>
<td>NA</td>
<td>0.3</td>
<td>Negative</td>
</tr>
</tbody>
</table>

a. In Sjögren’s syndrome AECG Diagnostic Criteria, ocular signs and ocular symptoms are separate criteria; in Table 4.6 both criteria were listed together in one column.

b. In Sjögren’s syndrome AECG Diagnostic Criteria, the critical value of SFR is defined as unstimulated whole salivary flow (≤1.5 ml in 15 minutes, that is ≤0.1 ml/min) (Vitali et al., 2002). The abnormal salivary flow rate were suggested as unstimulated whole saliva ≤0.1 ml/min (Edgar, 1990).
Table 4.7. Summary of the degree to which xerostomia subjects met the simplified Sjögren’s syndrome criteria.

<table>
<thead>
<tr>
<th>Sjögren’s syndrome criteria</th>
<th>Number of xerostomia subjects tested</th>
<th>Number of xerostomia subjects positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ocular Signs and Symptoms</td>
<td>20</td>
<td>6</td>
</tr>
<tr>
<td>Biopsy</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>SFR ≤ 0.1 ml/min</td>
<td>20</td>
<td>16</td>
</tr>
<tr>
<td>Antibodies tested</td>
<td>19</td>
<td>5</td>
</tr>
</tbody>
</table>

For xerostomia subjects with autoimmune disorders, other than the five xerostomia subjects with Sjögren’s syndrome, lupus was mentioned by another five xerostomia subjects. Other autoimmune diseases reported included Crohn’s disease, rheumatic arthritis and a connective tissue disorder. Two control subjects also reported arthritis. It should be noted that these other autoimmune illnesses are self-reported and therefore it is unknown whether they meet the objective assessment criteria for each disease.

4.7 Saliva flow rate in xerostomia subjects

The SFR of xerostomia subjects with and without a positive serum antibody test were compared (Figure 4.3). The results showed that all xerostomia subjects who tested positively for serum antibodies (-Ro/-La) have a SFR equal or less than 0.1 ml/min.
Figure 4.3. SFR in xerostomia subjects with and without antibodies to Ro/SSA or LA/SSB.

The orange bars represent xerostomia subject that tested positive for serum test antibodies and met four of the Sjögren’s Syndrome diagnostic criteria. Blue bars represent all other xerostomia subjects.
Chapter 5: Yeast Study
5.1 Introduction

Dry mouth can affect up to 30% of the population, and there are many factors that can cause dry mouth, including medications and Sjögren’s syndrome. The subjective sensation of oral dryness can be accompanied with a reduced production of saliva. Saliva flow and composition is an important part of protective mechanisms in the mouth, and the reduction in saliva flow could alter the micro-environment and may result in an increase in colonization by oral yeast.

*Candida* spp. are frequent colonizers of the oropharynx in humans, and colonisation is a crucial step in the development of *Candida*-related infections. High salivary *Candida* counts may predispose to (or reflect the presence of) oral candidosis, which not only results in localized pathological conditions, but can also serve as a source of systemic infection (Torres *et al.*, 2003). It has been shown that low SFRs are associated with higher oral *Candida* counts (Radfar *et al.*, 2003).

The objective of this project was to determine if there is a significant association between oral yeast carriage and a sensation of dry mouth as reported by a cohort of xerostomia subjects attending oral medicine clinics. The specific aims of this part of my thesis were as follows:

1. To compare the difference in oral yeast carriage between xerostomia subjects and age- gender-matched controls.
2. To determine if there was a significant association between xerostomia and oral carriage of yeasts.

3. To assess the host factors that may be associated with yeast carriage.

5.2 Yeast culture study

The yeast culture studies were carried out using the methods described in Chapter 3. Section 3.5.

5.2.1 CHROMagar™ Candida plate as a culture medium

Yeast were identified using the chromogenic agar plates (CHROMagar™ Candida, Fort Richard Laboratories, Auckland, New Zealand), as described in Section 3.5. CHROMagar™ Candida (Figure 5.1) is a differential culture medium that allows selective isolation of yeasts and simultaneously presumptive identification of the colonies as different Candida species (Pfaller et al., 1996). It can be used for isolation and differentiation of clinically significant Candida species.

Figure 5.1. The typical appearance of microorganisms on CHROMagar Candida plate (http://www.chromagar.com/).
The typical appearance of microorganisms (Figure 5.1) as described from the manufacturer’s website is (a) \( C. \) albicans→green, (b) \( C. \) krusei → pink, fuzzy (c) \( C. \) tropicalis→ Metallic blue (d) Other species → white to mauve. Based on a previous study using DNA sequencing for strain identification (Holmes A.R, unpublished observations) all light purple colonies were presumptively identified as \( C. \) glabrata.

![Figure 5.1. Typical appearance of microorganisms on CHROMagar™ Candida](http://www.chromagar.com/).

### 5.2.2 CHROMagar™ Candida yeast culture studies of participants

Yeast culture studies were carried out as described in Chapter 3 Section 3.5. Representative yeast colonies are shown in Figure 5.3. Numbers of types (colours) of colonies and total counts were recorded.

![Figure 5.3. Examples of yeast cultured on CHROMagar™ plates from saliva rinse samples from study subjects.](image)
As shown in Figures 5.4 and 5.5, 18 of the xerostomia samples contained yeast, and of these, seven contained more than one yeast species. Seven of the xerostomia subjects carried over 1000 CFU/ml saliva rinse. In contrast, only two control subjects were positive for yeast, and none of the control samples contained more than one type of yeast species.
Chapter 5. Yeast Study

Oral Yeast Carriage and Saliva Protein Profiles of Xerostomia Subjects and Matched Controls

Figure 5.4. Colony types and numbers (CFU/ml) in saliva rinse samples from xerostomia subjects.

Figure 5.5. Colony types and numbers (CFU/ml) in saliva rinse samples from control subjects.
Based on the Chromagar yeast cultures, the yeast species present were presumptively identified. As shown in Figure 5.6, one control subject carried *C. albicans* and another *C. glabrata*, whilst significantly higher numbers (18 of 20) of xerostomia subjects were found to be positive for *C. albicans* and 6 of the 18 *C. albicans* - positive xerostomia subjects also carried *C. glabrata*. 

**Figure 5.6.** CHROMagar™ *Candida* presumptive yeast identification in saliva rinse samples.
5.2.3 Factors that may influence yeast carriage

In the current study, xerostomia subjects who were serum – anti-Ro/La antibody positive, displayed objective oral signs of dry mouth with a SFR less than 0.1 ml/min (ranging from 0 to 0.1ml/min). The highest yeast counts were found in xerostomia subjects recording zero for SFR (Table 5.1).

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>#Colony types</th>
<th>Total Average CFU/ml</th>
<th>SFR (ml/min)</th>
<th>Positive Serum Antibodies Anti-Ro/La Tests</th>
</tr>
</thead>
<tbody>
<tr>
<td>P3</td>
<td>1</td>
<td>20</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>P4</td>
<td>2</td>
<td>1435</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>P5</td>
<td>2</td>
<td>1010</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>P6</td>
<td>1</td>
<td>700</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>P8</td>
<td>3</td>
<td>140</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>P9</td>
<td>2</td>
<td>20</td>
<td>0.07</td>
<td>Positive</td>
</tr>
<tr>
<td>P10</td>
<td>0</td>
<td>0</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>P11</td>
<td>2</td>
<td>20</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>P12</td>
<td>1</td>
<td>30</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>P13</td>
<td>1</td>
<td>40</td>
<td>0.1</td>
<td>Positive</td>
</tr>
<tr>
<td>P14</td>
<td>2</td>
<td>1380</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>P15</td>
<td>1</td>
<td>1385</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>P16</td>
<td>1</td>
<td>1835</td>
<td>0</td>
<td>Positive</td>
</tr>
<tr>
<td>P17</td>
<td>2</td>
<td>1295</td>
<td>0</td>
<td>Positive</td>
</tr>
<tr>
<td>P18</td>
<td>0</td>
<td>0</td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td>P19</td>
<td>1</td>
<td>1000</td>
<td>0.04</td>
<td>Positive</td>
</tr>
<tr>
<td>P20</td>
<td>1</td>
<td>250</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>P21</td>
<td>3</td>
<td>810</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>P22</td>
<td>4</td>
<td>1465</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>P23</td>
<td>2</td>
<td>390</td>
<td>0.3</td>
<td></td>
</tr>
</tbody>
</table>
5.2.3.1 Yeast carriage and SFR

The relationship between the salivary flow rate of xerostomia subjects and oral yeast carriage is shown in Figure 5.7. Although the relationship appeared to be quite weak ($R^2 = 0.16$, $r=-0.4$), there was a trend that an increased CFU/ml was associated with a lower SFR. As the control subjects were selected as not having self-reported dry mouth, the SFRs were not recorded and therefore the SFR data for the control subjects was not included.

![Figure 5.7. The correlation between yeast carriage and SFR.](image)
5.2.3.2 Yeast carriage and Sjögren’s syndrome subjects

The numbers of yeast species found in subjects who met four of the Sjögren’s syndrome diagnostic criteria (Vitali et al., 2002), and in the other xerostomia subjects was investigated (Figure 5.8).

Figure 5.8. Yeast colony types and the serum antibodies (-Lo/-Ra tests).

The xerostomia subjects who met four of the Sjögren’s syndrome diagnostic criteria were represented by the orange bars, and the other xerostomia subjects are represented by the blue bar.

Two out of the five Sjögren’s syndrome subjects had two types of yeast species in the saliva rinse, and the remaining three subjects only carried one yeast species.
species in their saliva. For the other xerostomia subjects, eight of fifteen subjects carried at least two types of yeast species, and among those, two subjects carried three and one with four types of yeast colony. The results show that yeast were found in all Sjögren’s syndrome subjects, but those subjects did not carry more yeast types in comparison to xerostomia subjects without Sjögren’s syndrome. The data do not indicate that xerostomia subjects who tested positive for serum anti-Ro/LA antibodies have more or less yeast types in their saliva than other xerostomia subjects.

The total yeast carriage of all xerostomia subjects is shown in Figure 5.9. Three out of the five subjects that met four of the Sjögren’s syndrome diagnostic
criteria had total yeast counts $\geq 10^3$ CFU/ml. Five of the other fifteen xerostomia subjects had yeast counts $\geq 10^3$ CFU/ml.

### 5.2.3.3 Yeast carriage and denture wearing

Yeast carriage and denture wearing in the xerostomia subjects is shown in Figure 5.10. Six xerostomia subjects wore dentures. For both the denture and non-denture groups, all but one participant carried *C. albicans*. Approximately one third of subjects in both groups also had other yeast species identified. Furthermore, a higher proportion of denture wearers had a total yeast average CFU/ml $\geq 100$ (83% compared to 57% for non denture wearers), see Figure 5.10 below, however a lower proportion of denture wearers had a higher level of carriage (CFU/ml $\geq 1000$) compared to the non-denture wearing xerostomia subjects.
5.3 Summary of yeast study findings and the statistical analysis

The percentage of experimental samples carried yeast, with more than one yeast type, with at least one yeast colony type $\geq 10^2$ average CFU/ml saliva, and with at least one yeast colony type $\geq 10^3$ average CFU/ml saliva rinse were summarized in the Table 5.2.

<table>
<thead>
<tr>
<th>Denture wearing xerostomia subjects (6)</th>
<th>% Colonized with yeast</th>
<th>% Colonized with C. albicans</th>
<th>% Colonized with other yeast types</th>
<th>% with Total average CFU/ml $\geq$ 100</th>
<th>% with Total average CFU/ml $\geq$ 1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denture wearing xerostomia subjects (6)</td>
<td>83</td>
<td>83</td>
<td>33</td>
<td>83</td>
<td>33</td>
</tr>
<tr>
<td>Non-denture wearing xerostomia subjects (14)</td>
<td>93</td>
<td>93</td>
<td>36</td>
<td>57</td>
<td>43</td>
</tr>
</tbody>
</table>

Figure 5.10. Denture wearing status and oral yeast carriage of xerostomia subjects.
### Table 5.2. Summary of yeast study data in experimental groups.

<table>
<thead>
<tr>
<th>Sample Group</th>
<th>No. of subjects</th>
<th>No. (%) of subjects with yeast</th>
<th>% of subjects with &gt;1 type of yeast species</th>
<th>% of subjects with at least one yeast species ≥ 10^2 CFU/ml saliva rinse</th>
<th>% of subjects with at least one yeast species and ≥ 10^3 average CFU/ml saliva rinse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xerostomia subjects</td>
<td>20</td>
<td>18 (90)</td>
<td>50</td>
<td>60</td>
<td>35</td>
</tr>
<tr>
<td>Controls</td>
<td>20</td>
<td>2 (10)</td>
<td>0</td>
<td>10</td>
<td>5</td>
</tr>
</tbody>
</table>

The Mann-Whitney test was used to analyse the difference in prevalence of oral yeast carriage between the xerostomia subjects and matched controls. There is a statistical significant difference of yeast carriage between xerostomia subjects and matched controls ($p<0.05$).

### 5.4 Conclusions

1. The results showed that there was a higher prevalence (18/20) of yeast carriage in saliva samples from xerostomia subjects than in control samples (2/20). More yeast species (two or more) and greater yeast numbers (>10^3 CFU/ml) were detected in a greater proportion of xerostomia samples than in control samples.

2. Based on statistical analysis (Mann-Whitney test) we conclude that there is a statistically significant difference ($p<0.05$) between oral yeast carriage in xerostomia subjects and matched controls.

3. Individuals with xerostomia may be more susceptible to oral yeast infections.
Chapter 6: Protein Study

A Novel Experimental Approach To the Analysis of Human Salivary Proteins with the Aid of Protein Separation Analysis Software
This chapter will be presented in two parts. In Part I, the development of experimental approaches to the cross-sectional study of salivary protein expression in individuals with self-reported dry mouth will be described; in Part II, the main study, the analysis of the salivary proteins detected in saliva rinse samples from both xerostomia and control subjects will be presented.

The goal is to determine whether sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) protein profiling can be used to detect consistent differences between the saliva proteins of study subjects and controls, which could be a contributing factor to colonization with yeast in dry mouth subjects, as described in Chapter 5.

Hypothesis: that the SDS-PAGE protein profiles of saliva rinse samples from dry mouth xerostomia subjects will show consistent qualitative differences from control subject sample profiles.
6.1 Part I: Development of methodology for analysis of the salivary proteins of study subjects

6.1.1 Introduction

The SDS–PAGE technique, established in the 1960s, permits the separation of proteins on the basis of surface charge and molecular mass (Righetti, 2005). SDS-PAGE, together with protein staining techniques, allows the qualitative and quantitative analysis of protein profiles (both one dimensional and two dimensional) representing the many polypeptides present in a complex mixture of proteins, such as human saliva. SDS-PAGE has been employed widely to study the protein composition of whole saliva (stimulated and unstimulated), specific major salivary gland saliva samples, and the separation of more than 30 protein bands has been reported (Banderas-Tarabay et al., 2002; Schwartz et al., 1995). Although electrophoretic profiles of human saliva polypeptides may differ markedly between different studies (Morales-Bozo et al., 2006) because of methodological variations and subject-specific polymorphisms, this does not necessarily preclude intra-study comparisons of protein profiles as proposed for the current study. This project took a novel experimental approach to study the salivary proteins present in saliva rinse samples by using SDS-PAGE in combination with analysis of the protein profiles with a computer programme: Gel
Chapter 6. Protein Study

Doc™ EZ system – Image Lab™ software (Life Science, Bio-Rad laboratories, Inc).

6.1.2 Outline of experiments conducted to develop and validate the experimental approach

A series of experiments was conducted to develop the experimental approach for the analysis of subject saliva proteins. A brief outline of the experiments conducted is shown in Figure 6.1.

1. Experiments to validate the use of SDS-PAGE to separate and detect proteins present in saliva rinse samples.

2. Experiments to evaluate two methods of SDS-PAGE gel production to be used in the main study; (i) ’in-house’ production of gels from individual components; and (ii) commercially available ‘ready-made’ gels.

3. Experiments to evaluate two different staining methods for the generation of protein profiles.

4. Experiments to determine the day-to-day variation in saliva rinse protein profiles.

5. Experiments to establish a standard saliva rinse sample that will be used as an internal standard on all SDS-PAGE gels in Part II.

6. Experiments to validate the use of the Gel Doc™ EZ system for saliva protein profile analysis.

Figure 6.1. Experimental steps undertaken to validate experimental approach.

The first experimental step was a pilot study in which salivary proteins of healthy individuals were separated using SDS-PAGE electrophoresis, and protein bands were visualised with EZBlue™ stain and silver stain as described in section
3.6 and section 3.7. The same methods were used to demonstrate that saliva proteins can be separated and visualised using saliva rinse samples collected from xerostomia subjects, and the experiments were conducted at least twice. To enable a comparison of salivary proteins from different individuals, an internal protein standard was established by combining the saliva rinse samples collected on consecutive days from two individuals of the pilot group. To ensure the consistency and adequacy of SDS-PAGE gels used in the main study, experiments were conducted to compare the lab-made and commercially available 'ready-made' gels (Ready Gel Precast Gels, Bio-Rad Laboratories, Inc) with both staining methods. The protein loading dye used was made in the molecular microbiology laboratory and as variations can be introduced into SDS-PAGE analysis when using loading dyes from different batches, therefore one batch of protein loading dye was chosen to be used throughout the main protein study, and was stored in freezer in aliquot.

6.1.3 Experimental aims for protein analysis part I:

1. To validate the use of SDS-PAGE to separate and detect proteins present in saliva rinse samples.

2. To evaluate two methods of SDS-PAGE gel production to be used in the main study: (i) 'in-house' production of gels from individual components; and (ii) commercially available 'ready-made' gels (Ready Gel Precast Gels, Bio-Rad Laboratories, Inc.).

3. To evaluate two different staining methods for the generation of protein profiles.
4. To determine the day-to-day variation in saliva rinse protein profiles.

5. To establish a standard saliva rinse sample that will be used as an internal standard on all SDS-PAGE gels in Part II.

6. To identify prominent protein bands in saliva rinse protein profiles.

7. To validate the use of the Gel Doc™ EZ system for saliva protein profile analysis.

6.1.4 Experimental approaches

6.1.4.1 Use of SDS-PAGE to separate and detect proteins present in saliva rinse samples

Over the past 50 years the physiological importance of saliva has been increasingly recognized and the development of new analytical techniques allows exploration of the biochemical and physiological properties of saliva. The use of metabolomics, genomics, proteomics and bioinformatics provides ways to study the roles of saliva in health and disease. However, the study of salivary components poses particular challenges in terms of its physical properties, and possible variations both between and within individuals (temporal variations). Various methods have been used to collect saliva samples; most commonly saliva is collected by expectorating into a container or chewing on an absorbent material.

Ideally whole saliva, either stimulated or unstimulated, would be used in a study of salivary protein content, however, given that the study population with self-reported dry mouth were likely to have low salivary flow rate, collection of
whole saliva was considered inappropriate. A method of collecting saliva rinse samples using commercially bottled water was developed (Chapter 3, section 3.2.3) as a procedure likely to cause least distress to the patient. For consistence all samples were frozen in duplicate (Chapter 3, section 3.4) and only thawed once before analysis. One issue with use of saliva rinses became apparent early in the study; saliva rinse samples have low protein concentrations. When initial samples taken from healthy volunteers were analyzed for protein content using the Bradford technique for detection of total protein (Chapter 3 section 3.3.1), low levels of protein were observed (Figure 6.2 and Table 6.1).

![Figure 6.2. Representative standard curve for Bradford assay](image-url)
Table 6.1. The total protein content of saliva rinse samples taken from seven healthy volunteers.

<table>
<thead>
<tr>
<th>Subject Code</th>
<th>Total Protein concentration*</th>
<th>(µg/20µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.33</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.183</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.403</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.35</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0.29</td>
<td></td>
</tr>
</tbody>
</table>

*as determined by the Bradford protein assay and calculated from a standard curve (Figure 6.2) as described in section 3.3.1

Therefore, for SDS-PAGE analysis, a constant volume of all samples was applied to the gel (i.e. 20µl plus 4 µl of 6 x loading buffer; see Chapter 3, section 3.2.6.3). This was the maximum amount of sample that could be loaded into wells of the polyacrylamide gels used in this study. Therefore, as can be seen in Figure 6.3, there was variation in the total amount of protein loaded into each lane. However, as shown in Figure 6.3, protein profiles could be observed in saliva rinse samples from 6 healthy volunteers, stained with either silver staining or EZBlue™.
Figure 6.3. A representative SDS-PAGE analysis of saliva rinse samples from 6 healthy volunteers, using laboratory made gels.

The above Figure showing that separated polypeptides were detectable by two staining methods: silver staining (a) or EZBlue™ (b).

6.1.4.2 Evaluation of two methods of SDS-PAGE gel production.

Protein electrophoresis involves the migration of charged protein molecules in an applied electric field. The electrophoretic mobility of an individual protein depends on a variety of factors including the aggregate charge on its surface, its size and shape, the strength of the applied electric field, and the nature of the SDS-PAGE gel medium in which the electrophoretic mobility takes place. Considering the variation that might be introduced in each batch of gels made at different times, we considered using commercially made gels, in order to reduce the inter-gel variation. Repeated experiments were conducted to compare the laboratory-made and commercially available Ready Gel Tris-HCl Precast Gels (Bio-Rad Laboratories, Inc.). Saliva rinse samples from two healthy individuals, denoted as individual S1 and individual S2, were used in these experiments, and the gels were stained with both EZBlue™ and silver stain (Section 3.2.6.4.1).
Although protein band profiles were observed both on laboratory made and commercially produced SDS-PAGE gels, the laboratory made gels showed a clearer protein band pattern (Figure 6.4 and Figure 6.5). However, there could be variation between laboratory-made gels which would affect the migration of proteins. So to minimise this possible variation we decided to use manufactured gels purchased in one batch to provide the best possible consistency.
Figure 6.5. Comparison of saliva rinse protein profiles generated by SDS-PAGE (either laboratory made, or commercially produced) and stained with Silver.

6.1.4.3 Evaluation of gel staining methods

The next consideration in the protocol development was centered on suitable staining methods to visualise separated protein bands. Two methods of staining were evaluated: a Coomassie Blue based commercial staining kit EZBlue™ (Sigma-Aldrich Co LLC) and a silver staining technique (see Chapter 3, section 3.2.6.4.2).
Coomassie Blue was first used to stain electrophoresis gels in the early 1970s to analyse the major polypeptides of the human erythrocyte membrane (Fairbanks et al., 1971). Detection of protein bands in a gel following Coomassie Blue staining depends on the non-specific binding of the dye, Coomassie Brilliant Blue R, to proteins. The detection limit is 0.3 to 1 µg/protein band (Sasse and Gallagher, 2009). The EZBlue Gel staining reagent is a ready-to-use solution containing Brilliant Blue G-250 at a low pH and, according to the manufacturer, can detect 5 ng of protein. Coomassie Blue is considered a common method used for simple, low cost protein visualization (Steinberg, 2009; Westermeier and Marouga, 2005).

Silver staining is widely regarded as the standard by which all other “ultrasensitive” staining methods are judged, and offers the advantage that it is considerably more sensitive than Coomassie Blue. As it enables the detection of more salivary proteins (Figure 6.5), it was the protein visualization method chosen for the project. Detection of protein bands in a gel by silver staining depends on binding of silver to various chemical groups (e.g., sulfhydryl and carboxyl moieties) in proteins. The detection limit is 2 to 5 ng/protein per band (Rabilloud et al., 1994). The basis of protein detection is reduction of protein-bound silver ions to metallic silver and, to a lesser extent, in some protocols, localized deposition of silver sulfide (Rabilloud et al., 1994). Silver staining is the most complex and variable protein gel staining methodology, which sets the standard for detection sensitivity but quantification of protein is not a simple matter, due to
the complex nature of the colour development step and differences between proteins in their silver signal sensitivity (Steinberg, 2009).

As shown in Figures 6.4 and 6.5, protein profiles were detectable using both staining techniques. Both methods were used in Part II of the study, but most analysis was done using the silver stained gels.

6.1.4.4 Demonstration of negligible day-to-day variation in the saliva rinse protein profiles of two healthy individuals

It was important to determine whether or not the SDS-PAGE protein profile generated from a saliva rinse sample from an individual showed day-to-day variation. If this was the case, then a cross-sectional study could not be used to compare the profiles from different individuals. Therefore saliva rinse samples were taken on five consecutive days at approximately the same time period in the day from each of two healthy volunteers.

As shown in Figure 6.6, there was some variation in intensity of the profiles for the samples from different days (eg day 1 and day 2), indicating that there was differing total protein concentration between samples. However, importantly, there was only minor variation in the banding patterns between days; ie, a similar protein profile was obtained each time a sample was obtained from an individual.
Figure 6.6. SDS-PAGE protein profiles for saliva rinse samples collected on five consecutive days from two individuals (S1 and S2) using laboratory made gels.

In the above Figure, D indicates day sample obtained.

6.1.4.5 Internal protein standard

As a matter of standard procedure, all polyacrylamide gels, in both Part I and Part II of this study, contained a lane of a commercially available mix of pre-stained marker proteins of known molecular masses (PageRuler™ Plus Prestained Protein Ladder, Fermentas, Thermo Scientific, USA). Use of the markers allowed calculation of the approximate molecular mass of separated saliva proteins. However, it was also important to include on each gel an internal control (internal standard) consisting of a standard saliva protein profile, to ensure consistent electrophoresis and protein staining from gel to gel and enable lanes from different gels to be compared directly. Saliva rinse samples collected from one individual (S1; Figures 6.7) on two days were combined to form an internal
standard (St). Aliquots were frozen so that an internal standard could be included with each gel. The lane is labeled St in all subsequent figures.

### 6.1.4.6 Identification of prominent bands in the saliva rinse protein profiles of two individuals

As shown in Figures 6.6, relatively consistent protein patterns were observed in two healthy individuals, denoted S1 and S2. To identify prominent protein bands in the saliva protein profile of both individuals that could be looked for in profiles from study participants, repeat gels of the five daily samples from each individual were prepared and stained with silver. Three independent examiners visually examined images of the stained gels and identified 11 prominent protein bands – SP1 to SP11 (Figure 6.7 and 6.8).
Figure 6.7. Gel image showing 11 prominent polypeptides identified from saliva samples obtained from individual S1 on five consecutive days (S1D1-S1D5).

In the above Figure, marker lane and internal standard lane are marked M and St respectively. Arrows indicate positions of the 11-polypeptide bands consistently identified in the SDS-PAGE separated internal standard saliva samples by three independent examiners.
Figure 6.8. Gel image showing 11 prominent polypeptides identified from saliva samples obtained from individual S2 on five consecutive days (S2D1-S2D5).

In the above Figure, marker lane and internal standard lane are marked M and St respectively. Arrows indicate positions of the 11 polypeptide bands consistently identified in the SDS-PAGE separated internal standard saliva samples by three independent examiners.

6.1.4.7 Validation of the use of the Gel doc™ EZ system for saliva protein profile analysis.

The consistent detection of prominent protein bands was supported by the lane profile analysis of a gel image (Figure 6.9) generated by the Gel Doc™ EZ system (Gel Doc). The Gel Doc™ EZ software identified the same proteins in the two lanes containing the internal standard (St, lanes 2 and 3, Figure 6.9). The software also measured the intensity of stained bands and calculated their molecular weights (Figures 6.9, 6.10 and 6.11).
Figure 6.9. Bands identified on the gel image using the Gel Doc™ EZ system.

Figure 6.10. Example of saliva protein profile analysis by Gel Doc EZ™ system.

In the above Figure, the molecular weights (kDa) of 11 prominent proteins in lane 2 of Figure 6.9 were identified by comparison with the pre-stained marker proteins, and are indicated by the numbers above each peak.
Figure 6.11. Example of saliva protein profile analysis by Gel Doc EZ™ system.

In the above Figure, the molecular weights (kDa) of 11 prominent proteins in lane 3 of Figure 6.9 were identified by comparison with the pre-stained marker proteins, and are indicated by the numbers above each peak.

The molecular weights of the 11 prominent saliva proteins as determined using the Gel Doc™ EZ system are shown in Table 6.2. There was good agreement between the calculated molecular weights of the proteins in lanes 2 and 3 of Figures 6.9, 6.10 and 6.11.
Table 6.2. Comparison of the molecular weights of prominent protein bands S1-S11 in saliva rinse samples as determined using the Gel Doc™ EZ system.

<table>
<thead>
<tr>
<th>Protein band identity</th>
<th>Calculated MW (kDa) Lane 2, Figures 6.9 and 6.10</th>
<th>Calculated MW (kDa) Lane 3, Figures 6.9 and 6.11</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP1</td>
<td>306.3</td>
<td>304.2</td>
</tr>
<tr>
<td>SP2</td>
<td>185.7</td>
<td>187.3</td>
</tr>
<tr>
<td>SP3</td>
<td>101.5</td>
<td>101.5</td>
</tr>
<tr>
<td>SP4</td>
<td>69.2</td>
<td>70.2</td>
</tr>
<tr>
<td>SP5</td>
<td>58.9</td>
<td>59.6</td>
</tr>
<tr>
<td>SP6</td>
<td>54.3</td>
<td>55.2</td>
</tr>
<tr>
<td>SP7</td>
<td>40.3</td>
<td>42.5</td>
</tr>
<tr>
<td>SP8</td>
<td>30.3</td>
<td>31.0</td>
</tr>
<tr>
<td>SP9</td>
<td>29.1</td>
<td>29.8</td>
</tr>
<tr>
<td>SP10</td>
<td>26.9</td>
<td>27.5</td>
</tr>
<tr>
<td>SP11</td>
<td>26.1</td>
<td>26.6</td>
</tr>
</tbody>
</table>

The analysis of SDS-PAGE gels stained with silver (Fig 6.9 lanes 2 and 3).

The use of the Gel Doc system allowed the calculation of the molecular weight of each band in a lane, however the image quality obtained with the Gel Doc™ EZ camera (Figure 6.12) was not as good as the gel images obtained using a Nikon digital SLR camera (Figures 6.13).
Figure 6.12. Example of a silver-stained gel image acquired with the Gel Doc™ EZ system.

In the above Figure, the image of silver stained gel captured by Gel Doc™ displayed in grey scale for comparison to Figure 6.13 below.

Figure 6.13. Example of a silver-stained gel image of the same gel as in Figure 6.12 obtained with a Nikon digital SLR camera.

In the above Figure, the images of silver stained gels was converted to grey scale for comparison to Figure 6.12.
6.1.5 Summary and conclusions for protein analysis part I

1. Despite the finding that the standard Bradford protein assay detected low protein concentrations in saliva rinse samples (Section 6.1.4.1), SDS-PAGE analysis of the samples demonstrated that proteins could be visualized when stained with either Coomassie EZBlue™ or silver stain.

2. To ensure inter-gel consistency, commercially available Ready Gel Precast gels – Bio-Rad (Life Science, Bio-Rad laboratories, Inc) were chosen for use in the next phase of the SDS-PAGE analysis of protein bands in xerostomia and control saliva rinse samples.

3. Two gel staining techniques were tested and silver staining was shown to have greater sensitivity of detection than the Coomassie-based EZBlue™ stain. Therefore silver-staining was chosen for use in the next phase of the project.

4. Importantly, a consistent protein profile was observed in samples taken on different days between from two individuals (Individual S1 and Individual S2). Thus SDS-PAGE protein analysis of saliva rinses samples was validated for the proposed cross-sectional study in Part II of the project.

5. Eleven prominent protein bands were consistently identified in silver-stained SDS-PAGE profiles using both visual inspection of gel images and the Gel Doc™ EZ image analysis system.

6. An internal standard was produced and stored in aliquots to allow direct comparison of several SDS-PAGE gels used to analyze the saliva rinse samples in Part II of this study. The internal standard also assisted assessment of the
presence or absence of the eleven identified prominent protein bands in subsequent saliva rinse samples.

7. The Gel Doc™ EZ analysis system was found to be suitable for qualitative and quantitative analysis of SDS-PAGE protein banding profiles. The image quality of the photographed gels obtained using the Gel Doc™ EZ system was however, not as good as that obtained using a digital SLR camera. Therefore in Part II, both methods of analysis were used.
6.2 Part II: Analyzing protein profiles of saliva rinse samples from xerostomia subjects and controls

6.2.1 Introduction

Saliva is an important body fluid for the maintenance of oral homeostasis and protection against extrinsic pathogens (Farnaud et al., 2010). A wide spectrum of components within saliva protects the integrity of oral tissues and also can provide clues concerning local and systemic diseases and conditions (Kawas et al., 2011). A promising approach to the study of saliva is the identification of its protein components using proteomics (Helmerhorst and Oppenheim, 2007). Moreover, a comparison between samples from healthy and diseased subjects may reveal unique or increased or decreased levels of specific proteins that may be used as disease biomarkers. (Amado et al., 2005; Rui et al., 2004). Although this was not the aim of the current study, the salivary protein profiles of study subjects were analysed.

In this phase of the study, two groups of individuals were recruited, 20 xerostomia subjects attending an Oral Medicine clinic with self-reported dry mouth and 20 age- and gender-matched control subjects attending other clinics in the School of Dentistry, University of Otago. Recruitment details and analysis of the information collected about the study participants is presented in Chapter 3. Subject groups were further divided into four subgroups to facilitate SDS-PAGE analysis, as only limited numbers of samples, together with a protein standard and
an internal standard as described in Part I, could be run on a single gel. The gels were electrophoresed as described in section 3.6.1.1.

6.2.2 Gel alignment: internal standard protein profiles

In each gel image, the internal standard lanes were extracted and aligned together to make a composite gel image, using the protein marker lanes on each gel as references (Figure 6.14). This alignment allowed direct visual comparison of protein profiles, and the 11 prominent protein bands, SP1-SP11 were identified on each gel as described in part I. Then attempts were made to study the protein band patterns of xerostomia and control subjects both individually and then as subject groups.
6.2.3 Internal standard lane images extracted from 8 gels

Figure 6.14. Composite image of the internal control saliva sample lanes from all experimental SDS-PAGE gels.

In the above Figure, gels C1-C4 were for control saliva samples; gels P1-P4 were for xerostomia saliva samples. Images of lanes were copied from each gel image. A representative marker lane and internal standard lane from one gel are labeled as M and St respectively and were used to align the lanes. Arrows indicate positions of the 11 polypeptide bands consistently identified in the PAGE separated internal standard saliva samples shown in Figure 6.7 and 6.8.

When the combined internal standard lanes from all eight silver stained gels were subjected to Gel Doc™EZ analysis, the MWs of the standard protein bands could be determined and mean values calculated (Table 6.3). The frequency of detection of the prominent bands was recorded and, although not all the bands were detected by the Gel Doc™EZ system on all gels, indicating its lower sensitivity compared to the digital camera image in Figures 6.12 and 6.13, at least 5 values were obtained for each band (Table 6.3). In all cases the standard deviations for protein MW values were less than 7% of the mean, validating the image analysis method.
Table 6.3. Mean molecular weights (MWs) of prominent protein bands (SP1-11) from internal controls on all experimental SDS-PAGE gels.

MWs are shown for the 11 prominent protein bands from individual gels (P1-4 for xerostomia saliva samples, gels C1-C4 for control saliva samples). Means and standard deviations (SD) are also given.

<table>
<thead>
<tr>
<th>Bands on Silver-stained gels</th>
<th>Gel P1</th>
<th>Gel P2</th>
<th>Gel P3</th>
<th>Gel P4</th>
<th>Gel C1</th>
<th>Gel C2</th>
<th>Gel C3</th>
<th>Gel C4</th>
<th>Mean MW (kDa)</th>
<th>SD</th>
<th>SD as % of mean</th>
<th>Number of gels on which protein was detected</th>
<th>Frequency of band detection (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP1</td>
<td>317</td>
<td>305</td>
<td>280</td>
<td>311</td>
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6.2.4 Visualization for SDS–PAGE analysis of xerostomia and control saliva rinse samples

The protein bands in samples from all experimental groups (xerostomia subject groups 1-4 and control subject groups 1-4) were separated in SDS-PAGE gels and visualized using both EZBlue™ and silver staining methods (Figure 6.15 and Figure 6.16). Gel images were aligned as described above to generate a composite image. Note that this figure is presented simply to show how all the gels can be aligned for direct comparison between gels; therefore individual lanes are not labelled.
Figure 6.15. Composite image of gels for all groups, stained with EZBlue™.

Figure 6.16. Composite image of gels for all groups, stained with silver stain.
Coomassie blue can detect 0.3 – 1 μg protein per band (Sasse and Gallagher, 2009). Figure 6.15 shows only a few major proteins of about 55-70 kDa that stain with EZBlue™ stain. Silver is 20-60 times more sensitive than Coomassie blue and can detect quantities of approximately 5 ng protein. Silver-staining offers the greatest sensitivity for nonradioactive protein detection (Wirth and Romano, 1995). With silver stained gels, proteins with a greater range of MW were detected than with EZBlue™, from over 250 kDa to less than 27 kDa. The composite images clearly supported the difference in protein visualisation sensitively between the two methods, as reported by other researchers (Morrissey, 1981).

6.2.5 Overview of the SDS-PAGE gels of xerostomia and control saliva rinse samples stained with silver

A closer look at the aligned silver stained gels (Figures 6.17 and 6.18), shows that a cluster of three or four protein bands with MWs of 55 kDa to 72 kDa was present in all samples. This is consistent with a 2D proteomic study of whole saliva that also used capillary liquid chromatography (LC) and tandem mass spectrometry (MS/MS) to identify proteins (Hu et al., 2005). This study reported a cluster of three proteins with MW in the range of 41,710 to 57,731 Da. It appears on a visual inspection that large protein bands with MW of 130-250 kDa are present in almost all control sample groups, but the band of ~130 kDa was not evident, or greatly reduced, in several xerostomia subjects (P 3, 12, 14, 15, 16, 17, 20). Previous studies have suggested under- and over-expression of salivary proteins in xerostomia subjects with Sjögren’s syndrome (Fleissig et al., 2009).
Xerostomia subjects recruited into the current study presented as a group with self-reported dry mouth (xerostomia).

For the main study, the xerostomia subjects and the age- and gender-matched controls were paired up in groups (a) Xerostomia group 1 (P9, 13, 16, 17, 19) and control group 1 (C9, 13, 16, 17, 19); (b) Xerostomia group 2 (P3, 4, 5, 6, 8) and control group 2 (C3, 4, 5, 6, 8); (c) Xerostomia group 3 (P10, 15, 20, 21, 22, 23) and control group 3 (C10, 15, 20, 21, 22, 23); (d) Xerostomia group 4 (P11, 12, 14, 18) and control group 4 (C11, 12, 14, 18).

Within the xerostomia groups, group 1 subjects P9, 13, 16, 17, and 19 met the Sjögrens syndrome diagnostic criteria (Vitali et al., 2002). Attempts were made to group other xerostomia subjects based on commonalities of subjects such as medical conditions and denture wearing status. However, due to the small sample size (20), that could not be attained.

While there was no clear difference in the protein bands visualized in samples from xerostomia subjects with Sjögren’s syndrome and the other xerostomia subjects, a more detailed analysis of the bands present in these samples will be presented in Figures 6.23, 6.24 and table 6.4. In addition, visually there were no obvious differences of band patterns between xerostomia and control subjects.
Chapter 6. Protein Study

Oral Yeast Carriage and Saliva Protein Profiles of Xerostomia Subjects and Matched Controls

Figure 6.17. Compilation of saliva rinse protein profiles from xerostomia subject groups 1-4, with silver staining of SDS-PAGE gels
Chapter 6. Protein Study

### Oral Yeast Carriage and Saliva Protein Profiles of Xerostomia Subjects and Matched Controls

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![Figure 6.18. Compilation of saliva rinse protein profiles from control subject groups 1-4, with silver staining of the SDS-PAGE gels](image-url)

Figure 6.18. Compilation of saliva rinse protein profiles from control subject groups 1-4, with silver staining of the SDS-PAGE gels
6.2.6 SDS-PAGE analysis of both xerostomia and control saliva rinse samples using Gel Doc™ EZ and silver staining

The silver-stained gels of saliva rinse samples from xerostomia subjects and controls were analysed using the Gel Doc™ EZ system (Figures 6.19 and 6.20). The software automatically detected the major protein bands present.

![Image of SDS-PAGE analysis](image)

**Figure 6.19.** Identification of the major polypeptides in saliva rinse samples from group 1 xerostomia subjects (P9, 13, 16, 17, 19) with the Gel Doc™ EZ system.

In the above Figure, the MW marker lanes and internal standard lane are marked M1, M2 and St respectively.
Figure 6.20. Identification of the major polypeptides in saliva rinse samples from group 1 control subjects (C9, 13, 16, 17, 19) with the Gel Doc™ EZ system.

In the above Figure, the MW marker lane and internal standard lane are marked M and St respectively.

In addition, each silver-stained gel image of saliva rinse samples was examined carefully by three investigators and the presence or absence of the previously identified prominent proteins (SP1-11) noted. Furthermore, bands additional to SP1-11 were also recorded (Figure 6.21 and Figure 6.22).
Figure 6.21. Identification of polypeptides in saliva rinse samples from group 1 xerostomia subjects (P9, 13, 16, 17, 19) (observer visualization).

In this Figure, the MW marker lane and internal standard lane are marked M and St respectively. Arrowheads on the St lane indicate the positions of the 11 prominent polypeptide bands consistently identified in the SDS-PAGE-separated internal standard saliva samples shown in Figures 6.7, 6.8 and 6.14. Stars indicate positions of additional saliva protein bands resolved by the Gel DocTM EZ system. Unfilled arrowheads indicate positions of additional saliva protein bands not resolved by the Gel DocTM EZ system.
Figure 6.22. Identification of polypeptides in saliva rinse samples from group 1 control subjects (C9, 13, 16, 17, 19) (observer visualization).

In this Figure, the MW marker lane and internal standard lane are marked M and St respectively. Arrowheads on the St lane indicate the positions of the 11 prominent polypeptide bands consistently identified in the SDS-PAGE-separated internal standard saliva samples shown in Figures 6.7, 6.8 and 6.14. Stars indicate positions of additional saliva protein bands resolved by the Gel DocTM EZ system. Unfilled arrowheads indicate positions of additional saliva protein bands not resolved by the Gel DocTM EZ system.

The xerostomia subjects in Group 1 were, in fact, those who met the Sjögren’s Syndrome diagnostic criteria. This allowed comparison of this subgroup with their age- and sex-matched controls to see if there were any differences in protein profiles associated with the Sjögren’s syndrome (Figures 6.21, 6.22 and 6.23).
Figure 6.23. Comparison of proteins present in saliva rinse samples from xerostomia group 1 and control group 1.
Xerostomia subjects (P9, 13, 16, 17, 19, represented in dark shaded bars) and control group1 (C9, 13, 16, 17, 19 in lighter shaded bars)
It was evident that some proteins (e.g. those detected within the ranges 55-57 kDa, 61-66 kDa and 79-88 kDa) are ubiquitous; they were present in all samples from Sjögren’s syndrome patients, other xerostomia subjects and from controls. Other protein bands were present in a portion of the subjects (e.g. bands in the range 143-155 kDa were found in eight control subjects, but were detected in only three xerostomia patient samples).

A group of high MW proteins were of interest. It was noted that a high MW band within the range 294-304 kDa band was absent from four of the five Sjögren’s syndrome patients, but was present in four of their matched controls (Figure 6.23). Conversely a smaller high MW protein band (within the range 261-285 kDa), was present in four of five Sjögren’s syndrome patients and in only one of their matched controls. Interestingly, if a band in the 261-285 kDa was present, the band in the 294-304 kDa range was absent, and vice versa. This could indicate that the protein in the 294-304 kDa range is in fact the same as the 261-285 kDa protein, but is post-transnationally modified in some of the subjects. This possibility could be further investigated by sequencing the protein bands and by looking at saliva rinses from a larger cohort of patients. Bands in these two ranges were also present in other xerostomia subjects and their controls (Figure 6.24 and Table 6.4). Again, it can be clearly seen in Table 6.4 that if the band in the 261-285 kDa was present, the band in the 294-304 kDa range was in most cases absent, and vice-versa, a further indication that these bands may represent the same protein.
Figure 6.24. Protein bands of saliva rinse samples from xerostomia groups 2, 3, and 4 (dark shaded bars) and matched controls.
Table 6.4. Comparison of proteins present in saliva rinse samples from xerostomia group 2, 3, 4 and matched controls.

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<td>249</td>
<td>194</td>
<td>125</td>
<td>92</td>
<td>78</td>
<td>72</td>
<td>78</td>
<td>65</td>
<td>59</td>
<td>52</td>
<td></td>
<td></td>
<td>41</td>
<td>30</td>
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<td>C22 MW (KDa)</td>
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<td></td>
<td>88</td>
<td>66</td>
<td>57</td>
<td>54</td>
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<td>42</td>
<td>30</td>
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<tr>
<td>C23 MW (KDa)</td>
<td>309</td>
<td></td>
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<td></td>
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<td></td>
<td>88</td>
<td>66</td>
<td>57</td>
<td>54</td>
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<td>42</td>
<td>30</td>
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<td></td>
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<tr>
<td>C27 MW (KDa)</td>
<td>250</td>
<td>217</td>
<td>163</td>
<td>143</td>
<td>96</td>
<td>86</td>
<td>85</td>
<td>86</td>
<td>65</td>
<td>54</td>
<td>51</td>
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<td>40</td>
<td>38</td>
<td>33</td>
<td>31</td>
<td>28</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>C32 MW (KDa)</td>
<td>290</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>84</td>
<td>75</td>
<td>62</td>
<td>56</td>
<td>54</td>
<td>49</td>
<td>38</td>
<td>35</td>
<td>31</td>
<td>28</td>
<td></td>
<td></td>
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<tr>
<td>C44 MW (KDa)</td>
<td>272</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>85</td>
<td>73</td>
<td>56</td>
<td>53</td>
<td>48</td>
<td>40</td>
<td>38</td>
<td>32</td>
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<td>24</td>
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<td>C48 MW (KDa)</td>
<td>279</td>
<td>240</td>
<td>198</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>80</td>
<td>63</td>
<td>58</td>
<td>54</td>
<td>47</td>
<td>41</td>
<td>38</td>
<td>29</td>
<td>23</td>
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</table>
Overall, 13 of 20 control samples contained protein bands in the 290-314 kDa range whereas 8 of 20 samples from xerostomia subjects' (including Sjögren’s syndrome patients) contained a band in this range. Bands in the 259-285 kDa range were present in 11/20 xerostomia subjects (including Sjögren’s syndrome patients) and in 6 of 20 controls.

**6.2.7 Summary and conclusions for protein analysis part II**

1. Consistent protein band patterns were observed for the profiles of the internal standards on repeated gels. This confirmed that the SDS-PAGE techniques used were reproducible.

2. Protein bands in the ranges ~70-90 kDa, 60-68 kDa, 50-58 kDa and 27-30 kDa were ubiquitous; such bands could be detected in salivary rinse samples from all xerostomia and control subjects.

3. Two high MW protein bands showed an interesting distribution, suggestive of post-translational modification of a single protein. When one of the two proteins was present in a given sample, the other protein band was consistently absent, except in a single sample (P23). One of these two protein bands, in the range 261-285 kDa, was present in samples from four of five Sjögren syndrome patients (Fig. 6.3) and in three other xerostomia subjects (Table 6.4). It was also detected in six control samples. The other protein (in
the range 290-314 Kda) was present in samples from eight xerostomia subjects (including one Sjögren syndrome patient) and in 13 control samples.

4. Due to the small number of subjects in each group, particularly the Sjögren’s syndrome group, it was not possible to detect consistent significant differences between any of the protein profiles from either the group of xerostomia patients or the matched controls. However, as noted in (3) above, there were some differences that would warrant further investigation, but this was not within the scope of this thesis.
Chapter 7: Discussion
7.1 Introduction

In this chapter, the yeast study and salivary protein study will initially be discussed separately. The key aspects of the findings will be examined in relation to the scientific literature described in Chapter 2. The methodological approaches used will also be evaluated; their limitations will be discussed. Finally, comments will be made on possible future research directions.

7.2 Discussion part I - yeast study

7.2.1 Introduction

The objective of the yeast study was to determine if there is a significant association between oral colonization by yeast species, such as *C. albicans*, and a sensation of dry mouth. This cross-sectional study (ethical approval: LRS/10/09/034) evaluated the oral yeast carriage in single saliva rinse samples from a group (20) of individuals attending the Oral Medicine clinic. Patients who reported dry mouth were recruited. Samples were also obtained from age- and gender-matched control subjects (20). Chromogenic, selective agar plates (CHROMagar *Candida*) were used to study yeast species and numbers, and the yeast were presumptively identified according to the manufacturer’s guidelines.
7.2.2 Sampling methods for isolation of *C. albicans* from oral cavity

Over the past 50 years the physiological importance of saliva has been increasingly recognized and the development of new analytical techniques allows exploration of the biochemical and physiological properties of saliva (Schipper et al., 2007a). The use of metabolomics, genomics, proteomics and bioinformatics provide a means to study the roles of saliva in health and disease. Saliva samples collected can be unstimulated whole saliva, unstimulated saliva from specific glands (such as parotid or submandibular-sublingual), or stimulated whole saliva or stimulated saliva from specific glands. Unstimulated whole saliva represents the usual, or baseline, saliva present in the oral cavity for the majority of a 24-hour period (Williamson et al., 2012). The composition of unstimulated whole saliva often correlates to systemic clinical conditions more accurately than stimulated saliva, since materials use to stimulate flow may change salivary composition (Navazesh, 1993). Various methods have been used to collect saliva samples. One of the most commonly used methods is to ask the participant to expectorate into a container or to chew on an absorbent material which collects the saliva. In other studies, *Candida* spp. have been isolated from the oral cavity using a swab of an infected site (Silverman et al., 1990), an imprint culture (Davenport, 1970), collection of whole saliva (Oliver and Shillitoe, 1984), an oral rinse (Samaranayake et al., 1986b) or mucosal biopsy. Each method has its advantages and disadvantages (Table 7.1) and the choice of sampling technique primarily depends on the nature of the investigation.
Table 7.1. Sampling techniques for *Candida* isolation from the oral cavity (Williams and Lewis, 2000).

<table>
<thead>
<tr>
<th>Method</th>
<th>Quantitative?</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imprint</td>
<td>Yes</td>
<td>Differentiates infected person from carrier</td>
<td>Difficult to undertake</td>
</tr>
<tr>
<td>Oral rinse</td>
<td>Yes</td>
<td>Quantification of other microbes</td>
<td>Not site specific. Less sensitive if not concentrated</td>
</tr>
<tr>
<td>Swab</td>
<td>No</td>
<td>Easy to use</td>
<td>May result in removal of tissue layer</td>
</tr>
<tr>
<td>Whole saliva</td>
<td>Yes</td>
<td>Sensitive in assessing <em>Candida</em> carriage</td>
<td>Lengthy collection time required for dry mouth patients</td>
</tr>
<tr>
<td>(Draining and spitting)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smear</td>
<td>No</td>
<td>Widely applicable and can reveal hyphae</td>
<td>Lack of sensitivity</td>
</tr>
<tr>
<td>Biopsy</td>
<td>No</td>
<td>Histological diagnosis of candidosis</td>
<td>Invasive and inappropriate for research</td>
</tr>
</tbody>
</table>

In a comparative study of saliva collection methods (Navazesh and Christensen, 1982), it was found that the methods involving swabbing introduced some degree of stimulation and thus are not recommended for unstimulated whole saliva collection. Draining and spitting of whole saliva provide similar samples of unstimulated whole saliva and both are reproducible and reliable. Smears are of value in differentiating between colonization with yeast and hyphal forms, but tend to be less sensitive than culturing methods (Silverman *et al.*, 1990). The concentrated oral rinse (in which the microorganisms in saliva rinses are concentrated by centrifugation prior to plating) and imprint culture methods of sampling both provide a quantification of *Candida* carriage. This is advantageous clinically since enumeration can differentiate between accepted levels of commensal *Candida* carriage and the presence of infection (Arendorf and Walker, 1979; Silverman *et al.*, 1990).
Samaranayake compared the oral rinse technique with imprint culturing for the detection of oral microbes and recommended the oral rinse technique as the preferred screening approach for detecting oral yeast carriage (Samaranayake et al., 1986b). The main reason for using the oral rinse technique in the present study was the fact that it is quantitative and collection of whole saliva is difficult and stressful for dry mouth patients. Saliva rinse samples were collected and analysed (without concentration by centrifugation for ease of sample handling) as described in Chapter 3 section 3.2.3, Yeast were detected in 18/20 patient samples and 2/20 controls.

It is worth noting that the yeast colonization of controls observed was less than that reported (20-70%) from other cross-sectional studies of healthy subjects (Odds, 1988). This may reflect the fact in the present study that oral rinses without concentration were used as a saliva collection method rather than whole saliva. Certainly the fact that protein levels (~0.2 – 0.8 mg/ml) detected in the saliva rinse samples (Figure 6.2 and Table 6.1) were much lower than the expected protein content of whole saliva (usually reported to be ~1mg/ml (Bandhakavi et al., 2009)) suggests considerable dilution and therefore a reduced sensitivity. Yet despite this reduced sensitivity for detection of yeast in saliva, 18 of 20 patient samples were yeast-positive, indicative of a high level of colonisation in this group.
7.2.3 Chromagar Candida as a yeast culture study medium

In the majority of clinical investigations yeasts are routinely cultured on Sabouraud dextrose agar (SDA) or malt extract agar. These media are reliable and permit the isolation of several different genera, but overall, the colonies on these media are very similar in appearance and their subsequent identification requires considerable investigation in the laboratory. These difficulties may explain the apparent discrepancies in the frequency of isolation of Candida species between clinical studies (Beighton et al., 1995), although C. albicans and C. dubliniensis can be readily distinguished from other Candida species for their ability to form germ tubes (early hyphae) in a serum-containing medium. The identification of yeasts from primary clinical samples was taken a step further by Odds and Bernaerts (Odds and Bernaerts, 1994), who reported the use of a differential and selective medium CHROMagar Candida (CHROMagar Ltd., Paris, France), that permitted the presumptive identification of C. albicans, other Candida spp., and isolates of related genera. Growth on CHROMagar Candida medium results in distinctive colony colours, such as C. albicans (green), C. tropicalis (blue) C. krusei (pale rose) (Figure 5-2) (Beighton et al., 1995).

A study comparing CHROMagar Candida with other commonly used media reported a 99.4-100% specificity and sensitivity value for identification of C. albicans strains and concluded that it was superior for the primary isolation of yeasts and in identifying mixed cultures of yeasts (Yücesoy and Marol, 2003). In
our study, *C. albicans*, *C. glabrata*, and other yeast species were readily detected and presumptively identified on CHROMagar plates (Figure 5.3. and Table 5.2.).

### 7.2.4 Age of the study participants and yeast carriage

It is reported that carriage of yeast increases with age (Narhi *et al.*, 1993; Percival *et al.*, 1991) In a study of 509 patient subjects with oral discomfort (Shimizu *et al.*, 2008), it was demonstrated clearly that “the prevalence of yeast in saliva significantly increases with older individuals”. Shimizu (Shimizu *et al.*, 2008) listed specific host factors associated with oral yeast carriage, and reported the percentage of the patients in age groups of 16-39, 40-59, 60-79, and ≥ 80 years of age that were colonised with yeast, as 29.9%, 49.3%, 69.5% and 73.7% respectively. In the present study, the median age of the patients was 59.5 years of age, and ranged from age 24 to age 86. The median age was the same for the controls, who were selected to match the ages and genders of the patient group, to eliminate the possible influence of age on yeast carriage. Again, our finding of only 2/20 control samples that were positive for yeast, despite the relatively high median age of the cohort, suggests that the dilution of saliva in the oral wash sample was a contributing factor to the low recovery rate. However, it does not invalidate the comparison of the patient and control groups as the same collection technique was used for both groups. Indeed, it accentuates the difference between the patient and control groups.
7.2.5 Medication and yeast carriage

Particular drug use (psychotropics, anti-asthmatics, antihypertensives and diuretics) has been correlated with subjective dry mouth and reduced unstimulated saliva flow rate (<0.1 ml/min) (Bergdahl and Bergdahl, 2000). Other studies have also found strong associations with oral dryness and particular drugs (Thomson et al., 2000). In Nederfors’ large scale survey of an adult population in Sweden (Nederfors, 2000) it was found that the probability of reporting dry mouth was not only strongly associated with continuing pharmacotherapy but also that the probability increased with the number of medications taken. In the current study, the xerostomia patient group used greater than triple the number of pharmaceuticals than the control group. However there could be a discrepancy between the reported and actual consumption of medications.

Scully noted that in Nederfors’ investigation no particular distinction was made between xerogenic or non-xerogenic drugs or drug groups (Scully, 2003) which means that the prevalence of dry mouth is correlated to polypharmacy rather than the type of medication taken. In the current study, 90% percent of patient subjects were taking at least one xerogenic medication compared to only 30% of control participants (Table 4.5). Furthermore, over half of the patient group was using two or more of such drugs, in contrast to only 10% (2) of the control subjects. This finding supports the statement by Field (Field et al., 2001), that medication is a better predictor for the occurrence of dry mouth than age or gender.
7.2.6 Denture wearing and yeast carriage

A recent systematic review evaluated studies on the association of oral candidosis (denture stomatitis (DS)) and the wearing of partial removable dental prostheses (RDP) (Emami et al., 2012). Eligible studies in this review were clinical trials and observational studies such as cross-sectional, case-control, and cohort studies. Only studies that had assessed at least one group of partial RDP wearers in adults (18 years or older) were included. All types of partial RDP designs were considered. Excluded studies dealt exclusively with other intraoral appliances, ie, complete RDPs, fixed rehabilitations. Studies with a sample population composed of partially and completely edentulous patients were considered only if separate data were provided for partial RDP wearers. The role of \textit{C. albicans} in DS has been investigated in two studies reviewed. Although \textit{C. albicans} has been reported as the main species on the palatal mucosa and in the saliva of individuals who wore partial removable prostheses and developed DS (Abaci et al., 2010), Shulman reported that only 7.2% of individuals with DS had a positive smear of \textit{C. albicans} (Shulman et al., 2005). Loster concluded that a large percentage of persons wearing dentures over a prolonged period of time (5 years) are colonised by \textit{Candida} species (Loster et al, 2011) and this could predispose to \textit{Candida} infections.

In the present study, 30% of the patient participants, but only 10% of control subjects, wore dentures (See Chapter 5, section 5.2.2.3) and this is a
possible confounder in our investigation of the role of dry mouth in oral yeast colonisation. We were not able to match controls to xerostomia samples for denture wearing due to the limited availability of age- and gender-matched subjects. However, the differences observed between the denture wearing and non-denture wearing patient subjects in respect to the percentages that were colonized with yeast, the yeast types, and the total CFU/ml (Table 5.2 and Figure 5.13) were negligible. The two control group participants who wore dentures were both over 75 years of age, and one was positive for yeast (Figure 4.4 and Figure 5.5).

7.2.7 Dry mouth and yeast carriage

Xerostomia is the subjective feeling of a dry mouth, a symptom that may or may not be accompanied by hyposalivation, an actual decrease in salivary flow. Although the most common symptom of hyposalivation is xerostomia, and patients that have a greater than 50% reduction in salivary flow usually experience xerostomia, studies have shown that the latter does not necessarily guarantee hyposalivation. Causes of xerostomia due to salivary gland dysfunction can be categorized into autoimmune exocrinopathies (most notably Sjögren’s syndrome), medication side effects, radiation-induced salivary gland dysfunction, and salivary gland trauma. Other, less common, causes that have been reported include salivary gland tumors, infectious processes, endocrine and renal disorders, dementia, cystic fibrosis, and amyloidosis. The distinction should be made between medication that causes dry mouth and those that cause hypo salivation, as
a subjective complaint of dry mouth does not always correlate to a reduced salivary flow rate. The variety of medications that cause dry mouth and the variety of medical conditions in these patients necessitating the medication complicates the analysis of dry mouth in these patients as commented on in Chapter 4, section 4.4.

Although the reason is not fully clear, there is enough evidence from the literature to suggest that dry mouth occurs more frequently in women than in men (Screebny and Vissink, 2010). Therefore, patient and control samples were gender matched. In this study, 18 subjects of each group were females and 2 were males, the proportion of female subjects in both groups was 90% due to limited availability of male dry mouth participants.

The relationship between SFR and Candida colonization reported in the literature varies. Navazesh and colleagues reported that higher Candida counts occurred in study subjects with decreased unstimulated whole saliva flow rates (Navazesh et al., 1995). Torres (Torres et al., 2002) evaluated the relationship between saliva flow rates and Candida colony counts in the saliva of patients with xerostomia, and reported a significant inverse relationship between saliva flow rates and Candida CFU count, when the CFU counts of xerostomia subjects were greater than 400 CFU/ml. Karbach (Karbach et al., 2012) evaluated the saliva flow rates and Candida colonization after head and neck radiation. Based on the SFR, hyposalivation was graded as: grade I (SFR 0.1–0.25 ml/min), grade II (SFR ≤0.1 ml/min), and grade III (SFR = 0.0 ml/ min). Karbach (2011) reported a
significant inverse correlation between the SFR and candidal colonization, suggesting radiotherapy can predispose to oral colonization of the oral mucosa by *Candida* species.

In current study, although the relationship between the salivary flow rate and total yeast count of xerostomia subject samples appeared to be quite weak (Figure 5.7). Samples from seven xerostomia subjects with SFRs of 0 to 0.04 ml/min (Table 5.1 and Figure 5.7) were found with a concentration of *Candida* greater than 1000 CFU/ml, and there was a trend that an increased CFU/ml was associated with a lower SFR in the xerostomia group (Figure 5.7). In the xerostomia group, seven subjects contained more than one colony type. In contrast, only two control subjects were positive for yeast, and none of the control samples contained more than one colony type of yeast. Only one control subject carried yeast of over 1000 CFU/ml. The results support the findings of a recent study of 509 subjects (Shimizu *et al.*, 2008), in which the salivary flow rate (SFR) under rest conditions, among other factors, was closely correlated with oral yeast numbers.

An alteration in the oral microbial flora caused by a reduction in salivary output was also indicated by a study of salivary output and *Candida* counts in the oral rinse of Sjögren’s syndrome patients and controls (Abraham *et al.*, 1998). The study found that using an oral rinse, the mean *Candida* count in Sjögren’s syndrome patients was 1672 – 1455 CFU/ml whereas no *Candida* was found in controls. Another study reported a statistically significant difference in oral yeast
carriage between Sjögren’s syndrome patients and healthy controls (Ergun et al., 2010). The present study included 5 patients who met four of Sjögren’s syndrome diagnostic criteria (Vitali et al., 2002) and all were colonised with *C. albicans* which is consistent with the Abraham report. Also the percentage of those patients (5/5) colonised with *Candida* (100%) was higher than the proportion of the other xerostomia subjects (13/15) colonised (87%). The current study is the first study to show the presence of multiple yeast species detected in the saliva of xerostomia subjects (Figure 5.3); of 20 xerostomia subjects, 7 carried more than one yeast type (Figure 5.4). In this study, and there was a statistically significant difference ($P < 0.05$) between oral yeast carriage in xerostomia subjects and in matched controls.

The presence of the multiple yeast species have been reported (Yan et al., 2011) to be associated with a high susceptibility to oral candidosis and a remarkable resistance to azoles in a group of Sjögren’s syndrome patients. Another study suggested that xerostomia has been reported in 75% to 92% of Sjögren’s syndrome patients (Boutsi, 2000). The detection of multiple *C. albicans* species in xerostomia subjects could lead to a better understanding and management of xerostomia subjects.

I conclude that xerostomia subjects in the present study were more susceptible than age- and gender-matched controls to oral colonisation with yeast, and in several cases were colonised with more than one yeast species. I also conclude that it was likely that a reduced saliva flow in most subjects contributed
to the observed increased yeast colonisation. One possible explanation is simply a reduced ability to clear the yeasts from oral cavity. Another possibility is that there is a reduced presence of host immune mechanism such as specific salivary IgA. However, it is also possible that an altered composition of saliva in xerostomia subjects could contribute to an increase in yeast colonisation in such individuals. Previous studies (Holmes et al., 2002; O'Sullivan et al., 1997) have shown that saliva proteins play an important role in adherence of *C. albicans* within the oral cavity. Thus, in the second part of this study, I investigated whether changes in saliva protein profiles could be detected in saliva rinse samples from dry mouth patients.

### 7.3 Discussion part II - protein study

#### 7.3.1 Introduction

The yeast study found that there are statistically significant differences in oral yeast carriage between xerostomia and control subjects. Colonization by *C. albicans* involves host recognition, binding of the organism to host cells, host cell defence mechanism, and microbial competitors that may affect clearance by the host. Adhesion is the crucial first step for Candida survival and proliferation in the oral cavity (Cannon et al., 1995a). The tooth surface is always coated in saliva even after tooth cleaning procedures; saliva proteins are quickly adsorbed forming a conditioning film or saliva pellicle. Edgerton (1993) reported that saliva proteins, such as mucins, bind to both yeast cells and germ tubes, as detected by
their presence in an extract of saliva-incubated *Candida* cells (Edgerton *et al.*, 1993). Jeng reported a *C. albicans* surface mannoprotein adhesin that binds to immobilized saliva components (Jeng *et al.*, 2005). Also, specific salivary proteins, such as the basic proline-rich proteins (O'Sullivan *et al.*, 1997) and salivary IgA (Holmes *et al.*, 2002) were found to promote the adherence of *C. albicans* to oral surfaces. Holmes and colleagues used PAGE analysis and a blot overlay adherence assay to reveal that certain salivary proteins were selectively adsorbed to silicone and that *C. albicans* yeast cells adhered specifically to the adsorbed salivary proteins (Holmes *et al.*, 2006b).

Despite these studies investigating particular salivary proteins, the complexity of saliva can make the study of the role of saliva in yeast adherence challenging. Using the study of adhesion of *C. albicans* to acrylic surfaces as an example, conflicting results have been reported: salivary coating can reduce or enhance the adhesion of *C. albicans* to acrylic (Edgerton *et al.*, 1993; Lain *et al.*, 2008; Park *et al.*, 2003). The conflicting data reported probably reflects differences in experimental methodologies - variations such as the use of stimulated or unstimulated saliva, (Veerman *et al.*, 1996), as well as intra- and inter-individual variations in the composition of the saliva (Elguezabal *et al.*, 2004; Lima *et al.*, 2006; Nikawa *et al.*, 2000). Therefore, in the present study, a consistent method of saliva collection (saliva rinse) was used for all subjects.

Because I found increased yeast carriage found in xerostomia subjects, the questions for the protein study were: (a) Do salivary patterns differ between the
xerostomia subjects and healthy controls? (b) Do saliva proteins play a role in the increased yeast carriage?

Numerous studies of xerostomia investigate the effects of medication (Chromagar, 2010), radiotherapy (Pontes et al., 2004), systematic conditions (Davies et al., 2006) or salivary gland performance; the role of salivary proteins in xerostomia is not well explored (Cowman et al., 1983). Human saliva contains a large array of proteins and peptides that have important biological functions. Investigation of the salivary protein profile alterations that may arise in xerostomia could provide insight to the understanding of the possible influences of such a condition. Moreover, a comparison between samples from healthy and from xerostomia subjects may reveal unique, decreased, or increased levels of specific proteins that maybe of clinical significance.

Recently, there has been considerable research into the use of saliva proteomics for the identification of biomarkers for various conditions including cancer (Schulz et al., 2012; Spielmann and Wong, 2011). Modern proteomic analysis is characterised by the use of advanced computer assisted analysis such as using 1D gel electrophrosis chromatographic steps and Mass Spectrometry analysis, or 2-D electrophoresis followed by protein digestion and MALDI-TOF analysis of the digest. These approaches are aided by automated data analysis, which helps to manage the huge amount of data, but those procedures are also characterized by potential sources of error due to the limitations of the technology itself (Messana et al., 2008). Our approaches - the separated saliva proteins by
SDS-PAGE followed by Gel Doc EZ™ system protein analysis shared a similar characteristic of an automated data analysis system, although those methods did not allow identification of individual proteins.

The combination of the well validated protein separation technique such as SDS-PAGE and automated Gel Doc™ EZ system, to allow a qualitative study of salivary protein, was a novel approach with advantages of simplicity over more complex proteomics. It provided a pathway for the study of salivary protein profile in a cost-effective manner. Salivary protein profiles of a group of xerostomia subjects were compared with those of age- and gender-matched controls. The goal of the protein study was to determine whether SDS-PAGE protein profiling can be used to detect consistent differences in saliva proteins of study subjects and controls, which may partially explain the susceptibility of dry mouth subjects to colonization with yeast, as described in Chapter 5.

7.3.2 SDS-PAGE protein study

7.3.2.1 Saliva rinse as a saliva protein study sampling method

Saliva is a complex mixture of proteins and other molecules, which originate from several sources, including several different salivary glands, serum, the oral microbiota and ingested material. The use of saliva as research material may pose particular problems due to its inherent variability and instability (Schipper et al., 2007b). One study (Michishige et al., 2006) reported the effects
of different saliva collection methods on the yield of protein components in saliva samples from normal volunteers. In that study, the saliva collected by suction, spitting or the swab method were investigated, and the results indicated that the volume of the saliva collected with suction method was two-fold greater than that by spitting or swabbing, which was possibly related to the stimulation of saliva flow. Ideally, whole saliva, either stimulated or unstimulated, would be the preferred sample for a study of saliva protein content. However, considering the likelihood of low saliva flow in xerostomia subjects, collection of whole saliva was considered inappropriate. Pramanik and colleagues reported protein and mucin retention on oral mucosal surfaces in dry mouth patients using saliva samples collected by paper strips (Pramanik et al., 2010). Saliva rinse samples are commonly used in studies of subjects with xerostomia or reduced salivary flow (Kindelan et al., 1998; Leung et al., 2007).

For the present study, a method of collecting saliva rinse samples using commercially bottled water was developed (Chapter 3, section 3.2.3) as a protocol least likely to cause distress for the patient. One issue with this approach became apparent when initial samples taken from pilot study volunteers were analyzed for total protein content using the Bradford technique (Chapter 3 section 3.3.1), as less than 0.0488 µg protein/µl was found in all samples studied (Figure 6.2 and Table 6.1). However it was also found that if the maximum volume of sample (25 µl) was loaded on a polyacrylamide gel, silver staining was sensitive enough to detect many proteins in the saliva rinse sample.
For consistency in any comparative study, a standard system for collection and storage of samples prior to analysis is essential. One major issue is that saliva contains high concentrations of proteases (Fingleton et al., 2004). Several studies have measured protein stability in saliva samples. Ng and colleagues investigated the effects of storage time on stability of salivary proteins (immunoglobulin A (IgA) and lysozyme), stored at -30 °C over 1, 2, 3, 8 and 12 months of storage and found a significant decrease in IgA levels (≥10%) after 8 months or more of storage (Ng et al., 2003). Morris et al., however, reported that total, and rubella-specific, IgG concentrations in oral fluid samples were stable for up to 7 days at different temperatures (Morris et al., 2002). Salivary protein degradation in saliva samples was demonstrated in a more recent study (Esser et al., 2008), where three degradation products with masses of 2937 Da, 3370 Da and 4132 Da were found to increase 2-7-fold over a period of 4 h storage at room temperature. Studies using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry and two-dimensional difference gel electrophoresis reported overall stability of salivary proteins stored at -80 °C over a 6 month period (Messana et al., 2008). Protein breakdown in saliva could be partly inhibited by a protease inhibitor cocktail targeting serine, cysteine and metallo-proteases. A complicating factor of using protease inhibitors is that the inhibitors themselves are peptides, and they may covalently bind to proteins thereby changing their masses.

In the present study, steps taken to reduce any effect of protein degradation included immediate storage of collected samples on ice and freezing of divided samples at -20°C within 4 h of collection (Section 3.4). All samples
were treated in the same way to promote consistency for protein profile comparisons of the two groups of individuals. Protein inhibitors were not added as they would affect the protein profiles. As shown in Figure 6.7 and Figure 6.8, protein profiles of saliva rinses from two individuals taken at 5 daily intervals, although varying in total protein content, did not show significant variation in the protein profile - supporting the storage approach used in the study. However, degradation of salivary proteins could partially explain the differences of protein bands observed in Figures 6.6, 6.7 and 6.8.

7.3.2.2 SDS-PAGE saliva protein profiling

SDS-PAGE (sodium dodecylsulfate polyacrylamide gel electrophoresis) is a technique widely used in biochemistry and molecular biology to separate proteins according to their electrophoretic mobility, and it predates many of the modern day proteomic technologies. Using electrophoresis, proteins can be separated based on their isoelectric point and apparent molecular mass (Laemmli, 1970). The electrophoretic mobility of an individual protein depends on a variety of factors including the aggregate charge on its surface, size, shape, and also strength of the applied electric field. Further, the pH of the electrophoresis buffer also influences protein migration since it affects the net charge on the protein surface. The proteins in the gel can be visualized by staining with Coomassie blue (Schleicher and Watterson, 1983) or with silver, a more sensitive technique (Morrissey, 1981). In this study both a Coomassie blue-based stain (EZBlue™, a commercially available protein stain, used to give greater consistency) and a silver
staining technique were used. Data from silver stained SDS-PAGE gels were used for the main protein profile analysis, as it was considerably more sensitive (Figure 6.15 and Figure 6.16) so that more proteins could be detected by silver stain.

Any variability that could have been introduced to the analysis during SDS-PAGE was reduced by strictly following the protocols in making the running buffer, running the gels and silver staining procedures. In addition, all buffers used for the main study were made as one batch and gels were from a commercial source to ensure consistency. Although silver staining is a challenging technique due to the complex nature of the colour development steps, which could introduce variations in staining (Merril, 1990), reviewed by Steinberg (Steinberg, 2009), a standard approach was developed as described in Chapter 3, section 3.6, and the consistency in profile staining is demonstrated by comparison of the standard lanes run on eight individual gels (Figure 6.14).

7.3.3 Validity of the cross-sectional study approach

To validate the experimental approach of comparing individual saliva protein profiles using a cross-sectional study approach, the initial experimental steps were to evaluate inter- and intra-individual variation. It was important to determine whether salivary protein profiles generated from a saliva rinse sample from an individual were consistent from day-to-day, and whether there was any similarity of profiles from two different healthy individuals. Therefore, saliva rinse samples from two healthy individuals, collected on five consecutive days as
described in Chapter 6 (section 6.1.4) were examined by SDS-PAGE (Figure 6.6). Using the combination of visual analysis of images and Gel Doc™ EZ analysis, 11 prominent protein bands were identified in both saliva profiles. This validated the use of a cross-sectional study for the xerostomia and control groups. The observation of a consistent saliva protein profile has been reported previously in a cross-sectional study (Morales-Bozo et al., 2006). In a study by Schwartz et al (Schwartz et al., 1995), using resting and stimulated whole saliva, 20 healthy subjects showed very similar electrophoretic protein profiles, but some samples had increased band densities. However, Morales-Bozo and colleagues, in a study comparing saliva protein profiles from groups of healthy young adults and elderly adults, found both invariant and variable proteins present in compared profiles (Morales-Bozo et al., 2006).

7.3.4 Salivary protein studies of xerostomia subjects and controls

A comparative study of salivary protein profiles for xerostomia subjects and matched controls has not been previously published. However, numerous studies have reported on salivary protein profiling of Sjögren’s syndrome patients, but mostly with a goal of identifying disease biomarkers. It seems logical to assume that the pathological changes that occur in illnesses such as Sjögren’s syndrome could result in the alteration of tissue structures, and breakdown, modification or over-production of salivary protein components. Such changes could be detected through the study of saliva protein variation. Many different techniques such as electrophoresis, Western blot, high-performance liquid
chromatography (HPLC) to 2D PAGE and mass spectrometry have been employed in studies of Sjögren’s syndrome patients (reviewed by (Giusti et al., 2007)). It was reported that a decrease in the fluid production rate and an altered expression of lactoferrin, lysozyme, salivary IgA and β2-microglobulin were detected in salivary secretions of Sjögren’s syndrome patients with several changes in protein glycosylation patterns. On the other hand, a lack of agreement for other typical salivary proteins, such as albumin, pre-albumin, α-amylase and cystatins, has been reported (Siqueira and Dawes, 2011). Yaltirik and colleagues reported that additional protein bands were detected visually using electrophoresis of stimulated parotid saliva samples from 10 Sjögren’s syndrome patients compared to 10 healthy controls, and suggested the possibility of using this technique as an aid to diagnosis of Sjögren’s syndrome (Yaltirik et al., 2005).

In the present study, protein-banding profiles were analysed for all subjects using two approaches, either by a visual, subjective comparison to a standard sample using digitally recorded photographs, or by the more objective, but less sensitive, GelDoc™EZ system.
7.3.4.1 Analysis by comparison to standard sample banding pattern.

Eleven protein bands (SP1-SP11) were identified by visual assessment of gel images in the standard sample, which was included on all gels. As shown in Figure 6.14 and Table 6.3, these bands were consistently identified in most of the standard samples on each separate gel. When all gels were aligned according to their standard lanes, then the eleven protein bands could be identified in many of the patient and control samples. However, the lower molecular weight bands SP8 and SP9 were often not detected, probably because of the low resolution in this part of the gels. For the readily identifiable bands 1-7, only two control samples did not show all these bands (C11 did not show band SP4 and C21 did not show SP7). Of the patient group samples, SP8 and SP9 were again often not detected, but in six samples, one or more of the higher molecular weight bands were not detected. However, such a difference could reflect differences in the total amounts of sample protein loaded onto gels. Thus it was not possible to differentiate patient and control saliva rinse profiles according to a comparison to the banding pattern of the standard.

This system of analysis also allowed detection of additional protein bands not observed in the standard samples. One or more (up to 4) additional bands were observed in 14 control samples, whereas additional bands (1-3) were observed in 17 of the xerostomia subjects. However, the additional bands were all in the same region of the gel, (between SP1 and SP2) regardless of the sample source, so
again, this analysis could not show consistent differences in protein profiles of xerostomia subjects compared to controls, and any differences observed could just reflect differences in total protein loaded onto gels.

7.3.4.2 Analysis by the Gel Doc™EZ system.

In a comparison of samples from Sjögren’s syndrome subjects with their matched controls, protein bands of 27-30 kDa, 55-57 kDa, 61-66 kDa and 79-88 kDa were ubiquitous; they were present in Sjögren’s syndrome patients and controls. In addition, protein bands of 27-31 kDa, 51-59 kDa, 60-68 kDa and 70-83 kDa were present in over 85% of the xerostomia subjects without Sjögren’s syndrome and matched controls tested. Also, for the protein study of xerostomia subjects without Sjögren’s syndrome and the matched controls, protein bands between 259-280 kDa were identified in seven xerostomia patient samples, and were detected in a similar number of control samples (5). In contrast, more control samples (8/15) than patient samples (3/15) possessed protein bands of estimated molecular weight between 143-155 kDa. When Sjögren’s syndrome patient samples were compared with their matched controls, protein bands between 261-285 kDa were detectable in four of five Sjögren’s syndrome patient samples and only one of the equivalent control samples (Figure 6.23), whereas there were clustered larger protein bands (294-304 kDa) detectable in four matched control samples and only one Sjögren’s syndrome patient sample. The differential presence of these high molecular weight proteins in the two subject groups may
represent post-translational modifications (e.g. different glycosylation) of the same proteins.

An additional analysis that the GelDoc™EZ system would allow is determination of the relative abundance of particular protein bands within each sample. However, given the the fact that no consistent changes in protein banding profiles were observed, and the already complex nature of the study, this analysis was not undertaken, although a future study could use this approach.

Thus, no major differences in protein banding patterns for any group of subjects were observed in this study. However, a detailed analysis of the protein bands detected on one-dimensional SDS-PAGE gels is inherently difficult, due to the possible co-migration of different proteins and differences in glycosylation of the same proteins, or other modifications of the protein bands present. Also, a positive identification of any particular protein band could not be obtained without using further analysis such as Western Blot with specific antibodies to known saliva proteins, or mass spectrometry.

Within the limitations of our project, the qualitative study of the salivary protein profiles of the two subjects groups did not detect consistent differences between the xerostomia patients and control subjects.
7.3.5 Limitations of the study

One of the major drawbacks of this study was the fact that there was a large variation in the possible causes of xerostomia for the patient group. For example, five out of the 20 subjects in the xerostomia group met four of the diagnostic criteria for Sjögren’s Syndrome (Vitali et al., 2002) with either a positive serum anti-Ro/anti-La antibody test or a positive histology report from labial salivary minor gland biopsy. Although the results showed that a higher level of yeast colonisation was present in three of the five possible Sjögren’s syndrome participants, lack of sufficient numbers of such subjects made it impossible to come to a definitive conclusion. Furthermore, for the other xerostomia subjects, the variety of other medical conditions/drugs, or the presence of dentures, factors known to affect saliva flow (Table 4.1, 4.2 and 4.3), may have had confounding effects on yeast carriage and/or on protein profiles. However we can say that all of those in the xerostomia group showed a much higher prevalence of yeast carriage and numbers of yeast species detected than the matched controls. Although yeast carriage was higher in the xerostomia subject group who had low SFRs an association between yeast colonisation and SFR could not be confirmed because the SFRs of the control group were not collected.

Methodologically, another limitation of this study was the low salivary protein concentration of saliva rinses (Figure 6.2 and Table 6.1). Due to the limited salivary protein content of the samples, with the SDS-PAGE analysis, instead of loading gel lanes with a fixed amount of salivary protein, the samples were loaded with a maximum fixed volume instead. This was less than ideal for
the accurate resolution of protein bands as it could result in overloading/under-loading of protein.

A recent study (Zoukhri et al., 2012) reported on the identification of a 7,606 Da biomarker peptide unique to the Sjögren’s syndrome subjects. Using mass spectrometry (MALDI O-TOF) and bioinformatics search algorithms, the study analysed saliva samples collected from parotid glands of 27 Sjögren’s syndrome subjects and age-matched controls. With the application of such a technique, a proteins range from 0.75 Kda – 7.5 Kda can be analysed. It is worth noting that, using the Gel Doc™EZ, the lowest molecular weights of the bands detected in internal standards generated were almost approximately 25 - 30 KDa (Chapter 6. Table 6.1), indicated the lack of sensitivity of SDS-PAGE analysis for the study of proteins with molecular weight less than 30-25 kDa.

7.3.6 Future studies

The sensitivity and consistency of the findings were optimised by the use of silver staining and combining calibrated visual protein band detection and the Gel Doc EZ™ image analysis system, but the inherent lack of sensitivity of one-dimensional gel electrophoresis was evident. Techniques such as 2D SDS-PAGE and MALDI-TOF analysis of separated proteins has revealed the complexity of the saliva proteome (Lopez, 2007; Spielmann and Wong, 2011). Such techniques were beyond the scope of the present study, however, future studies could utilise more sensitive techniques, such as two-dimensional electrophoresis (2D-PAGE).
Western blotting for specific saliva protein would also provide highly sensitive detection and identification.

Other worthwhile future studies include an investigation of an association between increased yeast carriage in xerostomia samples and salivary proteins using adherence assays, to explore the role of identified protein bands in promoting *Candida* adhesion. Previous studies (Cannon *et al.*, 2010; Holmes *et al.*, 2002; O'Sullivan *et al.*, 1997) found selected salivary proteins promoted *C. albicans* adhesion to oral surfaces.

### 7.4 Conclusions for both yeast and protein studies

Dental practice involves a vast number of materials with different physical and chemical properties that are exposed to the oral fluids and coated with salivary components. Saliva is a multifunctional biological fluid which plays an important role in maintaining oral health. Our findings show significant differences of yeast carriage between xerostomia and healthy controls, indicating that xerostomia subjects maybe more susceptible to *Candida* infections. This finding will add to the understanding of xerostomia. It may also contribute to the treatment planning and clinical management of this patient group.

### 7.4.1 Conclusions relating to study participant data

1. There is a significant difference between the number and type of medical conditions of xerostomia subjects when compared to matched controls.
2. The mean number of medications taken by xerostomia subjects was much greater than those taken by matched controls.

7.4.2 Conclusions for the yeast studies

1. There was a higher prevalence of yeast carriage in saliva samples from individuals reporting a dry mouth (18/20) than in control samples (2/20). More yeast species (two or more in 7/20 individuals) and greater yeast numbers (>10^3 CFU/ml in 7/20 individuals) were detected in xerostomia samples than in samples from control subjects (0/20 and 1/20 respectively).

2. Oral yeast carriage was significantly (p<0.05) higher in xerostomia subjects than in matched controls.

3. Individuals with xerostomia may be more susceptible to oral yeast infections.

7.4.3 Conclusions for the protein studies part I

4. SDS-PAGE analysis of the saliva rinse samples demonstrated that protein could be visualized when stained with either Coomassie EZBlue™ or silver stain.

5. Commercially available Ready Gel Precast gels (Life Science, Bio-Rad laboratories, Inc) were chosen for use in Part II of the protein study to ensure inter-gel consistency.
6. Silver staining was shown to have a greater sensitivity of detection than the Coomassie-based EZ™Blue stain. Therefore silver-staining was chosen for use in Part II of the protein study.

7. Importantly, although there was day-to-day variation in the concentration of protein in saliva rinses from individuals, there was, in general, a consistent protein profile observed with both staining methods. Thus SDS-PAGE protein analysis of saliva rinse samples was validated for the proposed cross-sectional study in Part II of the protein study.

8. Eleven prominent protein bands were consistently identified in silver-stained SDS-PAGE profiles using both visual inspection of gel images and the Gel Doc™ EZ image analysis system.

9. An internal standard was produced and stored in aliquots to allow direct comparison of several SDS-PAGE gels and was used to analyse the saliva rinse samples in Part II of the protein study. The internal standard also assisted assessment of the presence or absence of the eleven previously identified prominent protein bands in subsequent saliva rinse samples.

10. The Gel Doc™ EZ analysis system was found to be suitable for qualitative analysis of SDS-PAGE protein profiles. The image quality of the photographed gels obtained using the Gel Doc™ EZ system was, however, not as good as that obtained using a digital SLR camera. Therefore in Part II of the protein study, both methods of analysis were used.
Chapter 7. Discussion

7.4.4 Conclusions for the protein studies part II

11. Consistent protein band patterns were observed for the profiles of the internal standards on repeated gels. This confirmed that the SDS-PAGE techniques used were reproducible.

12. Protein bands in the ranges ~ 70-90 kDa, 60-68 kDa, 50-58 kDa and 27 - 30 kDa were ubiquitous; such bands could be detected in salivary rinse samples from all xerostomia and control subjects.

13. Two high MW protein bands showed an interesting distribution, suggestive of post-translational modification of a single protein. When one of the two proteins was present in a given sample, the other protein band was consistently absent, except in a single sample (P23). One of these two protein bands, in the range 261-285 kDa, was present in samples from four of five Sjögren syndrome patients (Fig. 6.3) and in three other xerostomia subjects (Table 6.4). It was also detected in six control samples. The other protein (in the range 290-314 Kda) was present in samples from eight xerostomia subjects (including one Sjögren syndrome patient) and in 13 control samples.

14. Due to the small number of subjects in each group, particularly the Sjögren’s syndrome group, it was not possible to detect consistent significant differences between any of the protein profiles from either the group of xerostomia patients or the matched controls. However, as noted in (3) above, there were some differences that would warrant further investigation, but this was not within the scope of this thesis.
The methodological approach of using SDS-PAGE in combination with a computer-aided automated data analysis system (Gel Doc EZ™ system), was a novel, inexpensive and easy-to-use protein analysis approach for salivary protein studies. Within the limitations of the project and technology, my study detected potentially variant protein bands in Sjögren’s syndrome patients. However, I conclude that there were no significant consistent differences in the salivary protein profiles between xerostomia subjects and healthy controls. The potential relationship between the variant protein bands detected and the increased yeast carriage could be explored further through an adhesion study. Also it will be essential in future studies to have a larger number of participants with the same cause of xerostomia to be able to identify statistically significant changes attributable to a particular cause of xerostomia.


Oral Yeast Carriage and Saliva Protein Profiles of Xerostomia Subjects and Matched Controls

Reference list


Oral Yeast Carriage and Saliva Protein Profiles of Xerostomia Subjects and Matched Controls


Shannon IL The biochemistry of human saliva in health and disease1972: Univ. of Michigan.


Appendices
Appendix I  Abstract for International Association for Dental Research Student Poster Competition on 15\textsuperscript{th} April 2012

Title: Oral Yeast Carriage of Xerostomia Subjects and Age and Gender Matched Controls

Authors: LXL. HOU*, A.R. HOMLES, A. NOLAN, K.M. LYONS, R.D. CANNON

Department: Oral Rehabilitation, Oral Sciences, and Oral Diagnostic and Surgical Sciences, University of Otago, Dunedin, New Zealand

Background: It has been reported that 17\%-29\% of the population experience a sensation of oral dryness. This has many causes, but generally results from a decrease in the production of saliva, which may alter the oral microenvironment, and the composition of the oral microbiota. \textit{C. albicans} is a common oral commensal and an opportunistic pathogen, causing serious mucosal and systemic infections in immunocompromised individuals. Colonisation of mucosal surfaces is the first stage for such infections.

Objective: To determine if there is a significant association between increased oral colonisation by yeast species, such as \textit{C. albicans}, and a sensation of dry mouth.

Methods: This was a cross-sectional study (ethical approval: LRS/10/09/034) in which oral yeast carriage was evaluated in single saliva wash samples (mouth rinsed with 10 ml of bottled water for 30 sec) from a group (20) of individuals attending the Oral Medicine clinic. Inclusion criteria were patient-reported dry mouth.
mouth without symptoms of candidosis. Samples were also obtained from age- and gender-matched control subjects (20). Yeast species and numbers were presumptively identified by culture on chromogenic, selective agar plates. Test of proportions was used to analyze the results.

**Results:** Yeast were detected in 18/20 patient samples and 2/20 controls. Seven patient samples contained more than one colony type, and >10^3 colony forming units (CFU)/ml and twelve contained >10^2 CFU/ml. In contrast, both positive control samples yielded a mono-culture and lower colony counts. **Conclusions:** Yeast were present at a statistically significantly higher prevalence (17/20) in saliva samples from individuals reporting a dry mouth than in control samples (2/20), and more yeast species (two or more in 7 individuals) and greater yeast numbers (>10^3 CFU/ml in 7 individuals) were detected. Such individuals may be more susceptible to oral yeast infections. We gratefully acknowledge a Fuller grant to Ms LH from the Sir John Walsh Research Institute of University of Otago.
Appendix II  
Abstract for The Academy of Australian and New Zealand Prosthodontics conference on 12th of July 2012

Title of Presentation: Oral yeast carriage and saliva protein patterns of xerostomia subjects and matched controls

Authors: LXL Hou, A.R. HOLMES, A. NOLAN, K.M. LYONS, R.D. CANNON

Department: Oral Rehabilitation, Oral Sciences, and Oral Diagnostic and Surgical Sciences, University of Otago, Dunedin, New Zealand

Aims: To investigate if there is a significant association between increased oral colonisation by yeast species, such as *C. albicans*, and a sensation of dry mouth, and if there is a difference in the saliva proteins detected in xerostomia subjects from those in age- and gender-matched controls.

Methods: This was a cross-sectional study (ethical approval: LRS/10/09/034) in which oral yeast carriage and saliva proteins were investigated in saliva wash samples. Mouth rinses (10 ml bottled water for 30 s) were obtained from 20 individuals attending an Oral Medicine clinic; inclusion criteria included self-reported dry mouth. Samples were also obtained from 20 age- and gender-matched controls. Yeast numbers and species were identified using the chromogenic agar CHROMagar Candida. Saliva samples were subjected to SDS-PAGE. Salivary proteins were visualized with both EZblue and silver stain, and
Appendices

provisionally identified by calibrated visual detection and the Gel Doc EZ gel analysis system.

**Results:** The prevalence of yeasts in saliva samples from individuals reporting a dry mouth was significantly greater ($p < 0.05$) than in control samples, with both a greater number of yeast species and more yeast cells in the patient saliva samples. There was, however, no significant difference in the salivary proteins detected in the two groups.

**Conclusion:** Within the limitations of this project, we conclude that patients with xerostomia may be more susceptible to oral yeast infections, but that there is no significant difference in the salivary proteins between xerostomia subjects and healthy controls. This project was supported by a Fuller scholarship.
Appendix III  Abstract for Research Day Presentation, Faculty of Dentistry – Sir John Walsh Research Institute on 2nd August 2012

Title of Presentation: Oral yeast carriage and saliva protein patterns of xerostomia subjects and matched controls

Authors: LXL Hou, A.R. HOLMES, A. NOLAN, K.M. LYONS, R.D. CANNON

Department: Oral Rehabilitation, Oral Sciences, and Oral Diagnostic and Surgical Sciences, University of Otago, Dunedin, New Zealand

Aims: To investigate if there is a significant association between increased oral colonisation by yeast species, such as C. albicans, and a sensation of dry mouth, and if there is a difference in the saliva proteins detected in xerostomia subjects from those in age- and gender-matched controls.

Methods: This was a cross-sectional study (ethical approval: LRS/10/09/034) in which oral yeast carriage and saliva proteins were investigated in saliva wash samples. Mouth rinses (10 ml bottled water for 30 s) were obtained from 20 individuals attending an Oral Medicine clinic; inclusion criteria included self-reported dry mouth. Samples were also obtained from 20 age- and gender-matched controls. Yeast numbers and species were identified using the chromogenic agar CHROMagar Candida. Saliva samples were subjected to SDS-
PAGE. Salivary proteins were visualized with both EZblue and silver stain, and provisionally identified by calibrated visual detection and the Gel Doc EZ gel analysis system.

**Results:** The prevalence of yeasts in saliva samples from individuals reporting a dry mouth was significantly greater ($p < 0.05$) than in control samples, with both a greater number of yeast species and more yeast cells in the patient saliva samples. There was, however, no significant difference in the salivary proteins detected in the two groups.

**Conclusion:** Within the limitations of this project, we conclude that patients with xerostomia may be more susceptible to oral yeast infections, but that there is no significant difference in the salivary proteins between xerostomia subjects and healthy controls. This project was supported by a Fuller scholarship.
Appendix IV

Ethical Approval Letter

Lower South Regional Ethics Committee
c/Ministry of Health 229 Moray Place Dunedin
Phone: (03) 4748562 Email:
lowersouth_ethicscommittee@moh.govt.nz

1 November 2010

Ms Leanne Xiaoli Hou Postgraduate Suite 3.74 Department of Oral Rehabilitation
Re: Ethics ref: LRS/10/09/034 (please quote in all correspondence)
Study title: Does saliva from dry mouth patients with Sjögren's syndrome promote greater Candida albicans adhesion than saliva from healthy individuals?
Investigators: Ms Leanne Xiaoli Hou, Professor Richard Cannon, Associate Professor Anita Nolan, Dr Ann Holmes, Mr Karl Lyons

Faculty of Dentistry University of Otago,
PO Box 647 Dunedin

Dear Ms Hou

This study was given ethical approval by the Lower South Regional Ethics Committee on 1 November 2010.

Approved Documents
— Amended Application Form
— Consent Form version 2, 19 October 2010

This approval is valid until 31 October 2012, provided that Annual Progress Reports are submitted (see below).

Access to ACC For the purposes of section 32 of the Accident Compensation Act 2001, the Committee is satisfied that this study is not being conducted principally
for the benefit of the manufacturer or distributor of the medicine or item in respect of which the trial is being carried out. Participants injured as a result of treatment received in this trial will therefore be eligible to be considered for compensation in respect of those injuries under the ACC scheme.

Amendments and Protocol Deviations All significant amendments to this proposal must receive prior approval from the Committee. Significant amendments include (but are not limited to) changes to:
— the researcher responsible for the conduct of the study at a study site
— the addition of an extra study site
— the design or duration of the study
— the method of recruitment
— information sheets and informed consent procedures.

Significant deviations from the approved protocol must be reported to the Committee as soon as possible.

Annual Progress Reports and Final Reports The first Annual Progress Report for this study is due to the Committee by 1 November 2011. The Annual Report Form that should be used is available at www.ethicscommittees.health.govt.nz. Please note that if you do not provide a progress report by this date, ethical approval may be withdrawn.

A Final Report is also required at the conclusion of the study. The Final Report Form is also available at www.ethicscommittees.health.govt.nz.

Requirements for the Reporting of Serious Adverse Events (SAEs) For the purposes of the individual reporting of SAEs occurring in this study, the Committee is satisfied that the study’s monitoring arrangements are appropriate.

SAEs occurring in this study must be individually reported to the Committee within 7-15 days only where they: — are unexpected because they are not outlined in the investigator’s brochure, and — are not defined study end-points (e.g. death or hospitalisation), and — occur in patients located in New Zealand,
and — if the study involves blinding, result in a decision to break the study code.

There is no requirement for the individual reporting to ethics committees of SAEs that do not meet all of these criteria. However, if your study is overseen by a data monitoring committee, copies of its letters of recommendation to the Principal Investigator should be forwarded to the Committee as soon as possible.

Please see www.ethicscommittees.health.govt.nz for more information on the reporting of SAEs, and to download the SAE Report Form.

We wish you all the best with your study.

Yours sincerely

Anna Paris
Lower South Regional Ethics Committee Administrator

dd (03) 474 8562 fax (03) 474 8090 Email: anna_paris@moh.govt.nz
Information sheet for dry mouth participants in the study:

Saliva from dry mouth patients with Sjögren’s syndrome *

You are invited to take part in this research project.

Introduction

Surfaces in the mouth are coated with saliva. Most people have the common microbe, the yeast *C. albicans* present in their mouths. This yeast sticks (adheres) to the saliva coating on oral surfaces and is usually totally harmless. However, sometimes it can cause a sore mouth (this is known as “thrush” or denture sore mouth).

People with the symptoms of dry mouth may have saliva with different types, or amounts, of saliva proteins. We want to study this and also find out whether this can affect the way *C. albicans* adheres to surfaces in the mouth.

You are invited to take part in this study. Please read this information sheet carefully before deciding whether or not to participate. If you decide to participate, we thank you. If you decide not to take part there will be no disadvantage to you of any kind and we thank you for considering our request.

*
What are the aims of this study?
Our aim is to find out whether saliva samples from people with dry mouth contain different proteins than those from people without dry mouth. We will also find out whether saliva samples from people with dry mouth promote a different amount of attachment of C. albicans to a representative oral surface, than saliva from people with a normal saliva flow.

Our goal is to use this information to improve oral health for people with dry mouth.

What types of participants are being sought?
Individuals with dry mouth attending the Oral Medicine clinic at the University of Otago dental school.

What will participants be asked to do?
Should you agree to take part in this project, and complete a consent form, you will be asked to rinse your mouth with 10 ml of water (commercially bottled) for 30 seconds without swallowing before dribbling the water into a sterile glass container. The sample will be collected in the clinic, and collection will take approximately one minute.

What are the benefits of the study?
The results of this study will improve our understanding of how the yeast C. albicans sticks to surfaces in the mouth. With this information we may be able to develop ways of preventing C. albicans from staying on the mouth and thus
reducing the chance of dry mouth patients getting oral thrush or denture sore mouth.

**What are the risks of the study?**

There is no risk of harm from rinsing your mouth with water.

**Do I have to participate in this study?**

Your participation is entirely voluntary (your choice). You do not have to take part in this study, and if you choose not to take part this will not affect any future dental care or treatment.

**What data or information will be collected and what use will be made of it?**

The investigator will collect information from your dental records on your age, gender, ethnicity and medical history, or directly from you if this information is not in your records. The results of this project will be written in a report, and may be published, but no material that could personally identify you will be used in any reports on this study.

You will have access to, and will be able to correct, any personal information gathered concerning you, and you are most welcome to request a summary of the results of the project when completed.

The data collected will be securely stored in such a way that only those people mentioned above will be able to gain access to it. At the end of the project any personal information will be destroyed immediately except that, as required by the University's research policy, any raw health data on which the results of the project depend will be retained in secure storage for ten years, after which it will
be destroyed. The saliva rinse samples will be tested for the presence of proteins and yeast. The remainder of the samples will be stored securely and after the completion of the project they will be destroyed by autoclaving.

As samples of human tissue will be taken during this study, there may be cultural issues associated with storing tissue that need to be discussed with your family/whanau. Some Iwi disagree with storage of human tissue citing whakapapa and advise their people to consult prior to participation in research where this occurs. To avoid problems at a later stage, we suggest your family/whanau is involved with you at all stages of the research. However, we also acknowledge that individuals have the right to choose to participate.

What if participants have any questions?
If you have any questions about this project or want more information, either now or in the future, please feel free to contact Professor Richard Cannon, Department of Sciences, School of Dentistry, telephone (03) 479 7081. If you have any queries or concerns about your rights as a participant in this study you may wish to contact a Health and Disability Services Consumer Advocate, telephone (03) 479 0265 or 0800 37 77 66.

In the unlikely event of a physical injury as a result of your participation in this study, you will be covered by the accident compensation legislation with its limitations. If you have any questions about ACC please feel free to ask the researcher for more information before you agree to take part in this trial.
This study has received ethical approval from the Lower South Regional Ethics Committee, ethics reference number XXX.

Thank you for considering this request to take part in this study.

Please feel free to contact the researcher if you have any questions about this study.

Principal Investigator: Leanne Xiaoli Hou, postgraduate student, Department of Oral Rehabilitation, University of Otago, Tel: (03) 479 7125
Supervisor: Professor Richard Cannon, Department of Oral Sciences, University of Otago, Tel: (03) 479 7081

* When the study was initially designed it was expected that all patients in the study would be diagnosed with Sjögren’s syndrome; the documents were not changed when given to xerostomia patients with other possible causes of dry mouth.
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Appendix VI  Control Information Sheet

Information sheet for control participants in the study:

Saliva from dry mouth patients with Sjögren’s syndrome*

You are invited to take part in this research project.

Introduction

Surfaces in the mouth are coated with saliva. Most people have the common microbe, the yeast *C. albicans* present in their mouths. This yeast sticks (adheres) to the saliva coating on oral surfaces and is usually totally harmless. However, sometimes it can cause a sore mouth (this is known as “thrush” or denture sore mouth).

People with the symptoms of dry mouth may have saliva with different types, or amounts, of saliva proteins. We want to study this and also find out whether this can affect the way *C. albicans* adheres to surfaces in the mouth.

You are invited to take part in this study. Please read this information sheet carefully before deciding whether or not to participate. If you decide to
participate, we thank you. If you decide not to take part there will be no
disadvantage to you of any kind and we thank you for considering our request.

What are the aims of this study?

Our aim is to find out whether saliva samples from people with dry mouth contain
different proteins than those from people without dry mouth. We will also find out
whether saliva samples from people with dry mouth promote a different amount
of attachment of *C. albicans* to a representative oral surface, than saliva from
people with a normal saliva flow.

Our goal is to use this information to improve oral health for people with dry
mouth.

What types of participants are being sought?

The project needs a control group of participants who do not suffer from dry
mouth. These participants will be recruited from the staff and students working in
the dental school and from patients attending dental school clinics.

What will participants be asked to do?

Should you agree to take part in this project, and complete a consent form, you
will be asked to rinse your mouth with 10 ml of water (commercially bottled) for
30 seconds without swallowing before dribbling the water into a sterile glass container. The sample will be collected in the clinic, or other part of the dental school (for staff and students) and collection will take approximately one minute.

**What are the benefits of the study?**

The results of this study will improve our understanding of how the yeast *C. albicans* sticks to surfaces in the mouth. With this information we may be able to develop ways of preventing *C. albicans* from staying on the mouth and thus reducing the chance of dry mouth patients getting oral thrush or denture sore mouth.

**What are the risks of the study?**

There is no risk of harm from rinsing your mouth with water.

**Do I have to participate in this study?**

Your participation is entirely voluntary (your choice). You do not have to take part in this study, and if you choose not to take part this will not affect any future dental care or treatment or, for staff and students, your future employment or academic progress.

**What data or information will be collected and what use will be made of it?**
The investigator will collect information from your dental records on your age, gender, ethnicity and medical history, or directly from you if this information is not in your records. The results of this project will be written in a report, and may be published, but no material that could personally identify you will be used in any reports on this study.

You will have access to, and will be able to correct, any personal information gathered concerning you, and you are most welcome to request a summary of the results of the project when completed.

The data collected will be securely stored in such a way that only those people mentioned above will be able to gain access to it. At the end of the project any personal information will be destroyed immediately except that, as required by the University's research policy, any raw health data on which the results of the project depend will be retained in secure storage for ten years, after which it will be destroyed. The saliva rinse samples will be tested for the presence of proteins and yeast. The remainder of the samples will be stored securely and after the completion of the project they will be destroyed by autoclaving.

As samples of human tissue will be taken during this study, there may be cultural issues associated with storing tissue that need to be discussed with your family/whanau. Some Iwi disagree with storage of human tissue citing whakapapa and advise their people to consult prior to participation in research where this occurs. To avoid problems at a later stage, we suggest your family/whanau is
involved with you at all stages of the research. However, we also acknowledge that individuals have the right to choose to participate.

What if participants have any questions?

If you have any questions about this project or want more information, either now or in the future, please feel free to contact Professor. Richard Cannon, Department of Sciences, School of Dentistry, telephone (03) 479 7081. If you have any queries or concerns about your rights as a participant in this study you may wish to contact a Health and Disability Services Consumer Advocate, telephone (03) 479 0265 or 0800 37 77 66.

In the unlikely event of a physical injury as a result of your participation in this study, you will be covered by the accident compensation legislation with its limitations. If you have any questions about ACC please feel free to ask the researcher for more information before you agree to take part in this trial.

This study has received ethical approval from the Lower South Regional Ethics Committee, ethics reference number XXX.

Thank you for considering this request to take part in this study.

Please feel free to contact the researcher if you have any questions about this study.
Appendices

Principal Investigator: Leanne Xiaoli Hou, postgraduate student, Department of Oral Rehabilitation, University of Otago, Tel: (03) 479 7125

Supervisor: Professor Richard Cannon, Department of Oral Sciences, University of Otago, Tel: (03) 479 7081

* When the study was initially designed it was expected that all patients in the study would be diagnosed with Sjögren’s syndrome; the documents were not changed when given to xerostomia patients with other possible causes of dry mouth.
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Appendix VII  Consent Form

Consent Form

For the research project:

Saliva from dry mouth patients with Sjögren’s syndrome*

I have read and I understand the information sheet dated 1 October 2010 for volunteers taking part in the study designed to find out more about the composition of saliva in patients with dry mouth and how the difference of the protein content might affect the adhesion of the yeast *C. albicans* to oral surfaces. I have had the opportunity to discuss this study. I am satisfied with the answers I have been given.

I understand that taking part in this study is voluntary (my choice) and that I may withdraw from the study at any time and this will in no way affect my future dental care or treatment.
I understand that my participation in this study is confidential and that no material that could identify me will be used in any reports on this study.

I have had time to consider whether to take part.

I know whom to contact if I have any questions about the study.

I consent to the researcher having access to medical information from my patient files that is relevant to this study.

I consent to the researcher storing my saliva rinse samples for their later use as part of this study.

I consent to my saliva rinse samples being destroyed at the end of this study.

I wish to receive a copy of the results. Yes/No

I (full name) .................................................. hereby consent to take part in this study.

Date: ..........................................................

Signature: ..................................................
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Full name of Supervisors: Professor Dr Richard Cannon, Department of Oral Sciences, University of Otago (03) 479 7081

Associate Professor Anita Nolan, Department of Oral Diagnostic and Surgical Sciences, University of Otago (03) 470 7046

Dr. Ann Holmes, Department of Oral Sciences, University of Otago (03) 479 7435

Mr. Karl Lyons, Department of Oral Rehabilitation, University of Otago (03) 479 7122

Project explained by: .................................................................

Project role: ...........................................................

Signature: ...........................................................

Date: ...........................................................

* When the study was initially designed it was expected that all patients in the study would be diagnosed with Sjögren’s syndrome; the documents were not changed when given to xerostomia patients with other possible causes of dry mouth.
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Appendix VIII  Patient Questionnaire and Clinical Sheet

Saliva from dry mouth patients

CONFIDENTIAL QUESTIONNAIRE

Name: ................................................ Unique identifier:

DOB:………………………………… Gender: Male / Female

Ethnicity: NZ European Other European NZ Maori Asian…………………………. Pacific Island (please specify) ……. Other (please specify) ………………….

Medical History

Have you ever had any of the following?

Dry mouth ........................................ YES / NO  If yes, do you currently have dry mouth? YES / NO

Diabetes...................................................YES / NO  Smoker…………… YES / NO

Asthma ....................................................YES / NO

Any other illness not mentioned above?
………………………………………………………………………………

Are you taking any kind of medications, pills or recreational drugs now?
YES / NO
If so, which ones?
……………………………………………………………………………………
………………………………………………………………………………
…………………………………….
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Have you taken any other medications in the past year?
YES / NO
If so, which ones?
........................................................................................................................................
...

Do you wear dentures? ......................... If so, for how long?
.................................
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Clinical Sheet

Sheet provided by Associate Professor Anita Nolan

Date of sample:

Patient ID:

Trial no:

Dry Mouth:

Cause: (circle)
Medication
Sjogren’s
Other connective tissue disease
Sarcoidosis
Radiation
Hep C
HIV

Investigations
Labial gland Bx:
Ro
La
IgG
SACE
Other

Unstimulated salivary flow rate:

Clinical type of Oral candidosis:

No clinical Candidosis
Denture Stomatitis:  N1  N2  N3
Atrophic
Pseudomembranous
Angular Cheilitis
MRG
Candidal leukoplakia

Current medications:

Smoking

Alcohol