Candida Albicans Adhesion and Biofilm Formation on Phosphated and Non-Phosphate Containing Poly(Methylmethacrylate) Polymers

Kathryn Elizabeth Ryan
Marquette University

CANDIDA ALBICANS ADHESION AND BIOFILM FORMATION ON PHOSPHATED AND NON-PHOSPHATE CONTAINING POLY(METHYLMETHACRYLATE) POLYMERS

by

Kathryn Ryan, D.D.S.

A Thesis submitted to the faculty of the Graduate School,
Marquette University,
in Partial Fulfillment of the requirements for
the Degree of Master of Science

Milwaukee, Wisconsin

May 2010

ABSTRACT

CANDIDA ALBICANS ADHESION AND BIOFILM FORMATION ON PHOSPHATED AND NON-PHOSPHATE CONTAINING POLY(METHYLMETHACRYLATE) POLYMERS

Kathryn Ryan, D.D.S.

Marquette University, 2010

Denture-induced stomatitis is prevalent among prosthesis users[1], particularly the elderly and institutionalized where it has been estimated to be present in over 60-65% of denture wearers.[2] Treatment of this clinical condition is problematic due to incomplete disinfection of the acrylic surface and rapid microbial re-colonization. Moreover, ingestion or aspiration of pathogens in denture plaque exposes the elderly and immunocompromised to unexpected infections.[3,4]

Acrylic polymers have generally been used to replace missing teeth and periodontal tissues in edentulous or partially dentate patients. Currently, edentulous adults in the US population are common, with complete edentulism greater than 41% among Americans aged 65 and older.[5] Poly (methyl methacrylate) [PMMA] is the most commonly used denture base material, and satisfies most esthetic, physical and mechanical criteria for an intra-oral prosthesis. However, the absence of ionic charge may inhibit the selective adsorption of salivary antimicrobials which could provide a more protective pellicle coat.[6]

Salivary antimicrobials such as defensins and histatins are cationic peptides, which are strongly adsorbed onto the tooth surface by electrostatic interaction.[7,8] Absence of an ionic surface on PMMA not only minimizes the adsorption of defense molecules on denture surfaces, attractive London-van der Waals forces actually facilitate adherence of *Candida albicans* and other microorganisms.[9] This is a prerequisite for denture biofilm formation and subsequent denture stomatitis.[10]

The goal of this research project is to assess the ability of phosphated polymers to inhibit *C. albicans* adhesion and biofilm formation, in the hope of preventing denture-induced stomatitis. The novel denture-base polymers incorporate a charge through bead suspension polymerization with poly (methyl methacrylate) [PMMA] and a phosphate containing monomer. Other groups have also been looking to alter denture base surfaces using other techniques.[9,11-13]

The possible effects of this project include a new generation of denture-base polymers with great potential for clinical and commercial application in a large population of denture and oral prosthesis users.

ACKNOWLEDGMENTS

Kathryn Ryan, D.D.S.

TO MY PARENTS

I thank my parents, Christine and Michael for supporting me throughout all of my successes and for always encouraging me to pursue every academic endeavor to the fullest. They have always been the driving force behind everything I've committed myself to. I would not be where I am today without their love, patience and continued faith in me.

TO MY HUSBAND

I thank my husband, Nicholas for always believing in me and staying patient throughout stressful times. He has always had faith in me and stayed committed to my educational goals. With his love, patience and incredible support I have been able to accomplish my dreams.

TO THE THESIS COMMITTEE

I would like to recognize Dr. Andrew Dentino, the thesis director for his profound knowledge, guidance, and unending support that he lent to this publication. I would like to thank Dr. April Harkins, for her insight, expertise and support. Without Dr. Harkins this project would not have been possible. I would also like to thank Dr. Gerald Ziebert, for his diligence and knowledge, Dr. Arthur Hefti, Dr. Abraham Lee, and Dr. Wen Guojiang for their suggestions and assistance.

TABLE OF CONTENTS

LIST OF TABLES

Table Page No.

LIST OF FIGURES

Figure Page No.

Figure Page No.

CHAPTER I

INTRODUCTION

Previous literature suggests that retention rates for natural teeth are on the rise, creating concern for significant reduction in the demand for removable prostheses.[14,15] Middle-aged populations have experienced improved health care and longer life spans compared to previous generations, therefore there is a possibility that edentulism could rapidly decline. More current information, however, refutes this idea and supports the fact that the number of older adults is on the rise and that the number of edentulous elderly will actually increase in the next two decades.[16] Douglass et al[17] calculated that the adult population in need of 1 or 2 complete dentures would increase from 33.6 million in 1991 to 37.9 million in 2020. There will be a constant increase in edentulism over the next ten years. Therefore at least in the near future there is going to be a growing need for denture prosthetics.

Denture acrylic resins historically have been the material of choice for the fabrication of complete dentures in the edentulous patient. Polymethyl methacrylate (PMMA) is the prosthetic material of choice, providing high esthetic quality, and substantial mechanical and physical properties to withstand the harsh oral environment. Acrylic is important to the replacement of lost hard and soft tissues as well as redistributing masticatory forces from the denture to the residual ridge. Use of acrylic is inherent for denture fabrication but microbial colonization predisposes denture wearers to a prevalent infectious disease called denture-induced stomatitis also known as chronic atrophic candidiasis or more currently referred to as Candida-associated denture stomatitis.[18,19]

A significant number of denture wearers develop a Candida-associated infection of the palatal mucosa underneath the upper denture.[20,21] Denture-induced stomatitis is the

description used for the pathologic changes found in the oral mucosa of the denture-bearing tissues. The observed mucosal changes present as a bright erythema, found under complete or partial dentures in the maxilla and the mandible but most commonly in the maxilla where denture contact area is much greater.[22] The mucosal lesions are multifactorial in nature but the most common significant pathogen isolated is the fungal species *Candida albicans.*[23,24] *C. albicans* has been found on and isolated more often from the actual fitting surfaces of dentures and in higher numbers then on the mucosal tissues.[20,25]

Successful colonization of the superficial epithelial cells and on acrylic surfaces is a prerequisite for infection. Lyon and Chick[26] have shown that in several patients a more profuse growth of Candida was collected on the denture surface than from the mucosa. Following this, Avon, et al[27]placed denture acrylic inserts into complete maxillary dentures and microscopically evaluated microbial colonization. They observed numerous spores, hyphes and germ tubes, implying the presence of Candida cells within the denture biofilm. This suggests that Candida may be present in high numbers and in a virulent stage of growth on the denture acrylics. Studying the acrylic surfaces and their relationship to *C. albicans* adherence is of significant importance for our investigation.

Acrylic surface topography and the presence or absence of saliva on acrylic surfaces directly affects adsorption of *C. albicans*. Several studies support the rationale that denture acrylics with a smooth surface reduce microbial adherence.[28-30] Comparatively, a roughened surface produces increased attachment of microorganisms but in a moist environment such as the oral cavity, transport and adsorption of organic molecules to a surface occurs more quickly than that of microorganisms.[31] Salivary

pellicle covers the surfaces within the oral cavity and serves to protect, lubricate, clear and adhere, normal resident flora.[6] Glycoproteins within saliva have demonstrated the ability to enhance microbial adherence on intraoral hard surfaces.[32] Studies have shown that acquired denture pellicle contains absorbed proteins, but this pellicle may not have the same protective proteins found in acquired enamel pellicle, therefore surface modified resins with components like an added phosphate group attract more positively charged salivary antimicrobial proteins and act as a deterrent for Candida adhesion and growth.[33] Irrespective of surface charge, studies have shown contradictory results regarding the effects of pellicle on *C. albicans* adhesion to PMMA surfaces. Some have shown less adhesion of *C. albicans* to denture pellicle while others have shown significantly increased adhesion.[6,28,34-37] Previous studies demonstrate conflicting results, possibly due to variation in protocol. Therefore, the focus of our study was to adopt a reliable methodology for adhesion and development of a *C. albicans* biofilm on denture pellicle *in vitro.*

A study by Edgerton et al [21]focused on the identification of salivary molecules involved in the adsorption of *C. albicans.* The type and quantity of bound salivary components on artificial PMMA surfaces may be influential for the colonization of different micro-organisms.[33] The idea to prevent fungal adhesion by loading salivary components in the form of antimicrobial peptides onto PMMA surfaces was proposed by Edgerton et al.[38] In particular the antimicrobial peptide histatin, found in the major salivary glands, has fungicidal and fungistatic effects on *C. albicans* which could regulate biofilm formation on PMMA. Due to the surface charge of histatin, it does not readily adhere to denture base resins. Edgerton et al[38] modified PMMA at the surface by

introducing a carboxyl group (negative charge) which resulted in double the adsorption of histatin to PMMA. Yoshinari et al[13] coated PMMA surfaces with cold plasma and changed the functional groups to absorb histatin 5. The modified PMMA surfaces adsorbed histatin 5 six fold and effectively reduced *C. albicans* biofilm formation. With this in mind, our study incorporated a phosphate group, giving PMMA a surface charge that could possibly enhance the attraction of protective salivary antimicrobials.

The purpose of our investigation was to examine the effects of surface roughness, protein pellicle and phosphate charge density on adhesion and biofilm formation of several *Candida albicans* strains to polymethyl methacrylate-based resins. These phosphated resins were compared to a control non-phosphate containing resin, Lucitone 199 (York, PA, USA). Four different strains of Candida were evaluated in our study and we wanted to determine if there was a variation in their ability to colonize various acrylic surfaces.

The null hypothesis was that there would be no change in *C. albicans* adherence to a negatively charged denture base material compared with the normal uncharged denture base material polymethyl methacrylate (PMMA).

CHAPTER II

REVIEW OF THE LITERATURE

Unmet need for provision of complete dentures in the U.S. populations:

Prevention of tooth loss is based on quality of care and the dental care providers' role in treatment. Multiple oral diseases can lead to tooth loss but dynamics such as access to care, motivation of patients, varying treatment philosophies of care and treatment modalities have changed our clinical outlook for care.[39] The goal of dentistry as a whole is to provide patients with oral function, maintain existing dentition, and provide adequate esthetic results. The current elderly population has not benefitted from modern advances in dentistry resulting in a 40% their population being edentulous.[40]

Studying the elderly is critical due to the prediction of growth in this population. The reason for the rise in this generation is the World War II baby boom, decreases in infant/childbirth mortality, deaths from infectious diseases, as well as life expectancy at birth and age 65 has greatly increased over the past ten years.[39] Education regarding healthy lifestyles, nutrition and advancements in modern medicine have helped the growth of the elderly population.[40] According to current population reports for the United States, the fastest growing population is the age 85 and up grouping. In 1994, 1 in 8 Americans were elderly and predictably by 2030, 1 in 5 Americans will be elderly.[16]

A study of a subset of the U.S. elderly population showed that edentulism was negatively correlated to both education level and annual income.[41] The majority of denture wearers had a high school education and an annual income of less than $5,000.00 per year. Of the subset, 90% of the completely edentulous had both maxillary and mandibular dentures, 80% wore both during the day, and almost a third of the participants slept with both dentures inserted.[14] This shows that based on low education and income,

denture wear is more prevalent. Furthermore, most edentulous patients have dentures and wear them during the night which may be the most significant factor for developing denture stomatitis.[42]

The NHANES I surveys, reviewed the need-for-treatment, and prosthodontic service data. Among adults 65-74 years of age, the greatest need was for treatment with complete dentures.[43] This study also showed that an increase in unmet edentulous treatment need is not due to an increase in the prevalence or severity of disease in the US population, but was due entirely to an increase in the number of older adults in the United States by the year 2000. There may be declining edentulism over time but the unmet need for prosthodontic services will remain high.[17] A review of elderly Iowans, showed that even with increasing populations of healthy elderly, the demand for dental care from the elderly groups will increase.[44] Provision of removable prosthetics in lower socioeconomic groups within elderly populations will remain pronounced.[39] As well, there will become a predictable increase in demand for prosthodontic services in the elderly when comparing several investigations.[17,45,46]

Growth in the population will occur with the groups older than 45 years of age substantially from 1991 to 2020. This increase will be 6% for ages 75 to 84 years and 8.7% for people 85 years and older.[17] Even with a 10% decline of edentulousness over the next 30 years, 24% of people aged 55-65 will need 1 or 2 dentures in 2020 and 41.5% of the population age 75 and up will need dentures by 2020. Even if there is a large group of the population that will never be edentulous in the future, the increasing population size for the elderly will lead to a rise in the number of dentures fabricated.[39]

Analysis of current elderly, age 65 and older, edentulous non-institutionalized individuals showed that 92% were unsatisfied with their dentures or felt they needed denture replacement.[46] The patients based their evaluations on denture fit, if sores or ulcerations were present, difficulty chewing food, and difficulty speaking. Chewing and fit both rated as needing improvement. In addition, the perceived need showed that 97% of the subjects felt they required dental therapy and 90% of the dentures were evaluated by examiners who determined the need for refinement or a remake.[41] Therefore it can be concluded that with a rise in elderly populations, there will be both clinical necessity and perceived needs by patients for denture care.

The current literature critically evaluates the present world populations and views the need for denture therapy to be on the rise. Adults aged 65 and above were universally viewed as elderly populations and this percentage of the population is ever increasing. Conventional denture therapy is critical to sustain this ever growing elderly population. Dental health care providers must incorporate denture fabrication into their practices as a standard of care. Denture fabrication is not a treatment modality of the past, it is the future. Care and education for the patient and maintenance of their dentures are imperative to sustain oral as well as overall health. .

Pathogenesis in the Elderly:

Elderly populations are at a heightened risk of oral mucosal diseases because with increased age comes hyposalivation caused by an added number of medications, used to treat increased numbers of diseases. Oral mucosa becomes more permeable to noxious agents and more susceptible to mechanical trauma.[47] These observations can increase the risk factors for abnormal changes of the mucosal tissues within the elderly. This also implicates that the elderly are at increased risk of yeast infections and possibly the development of oral cancers.[48]

Another area of concern involves the oral hygiene habits among the elderly populations. Their hygiene is considerably reduced when comparing younger generations. In a study which determined oral health related habits of the elderly, it was found that 91-96% of 303 dentate subjects above 60 years old needed improvement on their oral hygiene.[49] Changes in mental health and personal well being, as well as physical disabilities, reducing manual dexterity can alter an elderly patients' ability to maintain their oral hygiene. Again, the Budtz- Jörgensen et al[22] paper supports this idea that with age there is a higher prevalence of Candida infection and poor denture hygiene. Deterioration of general health compromises the immune defense system in the elderly leading to pathologic colonization of the opportunistic yeast, *C. albicans.*[50] Continued swallowing or aspiration of microorganisms exposes at risk elderly to numerous infections. For instance, once yeasts and other oral bacteria have colonized a denture, their release into salivary secretions can be aspirated into the respiratory tract which may lead to pneumonia. Pneumonia can be life-threatening and is a cause of morbidity and

mortality in the elderly. Pneumonia has been linked to aspiration of oralpharyngeal bacteria from dentures.[51,51,51] Another study by Sumi et al[4] showed that the predominant microorganisms found on dentures in 50 patients were Streptococcus and Candida. They concluded that denture plaque can be a reservoir for colonization and subsequent aspiration pneumonia.

Prevalence of denture wearing increases with age and with age there is an increased risk for general health complications.[3] More medications are used to maintain health but this can cause a reduction in saliva which is a natural protectant from oral infection.[40] Reduced saliva and poor oral hygiene are directly related to higher prevalence of Candida colonization on dentures.[42] Once colonization occurs this opens up further health risks for the elderly. Therefore we need to recognize this at risk group and develop a denture treatment that reduces microbial colonization.

Denture Induced Stomatitis:

The oral cavity is not impervious to bacterial infection in the edentulous patient. When wearing a removable denture, oral mucosal lesions can occur and become acute or chronic in nature. Mucosal irritations are associated with hypersensitivity reactions to residual monomer in denture acrylics, microbial colonization on the denture surface in the form of a biofilm or plaque, and mechanical trauma or irritation.[52] Denture induced oral lesions are categorized as: angular cheilitis, atrophic glossitis, pseudomembranous candidosis, traumatic ulcers, denture irritation hyperplasia, denture induced stomatitis, flabby ridges and oral carcinomas.[2]

Denture (Candida*)* induced stomatitis (DIS) or chronic atrophic candidiasis, associated with denture wear, are the terms used in the literature to constitute an inflammatory state of the denture bearing mucosa.[19,53] It is the commonest form of oral candidosis and is present in 24-60 per cent of denture wearers.[54] It can also be defined as a chronic erythema and edema of part or all of the palatal mucosa under maxillary dentures.[54] Elderly populations are the most susceptible and the most common location of occurrence is on the palatal mucosa.[25] The classification for denture induced stomatitis was proposed by Newton, et al[55] in 1962 and is categorized by severity into three forms: Type I is made up of localized (pin-point) erythematous lesions, Type II is a diffuse erythematous lesion and Type III, the most severe, is a hyperplastic granular lesion. The condition is usually symptom free but can present with mucosal bleeding, swelling, burning or other painful sensations, oral dryness, halitosis and unpleasant taste.[56]

Denture stomatitis can be caused by mucosal trauma, microbial colonization, continued denture wear, lack of denture hygiene, and denture plaque formation. Trauma was considered to be a dominant factor in denture stomatitis according to Nyquist.[57] Causes of localized stomatitis were associated with trauma from ill-fitting dentures and resolution of the lesions occurred after denture adjustments.[50]

Bacterial numbers are usually characteristic of an individual and there appear to be more aerobic than anaerobic clusters found on dentures and mucosal surfaces. Nyquist also found that dentures worn for longer periods of time showed increased bacterial counts.[57] High numbers but great variations in the type of Gram-positive bacteria were isolated from the palatal mucosa of denture stomatitis patients. In comparison, non-denture wearers had an absence of bacteria on their palatal epithelium.[58] Determination of whether or not bacterial colonization occurs before or after yeast adhesion has yet to be discovered.

Initial colonization has been linked to a fungal species called *C. albicans.*[24] The most recent literature shows that denture induced stomatitis has a direct correlation to chronic inflammation.[59] Many studies, past and present, have found the major oral isolate recovered from the mucosa in stomatitis patients to be *C. albicans.*[23] Initial adherence of the Candida species may lead to proliferation and complications due to infection. Risks include trauma, irritation, denture hygiene, night time wear, and smoking.[59] Predisposing factors for DIS development are systemic diseases such as diabetes mellitus and immunocompromised patients.[60]

Yeast cells have been isolated from the oral cavity in 25 and 50 per cent of healthy adults and children respectively. Comparing denture wearers, that value

increased to 60 to 100 per cent. *C. albicans* was found in 70% of denture wearers, by far the most common fungal species isolated from the oral mucosa. Other common species found in both the denture and non-denture wearers were *C. tropicalis, C. glabrata, C. parapsilosis and C. krusei,* stated in ascending order from the most prevalent to the least.[61,62]

Studies have shown that once *C. albicans* was isolated from the denture base of stomatitis patients, hyphae were present, determining that yeasts are in an active stage of growth. When dentures were removed from the mouth, the protective environment that the denture maintained was eliminated and the cleansing effects of the tongue and saliva become active, reducing yeast counts.[63] Research by Budtz-Jorgensen et al[2] and Van Reenen et al[58] showed a recovery of Candida cells higher in number on the intaglio surfaces of dentures then on the palatal mucosa. Therefore it is reasonable to assess the growth of Candida on denture plaques for a better understanding of the relationship between candida and denture stomatitis development.

Current surveys and evaluations of populations have shown and supported the claim that the elderly are predisposed to denture induced stomatitis. Although prevalence does vary due to disease threshold criteria, methods for diagnostic evaluation and other factors, these studies revealed the following: A German study with individuals in the 65 – 74 year age range and sample size of 90 were clinically evaluated for denture stomatitis and 18.3% of the individuals exhibited DIS.[1] A study conducted in Chile showed a prevalence of 22.3% of patients aged 65 and over to have denture stomatitis.[64] Another clinical evaluation in Finland showed that denture wearers, ages 76, 81 and 86 had a prevalence of 25% for a the oral mucosal lesion recognized as denture stomatitis.[65] The

highest prevalence of denture stomatitis was found in a Swedish study where 59.2% of the patients had the disease.[66] Denture stomatitis is also linked to institutionalized elderly and was reported in Brazil to have a prevalence of 20% of the cases.[62] These are some examples of the variation in world-wide prevalence rates. With so many elderly denture patients being infected by Candida, there is an ever present demand for therapeutic treatment options.

Candida biofilm development:

Biofilms are microbial communities encased in a matrix of extracellular polymeric substances, displaying phenotypic features that differ from their planktonic or free-floating counterparts.[67] *C. albicans* biofilm development has been observed by multiple researchers and structured into three developmental phases.[37,68,69]

Phase I (Early 1-11 hours): Adhesion of microorganism to a surface: Candida biofilms form after free floating Candida cells adhere to a substrate or surface. This process takes 1 to 2 hours and the *C. albicans* cells are blastospores.[37,70,71] Adhesion occurs because of non-specific interactions such as hydrophobic and electrostatic forces, between the cells and the substratum.[70,72] Next, specific adhesion molecules such as glycoproteins are expressed to facilitate stronger adhesion. At 3 to 4 hours, microcolonies appear and after 11 hours, *C. albicans* aggregations appear on substratum irregularities.[37]

Phase II (Intermediate/Developmental 12-30 hours): Candida biofilm structure now develops into a bilayer composed of yeasts, germ tubes and young hyphae with a matrix of extracellular polymeric substances (EPS).[73] EPS encapsulation matrix is composed of polysaccharides produced by the microbial components, and they often reside in a state of reduced metabolic activity.[69] The biofilm now has a non-cellular layer or EPS covering the fungal microcolonies.

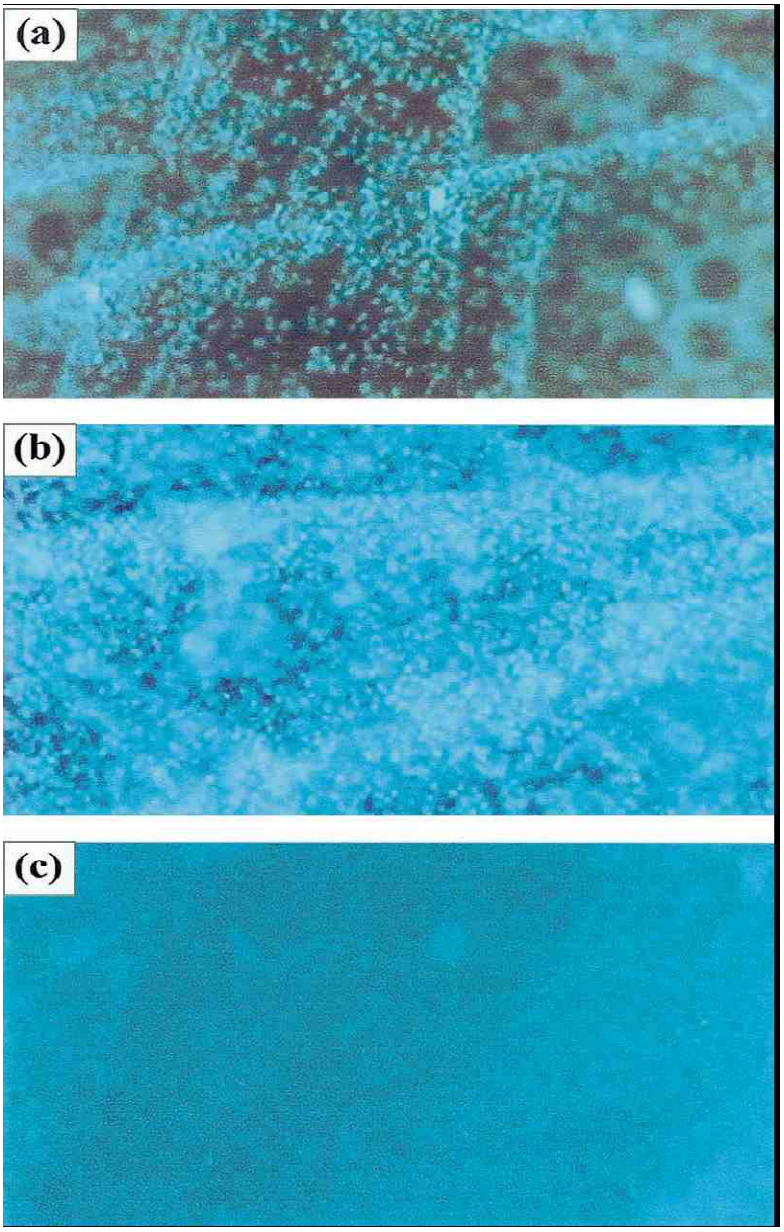
Phase III (Maturation 38-72 hours): Extracellular material increases with incubation time, until *C. albicans* yeasts, pseudohyphae and hyphae are entirely embedded in a matrix.

Phase I – III can be described as biofilms or fungal communities, encased in an extracellular polymeric substance.

Water channels between cells facilitate diffusion of nutrients from the environment to the bottom layers of the biomass and reversibly remove waste from the bottom layers to the environment.[74] This maintains the biofilm in an *in vitro* or *in vivo* environment.

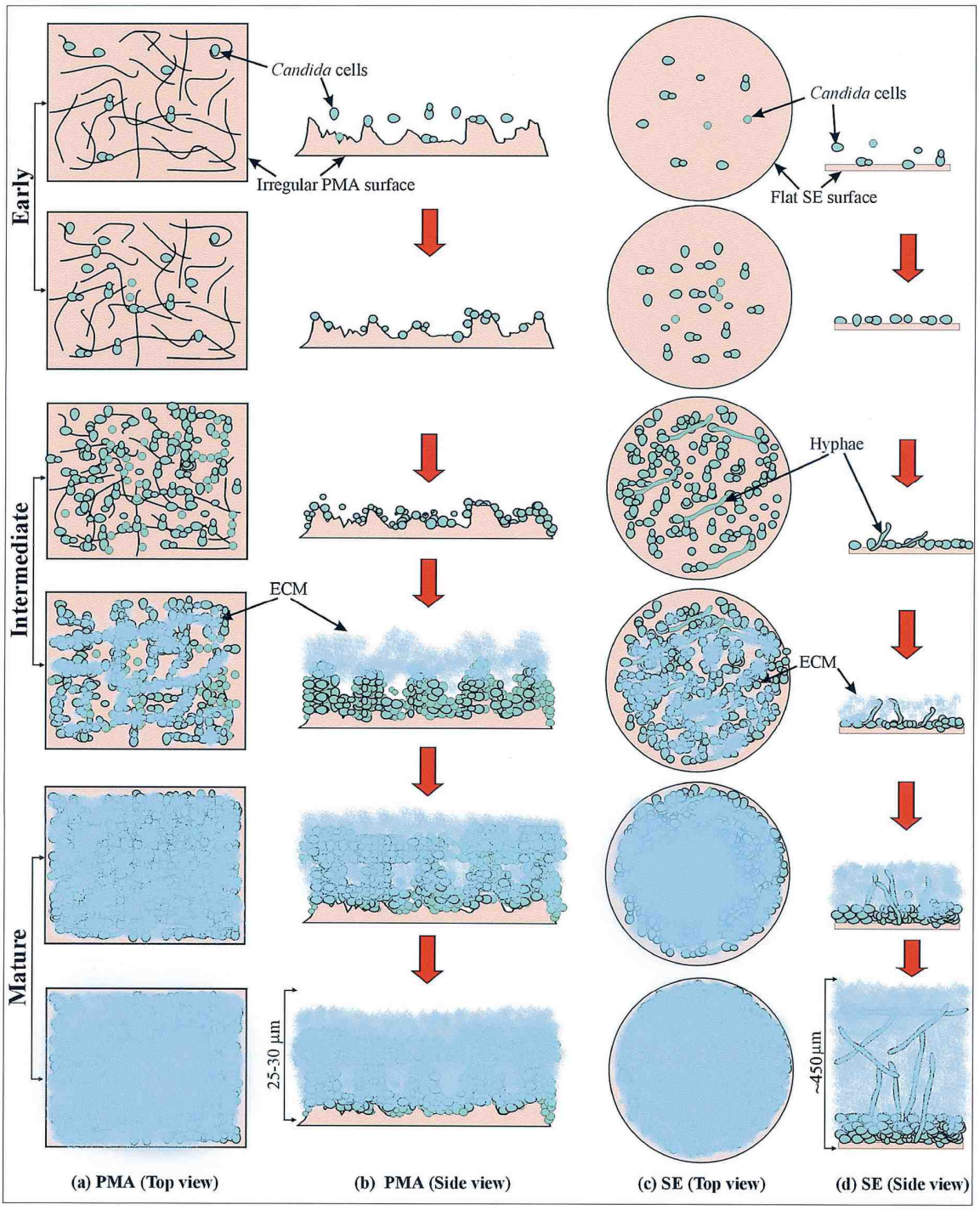
Drug resistance is strongly correlated with biofilm development. Resistance emerges relatively quickly after *C. albicans* substrate adherence.[75,76] Extracellular matrix could slow antifungal drug penetration, limiting access to growing cells. There could be an increased expression of drug efflux pumps but the specific pumps involved have not been elucidated. A change in membrane sterol composition during biofilm development may create resistance to only specific anti-fungal medications. There is a possibility of phenotypic changes resulting from limited availability of nutrients and a slow growth rate. Thus drug resistance has been recognized but the exact resistance mechanism is still unknown.

Figure 1: *Fluorescence microscopic images of biofilm development*



Development of *C. albicans* biofilm on polymethylmethacrylate strips. Fluorescence microscopy images show the three distinct developmental phases of *C. albicans* biofilms over a 71-h period: early (a), intermediate (b), and maturation (c) phases. Magnification, x10. (Images taken from Chandra et al[37] courtesy of *J Bacteriol*)

Figure 2: *Schematic representation of C. albicans biofilm development*



(a and b) Biofilm grown on polymethylmethacrylate (PMA) strips. (c and d) Biofilm grown on silicone elastomer (SE) disks. Panels a and c represent the substrate seen from the top, while panels c and d show the view from the sides of the PMA strip and SE disk, respectively. ECM, extracellular material. (Image taken from Chandra, et al[37] courtesy of *J Bacteriol*)

Treatment modalities for *Candida* infections:

Treatments for denture stomatitis are controversial due to the development of resistant strains. Relatively effective treatment protocols have been presented but a systematic review has not been conducted. Initial treatment with antifungals such as nystatin and amphotericin B in the form of a gel, mouthwash or lozenge are considered the treatment of choice.[60,77] Controlling denture stomatitis in the future is imperative but the methodology for treatment varies and the optimal treatment is not clear.

The most important therapeutic regimen for denture stomatitis in any form is efficient oral and denture hygiene. Most forms of oral candidasis can be treated with topical applications of antifungals. If there are chronic, nodular plaques present, long term antifungal treatment should be implemented.[78] Success with antifungals is often temporary, as there is a high recurrence rate with discontinued treatment.[61] If the patients predisposing factors are not corrected, there will be frequent recurrence of the infection.

Recolonization by Candida can occur unless the ill fit of the prosthesis is corrected or oral hygiene improved. Some studies showed that antimycotic drugs are effective while some determined it to be an inadequate treatment.[56] In most denture stomatitis cases, control of denture plaque, eliminating denture faults and discontinuation of denture wear are sufficient modes of treatment. Resistant strains to antifungal therapy have been determined therefore the first line of treatment should be denture hygiene education and discontinued nocturnal use.[79]

Treatment with antimicrobials continues to offer therapeutic benefits. For example, chlorhexidine and sodium hypochlorite have been used for the reduction of

denture plaque.[78] Fungicides and bactericidal agents have reduced bacterial colonization but not eradicated the disease.[56]

A study of copolymers of hydroxyethyl methacrylate (HEMA) and methyl methacrylate (MMA) was developed for an intra-oral drug delivery system.[80] Mirth and coworkers developed pellets of chlorhexidine within the HEMA and MMA which produced a controlled release of chlorhexidine.[80] This allowed for a reservoir-type controlled-release delivery system of the antiseptic, which was biologically active after 30 days *in vivo*. These oval shaped pellets were mixed and pressed into silicone rubber molds of the denture bases and allow a 30 day delayed release of chlorhexidine in non-compliant and physically or mentally compromised denture stomatitis patients. The results showed an extended release of biologically active chlorhexidine at a controlled rate for 30 days.

Miconazole lacquer was also tested in randomized control trials, and a significant reduction in Candida colonies over a longer period of time was observed when compared with miconazole gel or a placebo.[81,82] Fluconazole, another antifungal drug was studied by Kulak et al[83] in 1994 and they determined that fluconazole with chlorhexidine had improved efficacy toward denture stomatitis as compared to fluconazole alone.

Resistance of Candida to various antifungal agents is a major risk factor to our treatment modalities. Chandra et al[84] formed a biofilm on acrylic denture strips and tested Candida adherence *in vitro*. They added various concentrations of antifungal agents such as Fluconazole, Nystatin, Chlorhexidine and Amphotericin B to the biofilm matrices and planktonic cells. Metabolic activity recordings demonstrated that Candida cells within the biofilms became resistant to antifungal agents when compared with their

planktonic counterparts. Growth of planktonic cells, were inhibited at lower antifungal concentrations compared with biofilm-grown cells. Developed biofilms clearly have become a topic of discussion because of their resistance to standard antifungals. With this in mind, it is reasonable to consider methods that may block initial Candida adhesion. By understanding the composition of denture biofilms, more specific therapeutic approaches to the treatment of denture stomatitis may emerge.

Surface Roughness:

Denture acrylic resins are used for the fabrication of multiple forms of prosthetic dental appliances. In particular, polymethyl methacrylate (PMMA) resins have been the material of choice for the fabrication of complete denture prosthetics. The acrylic resin used in complete denture fabrications is necessary to replace the lost soft tissues and to distribute the forces of mastication from the denture to the residual ridge crest. During the fabrication of a complete denture, care is taken to provide a denture with a smooth surface in order to reduce the initial adherence and accumulation of microorganisms. Therefore adequately polishing and finishing of the external surfaces are regarded as essential to the clinical restorative protocol.

Once the denture is inserted, there are surface changes that occur, often promoting a more favorable roughened surface texture for denture plaque formation.[85] Adherence of Candida to denture base resin has been studied by a number of researchers yet there is limited research related to surface textures and denture base resin associated with denture plaque formation.[86-91]

Denture processing replicates the contours of the hard palate and the processed acrylic on the fitting surfaces are, excessively rough and never polished. This may alter microbial adherence considerably and Taylor et al[29] demonstrated how substratum topography influences adhesion of microorganisms to PMMA. Six acrylic surfaces were roughened with various methods and then measured by laser profilometry. The PMMA surfaces were sanded with silicone carbide paper of various coarseness and bead or shot blasted for the greatest roughening. The Ra values ranged from 0.04 to 7.89μm.

Results showed that surfaces with larger pits and gullies corresponded to increased Ra values and did not have the ability to retain bacteria as effectively as less abraded surfaces. A small increase in Ra values (0.04-0.1.24µm) resulted in significant increase in microbial attachment. Subsequently larger Ra values (1.89-7.89µm) resulted in a decrease of adhesion. Following these results, the extremely rough surfaces on the fitting side of a denture may not effect microbial attachment as much as we once thought.

Currently no studies reveal surface roughness for the intaglio area on a denture. Future investigations must measure processed dentures before clinical relevance of biofilm development can be assessed. Therefore the following literature only measures adherence based off of small Ra values and this must be taken into account before reviewing the following findings.

The purpose of polishing the outer surfaces of dentures after processing acrylic is to eliminate rough layers from the already rough processed surface. A study by Kuhar et al[92] found that polishing acrylic with a tungsten carbide cutter created the roughest surface but conventional polishing with silicon, pumice and a lathe produced the smoothest surfaces within the acceptable limit. The acceptable surface roughness (Ra) of hard surfaces in the oral environment should not exceed 0.2µm.[88,90,93] Producing an adequately smooth and glossy finish reduces the potential for bacterial plaque formation.[88,90,93,94]

Microbial adherence is facilitated best by a relatively rough surface topography. Specifically we wanted to study the adhesion and biofilm formation of *C. albicans,* the opportunist pathogen associated with denture induced stomatitis.[50,82] Many *in vitro* initial adherence studies, are unable to reproduce the oral cavity in part because their surface

roughness was smoother then the processing irregularities that are associated with dentures.[94-96,96,97] Differences in surface topography affect the attachment of microorganisms to acrylic surfaces as mentioned above, with higher numbers of cells generally adhering to rougher surfaces.[89,98,99] Cleaning of the denture may not be enough of a defense against the increased microorganisms remaining within a surface of irregularities.

Polishing PMMA for uniform surface texture can be done with sand paper of medium grade (P600) in a figure eight motion.[89] Comparing *C. albicans* cell number on rough and smooth surface acrylics, showed pronounced low adherence on a smooth surface. Cells seem to cluster around surface defects such as voids, depressions and scratches. It appears that microorganisms tend to find protection from shear forces by becoming entrapped within surface irregularities.[100] Ultimately dentures have an expected, patient specific lifespan before a remake is indicated. If a surface topography can minimize adherence during a dentures life expectancy, this could alter a denture acrylics carrying capacity for Candida and reduce mucosal infections.

An *in vitro* study by Yamauchi et al[91] looked at the adherence of oral microorganisms to differing surface textures of denture resins. Streptococci and Bacteroides adhered to a greater level than the *C. albicans* on the rougher surfaces. Polished surfaces seemed to have more Candida adherence but the presence of Candida on the coarsely polished surfaces was still high but not to the extent of the other species tested. All resin specimens were coated in saliva which may have played a role in altering Candida adherence. Overall, Yamauchi et al[91] showed that regardless of surface roughness on denture resins, various oral microorganisms have a greater adherence rate

then Candida alone. If there is selective binding of microorganisms to differing surface textures, it is possible, through deductive reasoning, to determine which microorganisms are the initial colonizer.

Due to the oral cavities constant contamination with varying species of microorganisms, non-shedding surfaces such as denture acrylics must maintain as smooth a surface as possible. Microorganisms maintain their existence intra-orally by two mechanisms, selective adhesion and stagnation.[101] In a review of the literature, bacterial retention was studied with respect to various intraoral hard materials.[90] Surface roughness was simultaneously compared with surface free energies and it was determined that surface roughness far outweighs the effects of surface free energy.[93] In 1984, Busscher et al[102] determined that sanding with silicon carbide sandpaper on acrylic resin surfaces caused a surface roughness to be below the 0.2µm threshold. This contradicts the other research which found increases in surface roughness after polishing was completed. As mentioned earlier, Verran et al[30] found a 10-fold increase in surface roughness after sanding acrylic with emery paper and Loney et al[103] found a two to five fold increase, in surface roughness when using burs, stones, sandpaper and then polishing with pumice or tin oxide.

Verran et al[100] showed that *Candida albicans* cells adhered in significantly higher numbers as the roughened surface Ra values increased. Along with that idea, if surfaces are rough regardless of type of acrylic used, promotion of Candida growth will occur.[34] Clinically these results are significant because the intaglio surfaces of dentures after processing are rougher then the highly polished outer surfaces. Moreover the repeated

cleansing procedures in which patients maintain their dentures can be abrasive and create rougher surfaces over time.[104]

Associated with surface roughness is the contact angle of water on a surface. Contact angle is the sum of the electronic and physical properties of polymers, also known as surface free energy. It has been stated that Ra values below 0.1µm do not affect contact angle and above 0.1µm the effect of contact angle depends on measurement made on the smooth surface. Busscher et al[102] points out that if the contact angle at the surface is between 60° and 86° then surface roughening has no influence. Quirynen et al[93] also showed that undisturbed plaque formation on polymer strips that were either rough or smooth resulted in a 4 fold increase in plaque formation on the roughened surfaces. These results show that surface roughness overrules the influences of surface free energies.

Research by Puri et al[105] wanted to determine if surface roughness of denture acrylic disks played a role in surface wettability based on measured contact angles. They found that with polished and unpolished PMMA surfaces, there was a trend of reduced contact angle with polished surfaces and increased contact angle with unpolished. Therefore polished surfaces were more hydrophilic then unpolished. Moreover, when they added increasing concentrations of phosphate to change the surface charge of either polished or unpolished surfaces, the contact angles were reduced. Therefore with heightened phosphate concentration, there was a reduction in contact angle constituting reduced surface free energy and increased hydrophilicity.

Specifically, the adherence and interaction of *C. albicans* on denture-base materials with various surfaces finishes was studied by Radford et al.[28] The research

method looked at the degree of surface roughness, type of denture-material and effect of saliva coating on bacterial adherence. Soft lining and heat-cured PMMA acrylic resins were selected for the experimental groups. Results showed a greater adhesion to the rougher surfaces, either machine roughened or bur roughened than to the control which was a smooth surface (Ra = 1.3-1.6 µm, s.d. 0.3-0.2 µm) processed against glass. Approximately two times as much adherence occurred with the rough surfaces. There was no significant difference in *C. albicans* adhesion between the tungsten carbide bur or steel bur roughened surfaces.

Salivary Pellicle:

Surface roughness clearly is a major factor in microbial adherence and specifically raises the adhesion of *C. albicans* to denture base acrylic resins. The literature supports this relationship but the addition of salivary pellicle to acrylic surfaces must be incorporated in order to more closely replicate the clinical environment. The literature presents mixed results regarding bacterial adherence to PMMA after exposure of unstimulated, pooled whole human saliva. Table 1 summarizes the findings in the literature for adherence variability of *C. albicans* to PMMA uncoated, saliva or serum coated surfaces. Current literature proves to have large variability in protocols, regarding differing chemical structures and fabrication of PMMA, various salivary collections, and experimental formulations which contribute to their non-congruent results.

Table 1: *Effects of salivary pellicle on C. albicans adhesion to PMMA and altered PMMA surfaces from the literature*

Pellicle	***Adhesion***	***Acrylic***	***Reference***
Mixed Saliva (pooled unstimulated frozen)	Enhanced	PMMA sheets	Nikawa, H. et al. 1997
Serum	Enhanced	PMMA sheets	Nikawa, H. et al. 1997
Mixed Saliva (whole freeze dried)	Enhanced	PMMA plate	Millsap, K.W. et al. 1996b.
Mixed Saliva (pooled unstimulated)	Reduced	PMMA against glass slide (Ra – 1.3-1.6)	Radford, D.R. et al. 1998
Mixed Saliva (pooled unstimulated)	Enhanced	PMMA strips	Chandra, J. et al. 2001
Parotid Saliva	Slightly Enhanced	PMMA beads	Edgerton, M. et al. 1993
Submandibular/Sublingual Saliva	Significantly Enhanced	PMMA beads	Edgerton, M. et al. 1993
Palatine Saliva	Significantly Enhanced	PMMA beads	Edgerton, M. et al. 1993
Mixed Saliva (pooled stimulated)	Enhanced	PMMA (320 grit sandpaper)	Vasilas, A. et al. 1992
Parotid Saliva	Enhanced	PMMA sheets (320 grit sandpaper)	Vasilas, A. et al. 1992
Submandibular	Enhanced	PMMA sheets (320 grit sandpaper)	Vasilas, A. et al. 1992
No Saliva	Reduced	mPMMA (-COO)	Park, S. et al. 2003
No Saliva	Reduced	mPMMA (-COO & KissCote)	Park, S. et al. 2008

Mixed Saliva (pooled unstimulated)	Reduced	PMMA strips	Samaranayake, L.P. et al. 1980
Parotid Saliva	Enhanced	PMMA strips	Samaranayake, L.P. et al. 1980
Serum	Enhanced	PMMA strips	Samaranayake, L.P. et al. 1980
Mixed Saliva (pooled unstimulated)	No significance due to large s.d.	Rhombic PMMA (Grinding paper 4000grit)	Waltimo, T. et al 2001
Mixed Saliva (single stimulated)	Reduced	PMMA against glass slide (Ra – 0.15)	Pereira, T. et al 2007

As previously mentioned, Radford et al[28] unlike his counterparts, determined that pooled unstimulated salivary pellicle played a significant role in the reduction of *C. albicans* to heat cured PMMA resins as well as diminishing the effect of surface roughness of the material. The pooled salivary collections were completed on participants that had eaten prior to collection which could have caused variation in saliva quality. In addition, the high centrifugation rates may have disrupted and separated high from low molecular weight mucins giving mixed results. The most important conclusion to be gained from this study is that, the effects of surface roughness are reduced because salivary pellicle causes alteration in surface free energy as well as dampening the roughness.

Salivary pellical functions as a lubricant and protectant as well as a clearance and adherence mechanism for oral flora.[32,106] Microbial colonization can be effected by the salivary glycoproteins on denture surfaces.[6] Nikawa et al[107] determined that mucins in whole saliva played a significantly higher role in the adsorption of *C. albicans* on PMMA glass processed sheets when compared with other proteins such as lysozyme, fibrinogen, albumin and whole serum which confirmed and extended the work of Edgerton et al.[6] Several articles pointed out that preincubating acrylic in whole filtered saliva tended to decrease *C. albicans* adhesion while coating denture acrylic with whole saliva along with parotid and submandibular-sublingual saliva enhanced *C. albicans* adherence.[36,86,108]

In 1993, Edgerton et al[6] demonstrated that adhesion of *C. albicans* to PMMA beads was specific to the location of salivary secretion. Human submandibular-sublingual saliva collection significantly enhanced adhesion of Candida cells determining that molecules specific to this saliva may act as receptors for *C. albicans* adhesins. More

specifically she found that high and low-molecular-weight salivary mucins served as receptors for a specific strain of yeast cell within this research. Mucins also have the ability to adhere to surfaces such as the intaglio surface of a denture. The entire palatal mucosa is covered by the denture base and is composed entirely of minor salivary glands which secrete high molecular-weight salivary mucins, therefore leading to improved adhesion of *C. albicans*. If the denture wearing patient does not remove the denture overnight or continues to wear it without cleaning its' surface, yeast cells will collect and replicate creating a reservoir for infection of the oral mucosal surfaces.

In order to understand the interaction of the salivary pellicle and denture resins, the surface free energy and wettability of the surfaces must be studied. Saliva is deposited as an organic film or acquired pellicle after exposure of the denture into the oral environment.[109] More specifically selective adsorption of salivary glycoproteins to the PMMA surface occur.[110] These protein layers change the original surface properties of acrylics leaving them with a changed state of surface wettability and surface free energy. Higher values of surface free energy (increased wettability) were noted once a salivary coating was introduced to acrylic resins and this in effect should give rise to greater adhesion of microorganisms.[111]

Selective adsorption of specific salivary glycoproteins on PMMA can assist in salivary bactericidal and fungicidal properties. Histatins for example are cationic proteins which were identified by Oppenheim et al[112] in the parotid and submandibular-sublingual glands but have not been found on denture pellicle. Their fungistatic properties may make them an important natural defense against *C. albicans.* Tsai et al[7] looked at the fungicidal and bactericidal activity by using recombinant salivary histatins.

The research found that histatin proteins have preponderance for the formation of alpha helical structures which have been shown to be important in antifungal activity. Baev et al[8] further showed that salivary histatins are involved in a loss of the control mechanism for Candida cell homeostasis which resulted in a loss of intracellular ATP leading to cell death. Therefore salivary histatins might be a useful addition to the denture pellicle. Giving PMMA a net negative charge could possibly attract cationic histatins, and ultimately reduce *C. albicans* adherence.

Edgerton et al[21] believed that there was a composition difference between enamel pellicle and acquired pellicle on denture acrylics. The belief was that within a denture pellicle are proteins such as albumin, lysozyme, fibrinogen, serum and mucins and they may increase fungal adhesion. Protein rich denture pellicle ADP (Adenosine Diphosphate) was evaluated by chemical and immunochemical methods and lacked proline rich proteins and cystatins. This was different from enamel pellicle. So they hypothesized that if surface modified resins were developed they might have an improved anti-Candida effect.

There is a preponderance of research favoring saliva as a proponent in the initial adhesion of *C. albicans* to PMMA resins or denture base materials. Precoating acrylic with pooled whole unstimulated saliva by a majority of investigations, showed an increase in *C. albicans* adherence, Table 1. Differing strains of Candida appeared to show altered binding in the presence of saliva due to their hydrophilic or hydrophobic nature. For example *C.* albicans, a hydrophilic yeast, bound much less on saliva coated PMMA than comparative hydrophobic yeasts.[113] This is also represented by another study that showed higher counts of *C. glabrata* than *C. albicans* on salivary coated

PMMA.[34] In general, studies show that *C. albicans* has an affinity for pellicle coated acrylics; therefore better standardization of future research will lead to better understanding of fungal adherence.

Edgerton et al[6] tried to ascertain which saliva if any would be a direct promoter of yeast adherence and found that sublingual-submandibular saliva had enhanced Candida binding. With this in mind, clinical salivary components are extremely variable with respect to secretions adjacent to maxillary or mandibular dentures and selective saliva collection is not a good representation of the oral cavity. *In vitro* studies may not replicate the entirety of the oral environment and can only touch on the intricacies between microorganisms and man-made prosthetic devices. Adherence of yeast cells is species and host specific as well as substrate dependent. More *in vivo* studies are needed to add further specification of which molecules within saliva are interacting with *C. albicans* to promote its adhesion.

Other factors influencing adherence of *Candida* to denture resins:

C. albicans has a unique ability to bind to inert polymeric surfaces but the exact mechanism that allows these organisms direct ingress into the human host is undefined. The biophysical forces involved in the adherence and these microorganisms may be hydrophobic interactions and electrostatic forces. Klotz et al[10] looked at hydrophobic and electrostatic forces and their relationship to Candida adherence. He found that if interfacial surface tension is reduced, total free energy is negative, causing, accumulation of yeasts to a polymer surface.

Minagi et al[85] studied the effects of hydrophobicities of surface substrates on microbial adherence by using hydrophilic *C. albicans* and hydrophobic *C. tropicalis* with 21 different denture base resins. When the solid surface free energy (of the substrate) increased so did the adherence of *C. albicans* and the reverse of this was true for *C. tropicalis*. These results were supported by Dexter et al[114] who showed increasing adherence of bacteria once solid surface free energy increased. Miyaki et al[98] also found an increase in adherence of *C. albicans* with an increase in surface energy of the resin. So the studies show that with an increase of surface free energy comes an increase in adherence of *C. albicans*.

Reduced bite force capacity and chewing efficiency can occur with denture wear, which may contribute to a soft diet high in carbohydrates.[40] This is significant because high carbohydrate diets are also high in sucrose. In lesion free denture wearers, stomatitis has been able to be induced through the introduction of sucrose rinses. Yeasts grown in sucrose containing media have a higher affinity for binding to acrylic.[86,115]

Samaranayake et al[116] also found that addition of sucrose and glucose to the incubation of *C. albicans* on acrylics significantly enhanced adhesion.

Another adherence study by Samaranayake et al[86] found that treatment of acrylic strips with saliva significantly reduced the number of attached Candida cells while the addition of serum significantly increased the adhesion and doubled the number of yeasts attached per unit area. Next acrylic strips were pre-coated with streptococci and then yeasts were introduced and a significant reduction in the attached yeasts resulted but by incubating the Candida with a bacterial streptococci culture, this caused enhanced adhesion of the yeasts. So, oral bacteria play a complex role in the regulation of Candida adhesion to denture acrylic surfaces.

Later, Milsap et al[117] showed that microbial colonization on acrylic is an ordered sequence of events with adhesive interactions between bacteria and yeasts playing a major role in development and maintenance of biofilms. Their research used a parallel plate flow chamber which allowed for different strains to be fixed onto a PMMA plate while *C. albicans* was flowed through the chamber. The results showed that yeast adhesion was suppressed by the presence of bacteria. A similar study used the same parallel plate flow chamber and found that co-adhesion of differing bacteria can occur on a surface.[118] It appears that if initial colonization by various bacteria occurs, it can suppress the initial adherence of *C. albicans*. The effects bacteria have on yeasts and vice versa are critical to our understanding of colonization and biofilm development on denture resins.

Summary:

The prevalence of *C. albicans* has been found to be up to almost 70% in denture wearers.[62] *C. albicans* appears to be an important pathogen involved in denture induced stomatitis which can lead to abnormal changes of the mucosal tissues within at risk elderly populations. Pathogenesis in the elderly is a major health concern and we have limited knowledge regarding the role *C. albicans* plays in the adherence to PMMA surfaces and its ability to resist treatment.

PMMA denture materials are universally used for the fabrication of complete dentures. The interactions that *C. albicans* and other microorganisms have with denture acrylic surfaces within the oral environment are complex and difficult to ascertain. Adherence to PMMA is the initiator of denture biofilm formation and the development of denture stomatitis.[37] Our study and several other groups are working under the hypothesis that surface alterations may reduce *C. albicans* adhesion.[9,11,13]

The purpose of our research is to incorporate phosphate polymer into PMMA denture bases in order to make the surface less hospitable to *C. albicans* adherence ultimately reducing the risk of denture induced stomatitis. Our research focuses on Candida adherence to PMMA but there are many regulators of yeast colonization in the oral environment and more *in vivo* studies need to be performed.

CHAPTER III

METHODS AND MATERIALS

Statement of Procedure:

PMMA Disk Fabrication:

PMMA fabrication technique follows that of Dhir et al[119] and Puri et al.[105]

Three groups of acrylic resin were investigated in this study, a control group and two experimental groups. The acrylic denture base material Lucitone 199® (Dentsply International Inc. York, PA, USA) was used as the control and the basis of the three experimental groups. Lucitone 199 was composed of a polymer powder containing polymethylmethacrylate (PMMA) prepolymer, initiator, other additives and a liquid monomer containing methylmethacrylate (MMA) with a cross-linking agent, ethylene glycol dimethacrylate (EGDMA). The materials were formulated in a powder:liquid ratio (3:1) according to the Lucitone 199 manufacturer's instructions for use (Table 2).

Table 2: *Components of the Control and Experimental groups tested in this study*

Acrylic Groups	**Polymer**	**Monomer**
Group A (Control) Lucitone 199	3 Parts Polymethylmethacrylate	1 part Methylmethacrylate
Group B	3 Parts Polymethylmethacrylate	1 part Methylmethacrylate with 5% by volume of phosphate compound substituted in liquid (95 MMA : 5 PO4)
Group C	3 Parts Polymethylmethacrylate	1 part Methylmethacrylate with 15% by volume of phosphate compound substituted in liquid (85 MMA : 15 PO4)

Table 3: *Instructions for Lucitone 199 fabrication based on manufacturer's recommendations*

Instruction number	Property	Manufacturer's Suggestions
1	Powder : Liquid	10 mL by volume/32 cc
2	Mixing Time	30 sec.
3	Gelation time	9 min.
4	Working time	10 min.
5	Packing temperature	Room Temperature
6	Processing cycle	163°F for 90 min. and 212°F for 30 min.

Mixing and Processing of the Acrylic:

PMMA samples were compression packed into a circular metal 2 piece stainless steel processing recessed jig (Figure 3) with dimensions of 3mm in height and 50mm in diameter. The processing jig was fabricated courtesy of Marquette University School of Engineering Discovery Learning Center. 30 grams of polymer was slowly added to 10 mL of monomer until the polymer was entirely saturated in monomer.

After 10 minutes, the PMMA was in the dough state and 1/5 portions were fractioned out. PMMA was hand-pressed into the depressed jig using aseptic technique. A flattened stainless steel top was placed over the PMMA filled depression and compressed to 3,000 psi. Five jigs were filled for each PMMA mixture, compressed and entered a curing cycle (Table 2).[119] 15 PMMA disks for each phosphate concentration (0%, 5%, 15%) were completed for a total of 45 PMMA disk samples.

The polymer and monomer used for the experimental groups were from the same batch of Lucitone 199 control group. Experimental formulations contained phosphate-containing monomer (phosphoric acid hydroxymethylmethacrylate ester), substituted for the liquid monomer in increasing concentrations of 5% and 15% by volume, respectively (Table 1).

The PMMA heat processed disks were removed from each of the jigs. A laser was used to label and cut out seven 15mm diameter disks from each sample (Marquette University School of Engineering, Discovery Learning Center). A total of 105 disks per phosphate concentration were cut. The 15mm disks were mechanically sanded according to a technique by Quirynen et al.[88] Then disks were placed in an epoxy prefabricated

polishing jig with a 1mm depression and a rectangular 30 X 50 mm handle. They were polished to a 600 (smooth) grit surface roughness on a rotational table with one-sided, adhesive carborundum sandpaper to a 2mm thickness.

After sanding, one examiner determined Ra values using a profilometer and surveyed six different locations on the polished surface. Ra values were then averaged for each disk. The range of average surface roughness for all the disks was: 0.3 – 0.5 µm. 10% of the disks were discarded because they had excessive voids, fractures or pitting. The remaining 90% of the disks were used for the experiments.

Figure 3: *Stainless steel processing jig for resin disk fabrication*

Saliva Collection: Saliva collection protocol was taken from Leung et al[120]

Briefly, collection of human saliva began at 8:00 am and was obtained from 4 different individuals with clear medical (no systemic disease) and dental histories (no periodontal disease). Participants refrained from intake of food or drink overnight before collections. Parafilm was used to stimulate saliva flow. Samples were collected into sterile Falcon tubes and pooled.

Samples were processed by centrifugation twice: 800 rpm for 10 min. and once at 1000 rpm for 10 min. Each time the supernatant would be collected and the mucus and debris discarded. The saliva was then filter (0.2µm) sterilized and used immediately.

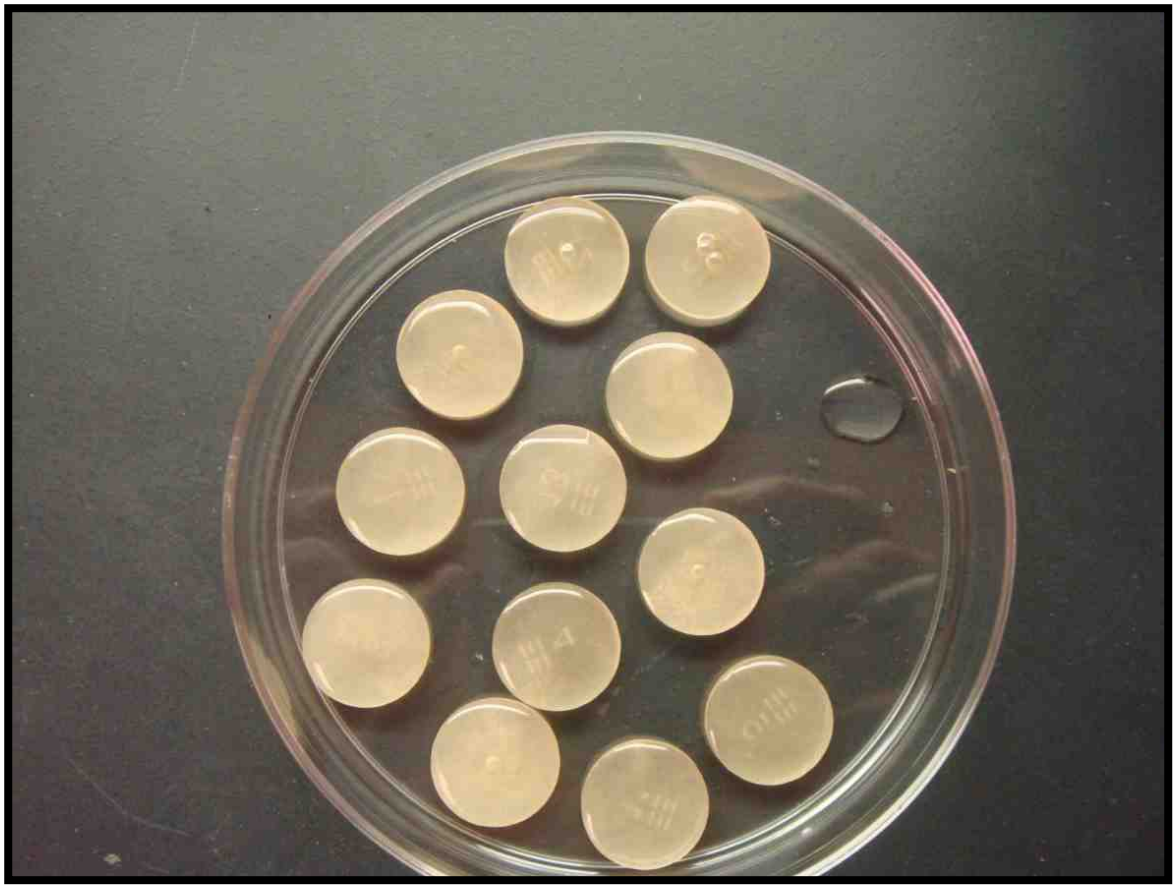
Washing and Disinfecting Disks:

Polished disks were disinfected before every experiment at room temperature. 15mm diameter, 0%, 5% and 15% phosphate disks were transferred to 50mL polypropylene tubes. Each tube held 30 disks. 20 mL of sterile distilled water was added and the disks were leached of any residual monomer for 48 hours. Next, liquid was removed and replaced with 70% Ethanol. Tubes were placed in an ultrasonic bath for 5 minutes. Ethanol was removed and disks were rinsed with 25 mL of sterile distilled water three times. On the third wash, the tubes with disks were placed into an ultrasonic bath for 15 minutes. Distilled water was removed and two more consecutive sterile distilled water rinses were completed. The disinfected disks were placed in 25 mL of distilled water and allowed to sit over night. This procedure was executed before each experiment started.

Pellicle Formation Procedure:

4 disks each of 0%, 5% and 15% phosphate were placed into a petri dish with their polished side up. A total of 48 disks were distributed onto 4 separate petri dishes for each experiment. They were allowed to air dry for 15 minutes in a 37°C incubator. Each of the disks was pretreated with PBS (phosphate-buffered saline), whole stimulated pooled saliva or fetal bovine serum (FBS – Atlanta Biologicals stored at -25°C) (Figure 4). 100 µL PBS, filtered saliva or FBS was micropipetted onto the polished surfaces. The liquid volume was sufficient to cover the entire surface of the disk with liquid. Covers on petri dishes were closed and disks were put into an incubator for 90 min. at 37°C.

Figure 4: *Pretreatment of disks with whole stimulated pooled saliva*



Biofilm Formation Procedure:

Strains and Media:

C. albicans strains included isolates from a bronchial lavage (strain A), sputum sample (strain B) and two denture stomatitis isolates (strain C, D). Isolates were streak plated and maintained at 4°C on YPD (1% yeast extract, 2% peptone, 2% dextrose and 2% agar) agar plate (Figure 5).

C. albicans was subsequently grown overnight in 2mL of YPD media in a glass test tube to an exponential phase at 37°C with vigorous shaking (Figure 6). Identification of *C. albicans* was based on germ tube formation in the presence of serum.

Cell Counting: (cell counting procedures illustrated in Figures 5-7)

Preparing of the inoculum cell suspension was done by adding 1µL of the YPD stock culture into 99µL of sterile distilled water. 20 µL was removed from the suspension and placed on a hemocytometer (Figure 7). Cells were counted using a light microscope at 40X magnification. Cell counts were repeated 3 times; the average was calculated and used to adjust the suspension density to 10^6 cells/µL. A standard inoculum of 1 X 10^5 cells from the overnight culture was used for inoculation.

The control groups without saliva or serum pellicle were placed in petri dishes with 4 disks of 0%, 5% and 15% phosphate groups. For pellicle groups, each disk was removed from the petri dish with a sterile forcep and the excess saliva or serum was tapped off on the petri dish surface. The disks were then transferred polished side up into a new sterile petri dish. All the pellicle coated disks were transferred into the same groups of 4 disks of 0%, 5% and 15% phosphate groups, totaling 12 disks per petri dish. Each disk was then inoculated with *C. albicans* (100 µl @ 1 x 10^6 cells/ml) and incubated for 90 min at 37°C to allow cells to adhere.

Disks were removed from the petri dish and gently rinsed with sterile phosphate-buffered saline (PBS) to remove nonadherent cells. They were then transferred to a sterile 12 well microplate (Corning) plate containing 2 ml SD medium with 50 mM glucose per well. Biofilms were allowed to form for 48 hrs at 37°C in an incubator.

Determination of metabolic activity using the XTT assay:

The next step included the quantification of biofilm, metabolic activity. XTT solution was composed of 2mL PBS mixed with 5mg XTT powder. Menadione solution was composed of 10µL menadione, and 90µL acetone. PBS solution was composed of 80 mL PBS and 1mM glucose. The XTT solution, menadione solution and PBS solution were mixed together.

First, the biofilm covered disks were transferred to a 12 well microplate with 2mL of the mixed XTT solution per well. Plates were incubated at 37°C for 5 hours. For each well the fluid was transferred to a 5mL tube and centrifuged for 10 min at 6000g. 200µL samples of the supernatant were transferred to a 96-well microtiter plate and XTT formazan formation was determined colorimetrically using a spectrophotometer at 492 nm (Figure 8).[84]

Disks with no pellicle and no biofilm served as the controls. Assays and experiments were carried out in 3 replicates and were repeated on different days. Heat-inactivated Candida cells (80°C for 2 hrs) were used as an additional negative control.

Table 4: *Candida albicans isolates for each experimental group*

Isolate	**Location**
A	Bronchial Lavage (VA Hospital / Clinical Lab. Sciences – Dr. A. Harkins)
B	Sputum Sample (VA Hospital / Clinical Lab. Sciences – Dr. A. Harkins)
C	Oral Isolate (MUSoD) *Denture Induced Stomatitis – Newton II*
D	Oral Isolate (MUSoD) *Denture Induced Stomatitis – Newton II*

Figure 5: *Cell counting procedure: a) C. albicans strains were inoculated, streak plated and maintained at 4°on a YPD growth agar plate.*

a)

Figure 6: *Cell Counting Procedure:*

b) C. albicans was transferred from agar plate to YPD growth medium and maintained in a test tube for 12 hrs. at 30°C with vigorous shaking.

c) C. albicans was added to sterile distilled water. A 1:100 dilution of the original cell suspension was prepared before counting cells.

b) *c)*

Figure 7: *Cell counting procedure:*
d) A hemocytometer chamber was used at 40X magnification

d)

Figure 8: *96 well microtiter plate used to read metabolic activity of the biofilms.*

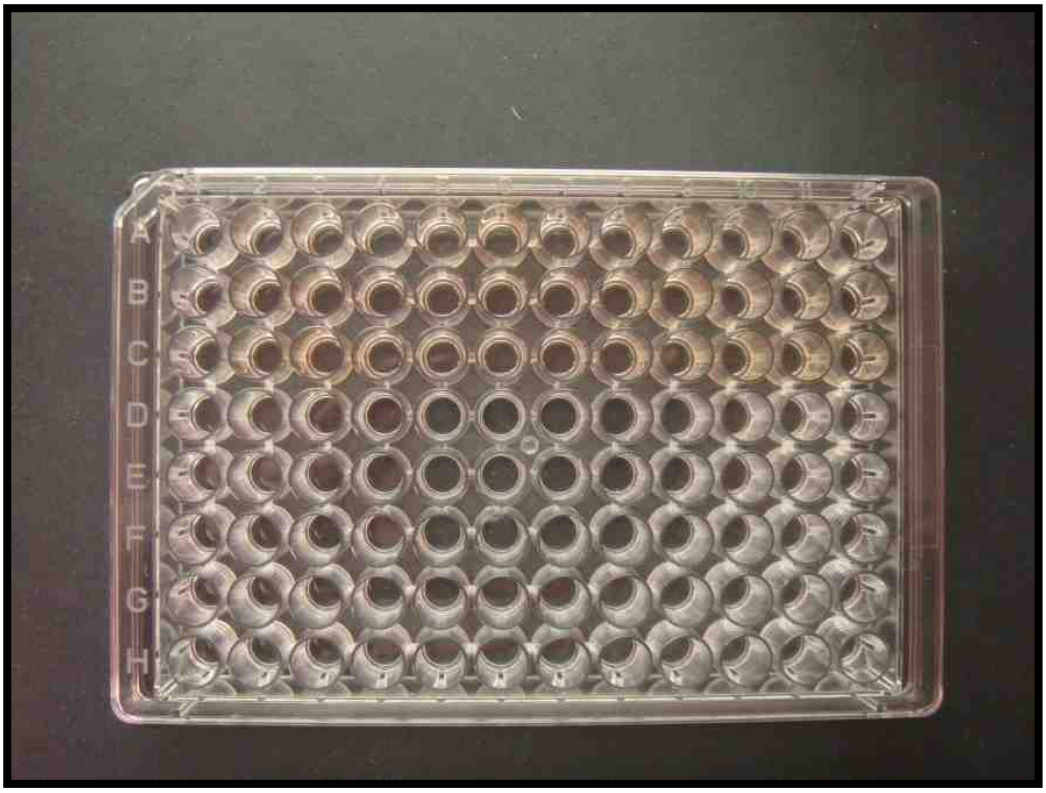
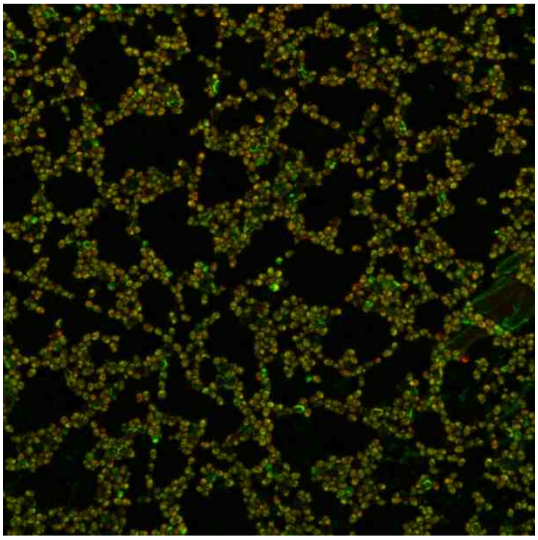
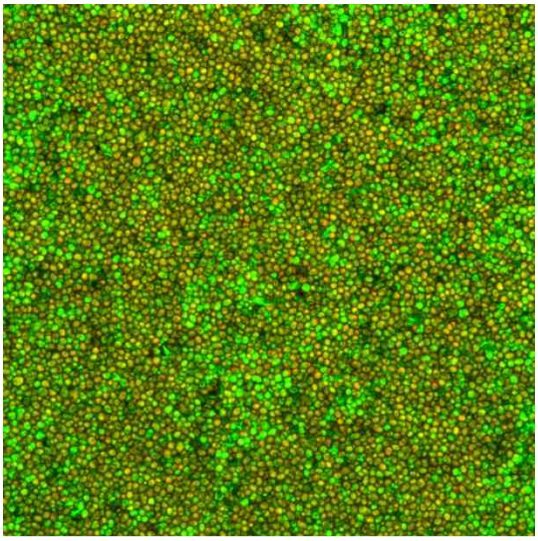


Figure 9: *Confocal fluorescence microscopy. 40 X magnification. Live cells of C. albicans are stained green, dead cells are red.*
a) 24 hr. biofilm of C. albicans strain AD-1 adherence on 0% phosphate disks without salivary pellicle
b) 24 hr. biofilm of C. albicans strain AD-1 adherence on 0% phosphate disks with salivary pellicle

a)



b)



CHAPTER IV

RESULTS

I. Effects of PMMA phosphate content on biofilm formation:

We examined whether increasing the amount of phosphate incorporated in PMMA would increase the rate of *C. albicans* adherence or biofilm formation on the naked surface. Changing the phosphate content of PMMA showed no difference in biofilm metabolism/formation. Comparing PMMA with 0% (control) and 5% phosphate ($p = 0.329$), 0% and 15% phosphate ($p = 0.345$) and 5% and 15% phosphate ($p = 0.999$) showed no statistically significant differences (Table 5). Therefore, increasing the quantity of phosphate incorporated in PMMA resins did not affect biofilm in our model system.

II. Effects of PMMA surface coating on biofilm formation:

Our goal was to examine whether coating various PMMA surfaces with either whole stimulated clarified filtered saliva or fetal bovine serum (FBS) would modify their ability to bind *C. albicans* and form a biofilm when compared with the formation of biofilm on uncoated surfaces. Coating the PMMA surfaces with saliva or serum showed significantly enhanced biofilm metabolism/formation (Figure 14, Table 5). PMMA surfaces coated with saliva significantly increased ($p < .0001$) the biofilm metabolism when compared to the biofilm formed on uncoated PMMA. PMMA surfaces coated with serum significantly increased ($p = 0.029$) the biofilm metabolism when compared to the biofilm formed on uncoated PMMA (Table 5). The difference between saliva-coated and serum-coated surfaces was statistically not significant (Table 5) although there was a clear trend towards a stronger effect for saliva. Overall, coating the surfaces of PMMA with saliva or serum enhanced *C. albicans* adhesion and biofilm metabolism.

III. *C. albicans* strain dependent effects on adherence and biofilm metabolism on PMMA surfaces:

Four strains of *C. albicans*, designated strains A, B, C and D, were used in this experiment. The bronchial lavage sample (strain A) showed the greatest metabolic activity followed by the sputum sample (strain B). There was no statistically significant difference ($p = 0.274$) between these two strains. Oral isolates (strains C, D) were less metabolically active when compared with the bronchial and sputum strains. Comparing the two oral isolates with one another showed no statistically significant difference ($p = 0.842$) (Table 5). These results suggest that biofilm development, as reflected by metabolic activity, is strain dependent and that *C. albicans* strains isolated from denture stomatitis patients, showed less ability to form a biofilm then bronchial or sputum strains in this model system.

IV. Change in PMMA surface roughness values

After several experiments which showed equivocal results, we began to remeasure our Ra values. We observed an increase in roughness over time. Surface roughness increased on the same PMMA surfaces measured throughout our experiments and this could have been due to mineral deposition which was not removed by our cleaning procedures, or possibly could be due to remaining biofilm matrix that was not completely dissolved in our washing/sterilizing technique. It appears that as surface roughness increased over time, so did adherence/biofilm metabolism of our *C. albicans* strains (Figure 10, 11 & 12).

Figure 10: a) *Positive correlation between surface roughness and biofilm metabolic activity for 0% Phosphated PMMA*

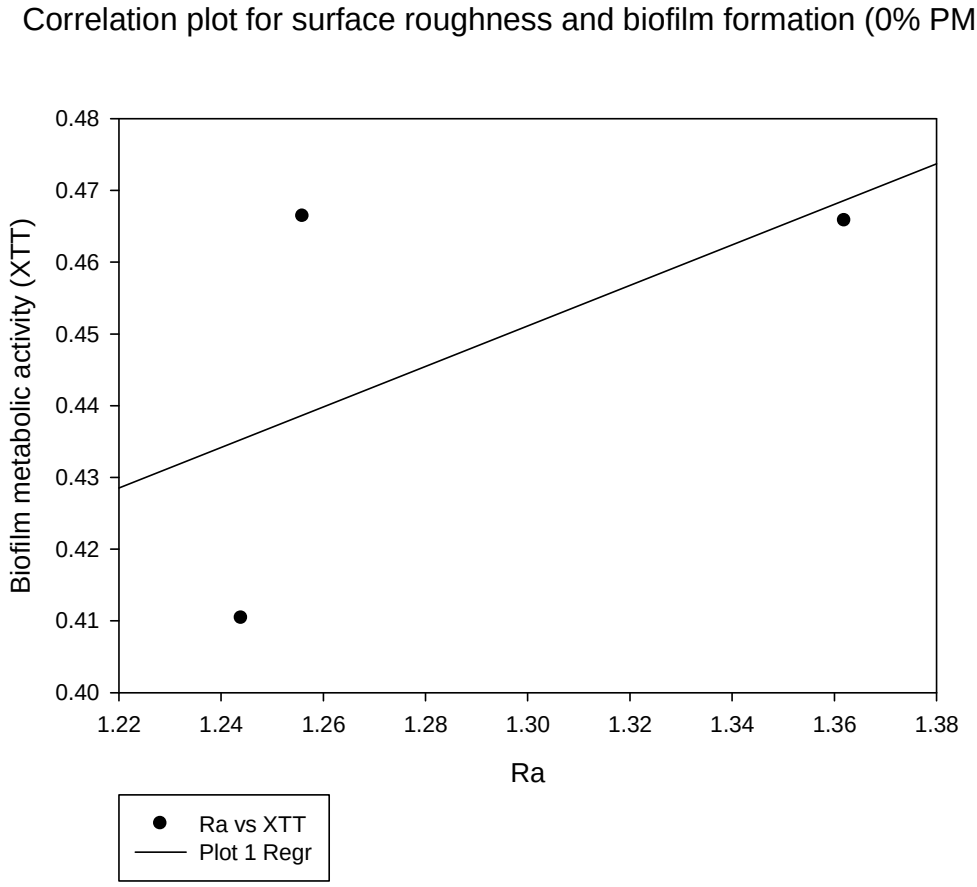


Figure 11: b) *Positive correlation between surface roughness and biofilm metabolic activity for 5% Phosphated PMMA*

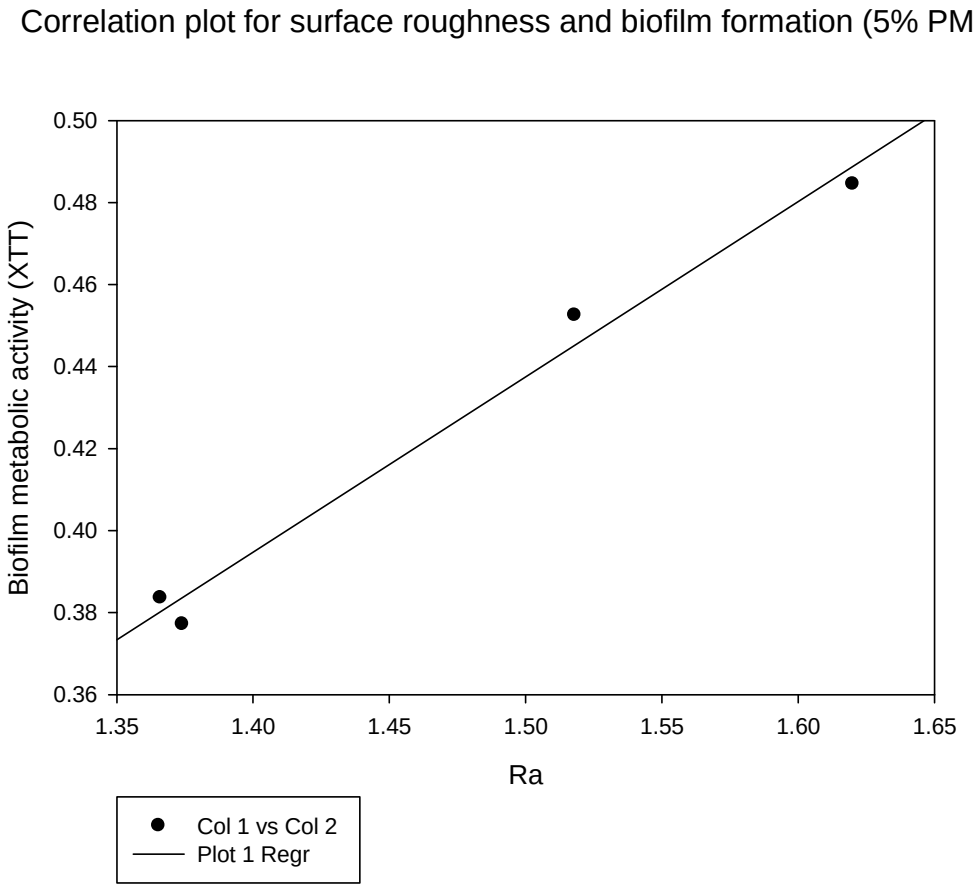
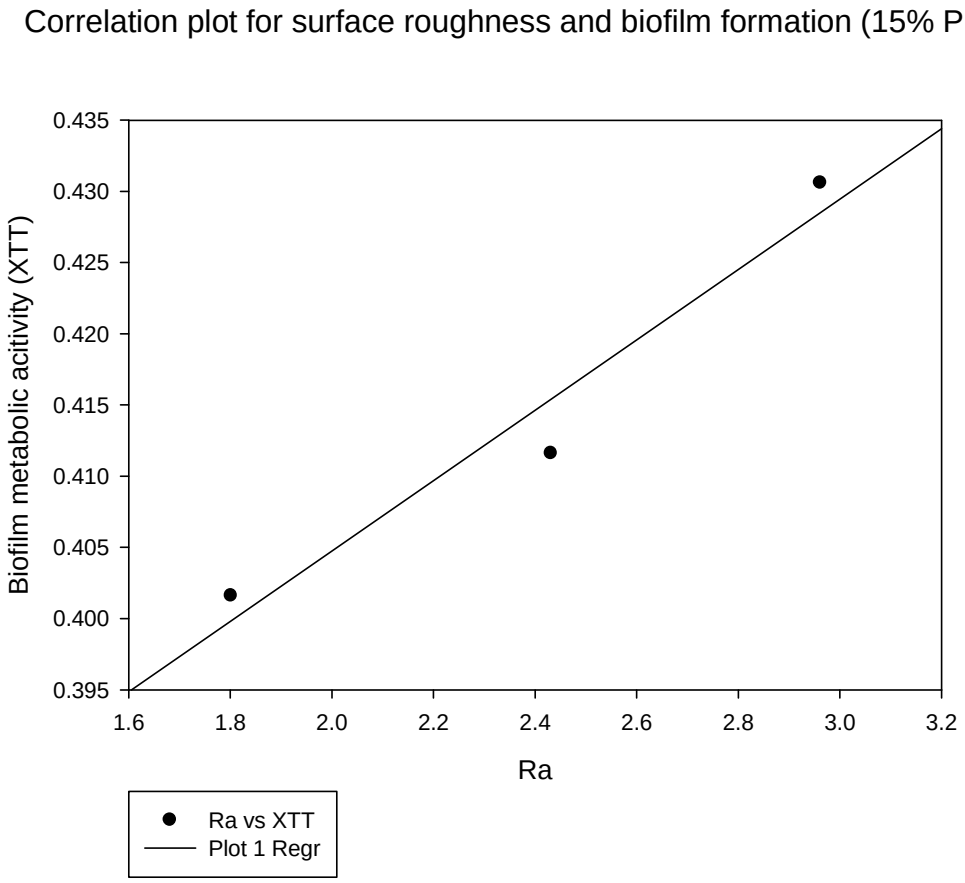


Figure 12: c) *Positive correlation between surface roughness and biofilm metabolic activity for 15% Phosphated PMMA*



Statistical Analysis (Table 5)

A factorial design was used to execute the experiments. It included 3 main factors. Each factor included several levels:

1. Candida strain – bronchial, sputum, two oral isolates (Table 4)
2. Surface coating – saliva, serum or naked surface (control)
3. Phosphate content – 0%, 5%, 15% ($^{vol}/_{vol}$)

A three-way ANOVA was used to analyze the data (PROC GLM, SAS Cary, NC). The main analysis was followed by a series of pairwise comparisons using Tukey's test. This procedure adjusts for multiple comparisons. For each pairwise comparison made, the mean difference and its 95% confidence interval are reported (Table 5), as well as the probability that the difference was due to chance.

Main effects were found to be significant for surface coating and *C. albicans* strain. No two and three-way interactions were significant statistically. Therefore, comparisons were limited to among the levels of each main effect.

Results of the statistical test showed that saliva and serum had significantly increased optical density (measured spectrophometrically in nanometers (nm)) compared to the control. This implies that saliva-coated and serum-coated surfaces had a greater ability to support biofilm formation than the untreated surfaces. Oral isolates of *C. albicans* resulted in less metabolic activity than bronchial lavage or sputum sample isolates although all four strains were capable of adherence and biofilm development/metabolism.

Table 5: *Comparisons among levels of three major factors involved in biofilm formation. Surface coating, phosphate content, and Candida strain. The difference in optical density (Δnm), the 95% confidence interval, and the p-value are listed for each comparison.*

Effect	Comparison	Difference	LowerCL	UpperCL	p-value
TREATMENT	Saliva - Serum	0.015481	-0.003977	0.034940	0.1480
TREATMENT	Saliva - Uncoated	0.036667	0.017209	0.056125	<.0001
TREATMENT	Serum - Uncoated	0.021185	0.001727	0.040643	0.0291
PHOSPHATE	0 – 5	-0.011778	-0.031236	0.007680	0.3290
PHOSPHATE	0 - 15	-0.011519	-0.030977	0.007940	0.3451
PHOSPHATE	5 - 15	0.000259	-0.019199	0.019717	0.9995
CANDIDA	A - B	0.017198	-0.007446	0.041841	0.2740
CANDIDA	A - C	0.048667	0.024023	0.073311	<.0001
CANDIDA	A - D	0.040790	0.016146	0.065434	0.0001
CANDIDA	B - C	0.031469	0.006825	0.056113	0.0060
CANDIDA	B - D	0.023593	-0.001051	0.048236	0.0662
CANDIDA	C - D	-0.007877	-0.032520	0.016767	0.8423

Figure 13: *Chart showing all the pooled data for phosphate percentage treatment groups. Means and standard deviations corresponding to: Candida strain and coated or naked PMMA disks*

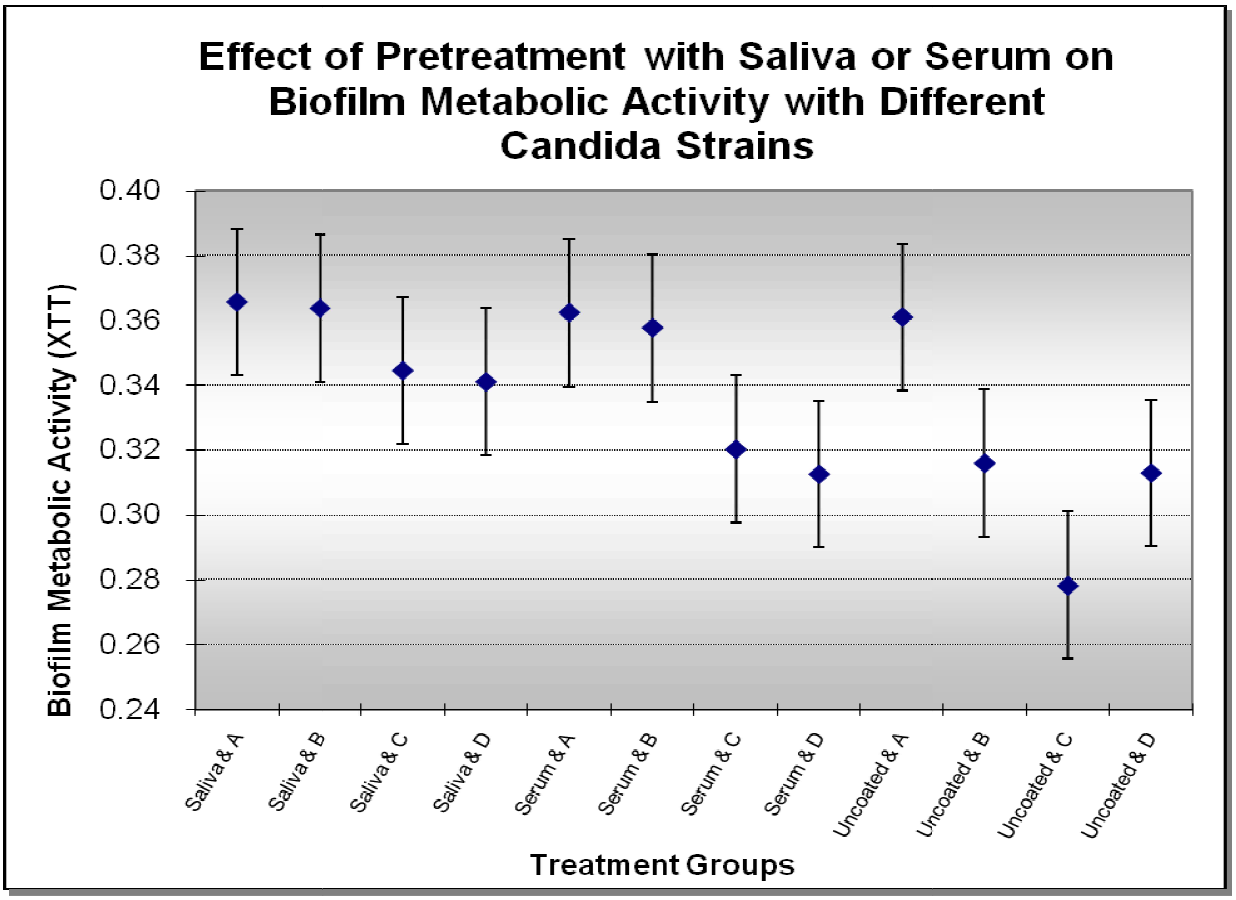
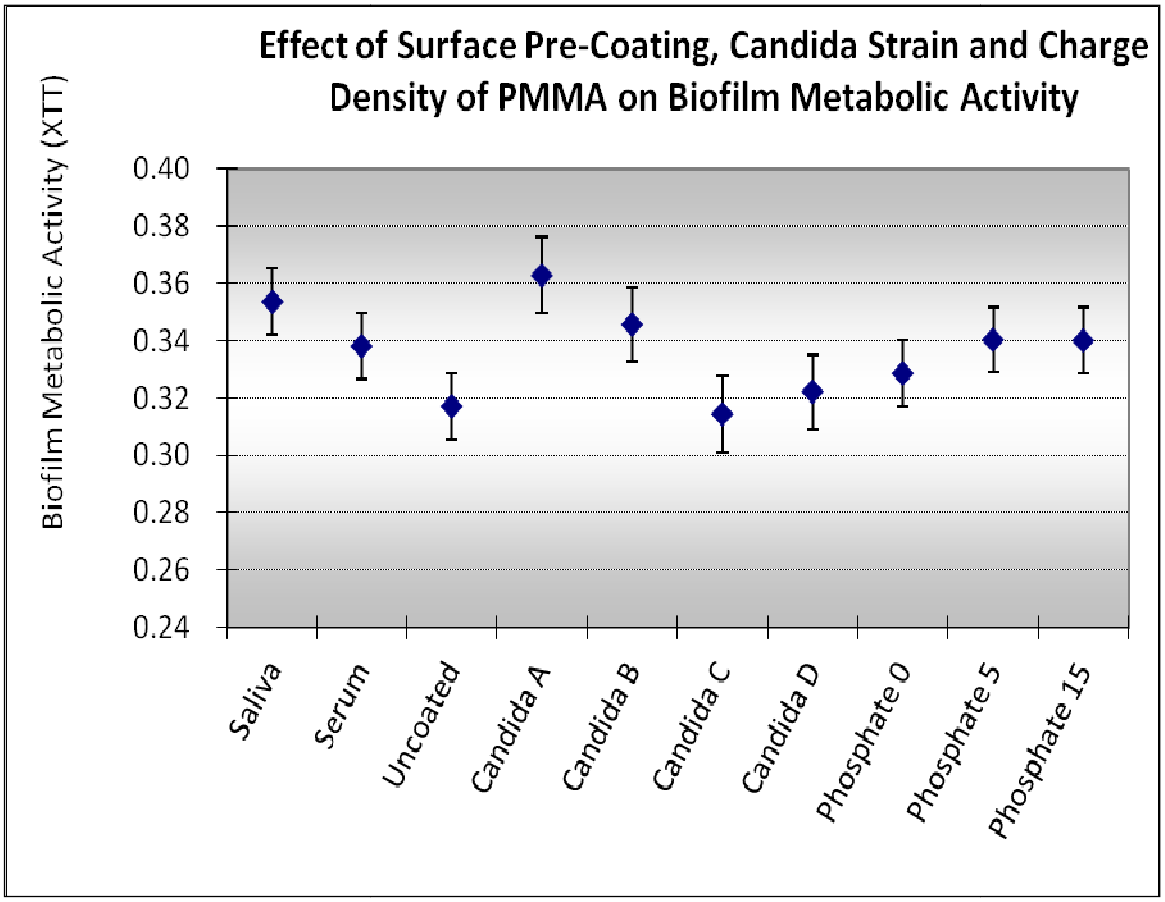


Figure 14: *Chart showing pooled means and standard deviations corresponding to each treatment group individually*



DISCUSSION

CHAPTER V

This thesis reproduced an *in vitro* model for investigating the interactions between *C. albicans* biofilms and various modification of a PMMA denture base material. Chandra et al[37], developed the original model with the goal to grow biofilms on acrylic. With this knowledge we reproduced a sustainable and active monospecies fungal biofilm system that allowed us to test three variables that may affect biofilm formation on PMMA surfaces.

In addition an effort was made to gain a better understanding of the interactions between various surface properties of PMMA and the adherence ability of *C. albicans.* The principle that the oral environment creates a proteinacious pellicle on denture surfaces through salivary secretions was maintained. Similarly, the effects of a serum pellicle were studied, which may have relevance in areas of trauma and inflamation. Acquiring a colony of microorganisms on a denture base is believed to produce specific receptors that promote the adherence of Candida cells.[10] This suggests that PMMA surfaces coated with salivary pellicle will have surface receptors for adhesins on *C. albicans* which promote anchorage and subsequent biofilm formation.

In vitro studies of denture pellicle formation on acrylic surfaces have shown the direct influence saliva has on the adherence of yeasts.[36,84] In the present study, results suggested that with the addition of whole stimulated pooled saliva, there was an increase in biofilm metabolism and presumably adherence of all four strains of *C. albicans* (see figure 9). This heavily suggests that PMMA surfaces coated with a salivary pellicle create an attractive environment for microbial adherence and subsequent biofilm development.

There are multiple ways of collecting saliva. In this study, whole

stimulated fresh pooled saliva was obtained from four healthy individuals to represent our clinical scenario. Almost all previous research (Table 1) studying salivary pellicle on acrylic resins has demonstrated different saliva collection techniques, which may have contributed to widley varied findings. For all studies in this body of work, saliva was collected, filtered, and immediately used to best represent a clinical case scenario and maintain the integrity of the saliva as much as practically possible. The present results demonstrated improved adherence with saliva. They may be comparable to previous research in that whole stimulated saliva was shown to mildly enhance *C. albicans* adherence as compared to untreated/unconditioned PMMA.[6,36] The fitting surface of a maxillary denture covers the minor salivary glands and therefore collecting this form of saliva in the future, though difficult, may be more clinically appealing.

Inflammation from denture trauma or mucosal irritation may cause compositional changes in pellicle and possibly propogate denture induced stomatitis.[121] Denture trauma can release serum transudate, a form of extracellular fluid, leaking through the mucosal epithelium and coating the intaglio surface of the denture. With this in mind, this study looked at the effects serum has on *C. albicans* adherence and biofilm metabolism as well. The results indicated a significant increase in metabolic activity of cells grown on serum-coated disks, suggesting, along with other studies, that PMMA surfaces coated with serum enhance the adhesion of *Candida*.[35,86]

Surface charge is not present in denture resins used today and may be an important alteration to PMMA surfaces.[9,11,13,122] *C. albicans* species have an ability to colonize polymeric surfaces based on hydrophobic and free energy consideration.[85] The surface of PMMA objects is hydrophobic. This appears to attract other particles with

hydrophobic surfaces, such as *C. albicans*. A denture base having a negative surface charge may hinder the initial adhesion interaction via repulsive electrostatic forces. In addition, a negative charge may increase the presence of protective cationic anti-microbial peptides in the pellicle.[38] Giving PMMA an anionic surface charge is a way of increasing its hydrophilicity and preventing adhesion of less hydrophilic microorganisms, reducing the patients risk of developing denture induced stomatitis.

Surface charge was modified through the addition of phosphate groups within the monomer of the PMMA mixtures. The PMMA phosphate concentration levels used in this study were 0%, 5% and 15%. These levels were based on previous research that confirmed adequate physical strength for the fabrication of denture bases with phosphate containing resins.[105,121] The results of this study showed that incorporation of a negative surface charge, at varying concentrations, in PMMA resins had no effect on the ability of different strains of Candida to develop a biofilm. This is in contrast to the original hypothesis that surface charge would reduce adhesion and biofilm metabolism, originally postulated by Park et al[11] who found a decrease in *C. albicans* adherence by adding a negative carboxyl group to PMMA.

Several factors may explain the difference observed. They include i) the functional groups, ii) assays to measure biofilm metabolism, iii) Candida strains and iv) a different biofilm model. Explanations could include also differences in PMMA surface roughness. The present investigation showed that surface roughness can change over the course of an investigation.

The literature shows that surface roughness may override any electrostatic effects that the negative phosphate charge in our PMMA provided.[93,98-100] Surface roughness

allows microorganisms to settle in areas that are protected from fluid shear forces. Moreover if the roughness was due to calcium deposition then the surface would also show a drop in net negative charge. In a personal communication by Dentino et al, 2010, which used a similar protocol, they found that by adding a washing step and soaking the PMMA disks in EDTA (ethylenediaminetetraacetic acid) after every experiment, one could maintain the original surface roughness (Ra) of the disks even after several experiments. EDTA is a chelating agent and has the ability of sequestering metal ions such as Ca^{2+}, Mg^{2+} and Fe^{3+}.[123] It was determined that once the deposits had been removed with EDTA, phosphate containing PMMA co-polymers were in fact capable of reducing *C. albicans* adherence and/or biofilm metabolism to the naked surface in a phosphate dependent manner. Once surface roughness is eliminated or greatly reduced to near the postulated threshold roughness value (0.2 µm)[90] below which no effect on adhesion should be expected, then surface charges on PMMA denture bases may have an effect on *C. albicans* adherence and/or biofilm metabolism. However, the clinical utility of this approach is not certain and more studies need to be done.

Various strains of *C. albicans* have differing adherence capabilities and cell surface hydrophobicities. For example Minagi et al[85] compared two species, *C. tropicalis* and *C. albicans,* and each demonstrated variation in adherence to hydrophobic surfaces such as heat-cured resins. Adherence of *C. albicans* was far lower than of *C. tropicalis*, and it was determined that the closer the surface free energy is between substrate and microorganism, the greater the probability of adherence. Another factor, as demonstrated by Klotz et al[10], is that size and shape of Candida species varies, resultsing in variable adherence to different substrates. This study demonstrated strain-dependent differences

in adherence behavior for *C. albicans*. Statistically significant differences were found when comparing bronchial and sputum strains to both oral isolates. *C. albicans* isolated from denture stomatitis patients appeared to have less XTT activity when compared to the bronchial wash and sputum isolates. This corresponds with previous research in that microorganisms demonstrated specific species characteristics that can moderate their capacity to adhere to surfaces substrates at varying rates.[10,124]

CONCLUSIONS

CHAPTER VI

The objective of this study was to invesitgate the the effect of protein pellicle and phosphate charge density on adhesion and biofilm formation of *C. albicans* to polymethyl methacrylate-based resins. Four different strains of *Candida* were incorporated in this study, and we wanted to determine if there was a variation in their ability to colonize various acrylic surfaces. The methods employed in this study for the formation of a biofilm on PMMA were taken from Chandra et al.[84] The methods for disk preparation were taken from Dhir et al and Puri et al.[105,119]

Within the limitations of this *in vitro* study, it can be concluded that with an increase in phosphate charge density (increased anionic charge capacity), there was no significant change in the amount of *C. albicans* adherence and/or biofilm metabolism. The introduction of a salivary pellicle as well as the addition of serum coating to the PMMA surfaces did, however, show statistically significant increases in *C. albicans* biofilm metabolism which was later confirmed microscopically. The proteinacious salivary pellicle showed greater *C. albicans* adhesion compared to that of the serum. The level of biofilm metabolism on PMMA surfaces was strain dependent. Oral isolates, collected from infected denture stomatitis patients, had significantly reduced biofilm formation when compared with that of bronchial or sputum strains.

Overall the ability of *C. albicans* to have significant biofilm metabolic activity on PMMA surfaces was dependent on strain and surface coating. Although the results of this study did not support that phosphate containing PMMA surfaces can reduce *C. albicans* adherence, further testing of biofilm formation on uniform smooth surfaces (Ra ≤ 0.2 µm) and studies *in vivo* need to be undertaken to determine if negatively charged PMMA could still be an effective inhibitor of Candida adhesion.

REFERENCES

BIBLIOGRAPHY

1. Reichart PA. Oral mucosal lesions in a representative cross-sectional study of aging Germans. *Community Dent Oral Epidemiol*. 2000;28(5):390-398.

2. Budtz-Jorgensen E. Oral mucosal lesions associated with the wearing of removable dentures. *J Oral Pathol*. 1981;10(2):65-80.

3. Yoneyama T, Yoshida M, Ohrui T, et al. Oral care reduces pneumonia in older patients in nursing homes. *J Am Geriatr Soc*. 2002;50(3):430-433.

4. Sumi Y, Kagami H, Ohtsuka Y, Kakinoki Y, Haruguchi Y, Miyamoto H. High correlation between the bacterial species in denture plaque and pharyngeal microflora. *Gerodontology*. 2003;20(2):84-87.

5. Shay K, Ship JA. The importance of oral health in the older patient. *J Am Geriatr Soc*. 1995;43(12):1414-1422.

6. Edgerton M, Scannapieco FA, Reddy MS, Levine MJ. Human submandibular-sublingual saliva promotes adhesion of Candida albicans to polymethylmethacrylate. *Infect Immun*. 1993;61(6):2644-2652.

7. Tsai H, Raj PA, Bobek LA. Impact testing of polysulphone denture base polymer. *Aust Dent Journ*. 1984;29(6):398-403.

8. Baev D, Li XS, Dong J, Keng P, Edgerton M. Human salivary histatin 5 causes disordered volume regulation and cell cycle arrest in Candida albicans. *Infect Immun*. 2002;70(9):4777-4784.

9. Park SE, Blissett R, Susarla SM, Weber HP. Candida albicans adherence to surface-modified denture resin surfaces. *J Prosthodont*. 2008;17(5):365-369.

10. Klotz SA, Drutz DJ, Zajic JE. Factors governing adherence of Candida species to plastic surfaces. *Infect Immun*. 1985;50(1):97-101.

11. Park SE, Periathamby AR, Loza JC. Effect of surface-charged poly(methyl methacrylate) on the adhesion of Candida albicans. *J Prosthodont*. 2003;12(4):249-254.

12. Yildirim MS, Kesimer M, Hasirci N, Kilic N, Hasanreisoglu U. Adsorption of human salivary mucin MG1 onto glow-discharge plasma treated acrylic resin surfaces. *J Oral Rehabil*. 2006;33(10):775-783.

13. Yoshinari M., Kato T., Matsuzaka K., Hayakawa T., Inoue T., Oda Y., Okuda K., Shimono M. Adsorption behavior of antimicrobial peptide histatin 5 on PMMA. *Journal of Biomedical Materials Research Part B, Applied Biomaterials*. 2006;77(1):47-47-54.

14. Marcus PA, Joshi A, Jones JA, Morgano SM. Complete edentulism and denture use for elders in New England. *J Prosthet Dent*. 1996;76(3):260-266.

15. Marcus SE, Drury TF, Brown LJ, Zion GR. Tooth retention and tooth loss in the permanent dentition of adults: United States, 1988-1991. *J Dent Res*. 1996;75(Spec No):684-695.

16. Jennifer Cheeseman Day. Population Projections of the United States, by Age, Sex, Race, and Hispanic Origin: 1993 to 2050, Current Population Reports. . 1993:25-1104.

17. Douglass CW, Shih A, Ostry L. Will there be a need for complete dentures in the United States in 2020?. *J Prosthet Dent*. 2002;87(1):5-8.

18. Moskona D, Kaplan I. Oral lesions in elderly denture wearers. *Clin Prev Dent*. 1992;14(5):11-14.

19. Samaranayake LP, Keung Leung W, Jin L. Oral mucosal fungal infections. *Periodontol 2000*. 2009;49:39-59.

20. Davenport JC. The oral distribution of candida in denture stomatitis. *Br Dent J*. 1970;129(4):151-156.

21. Edgerton M, Levine MJ. Characterization of acquired denture pellicle from healthy and stomatitis patients. *J Prosthet Dent*. 1992;68(4):683-691.

22. Budtz-Jorgensen E, Bertram U. Denture stomatitis. I. The etiology in relation to trauma and infection. *Acta Odontol Scand*. 1970;28(1):71-92.

23. Radford DR, Challacombe SJ, Walter JD. Denture plaque and adherence of Candida albicans to denture-base materials in vivo and in vitro. *Crit Rev Oral Biol Med*. 1999;10(1):99-116.

24. Pires FR, Santos EB, Bonan PR, De Almeida OP, Lopes MA. Denture stomatitis and salivary Candida in Brazilian edentulous patients. *J Oral Rehabil*. 2002;29(11):1115-1119.

25. Olsen I. Denture stomatitis. Occurrence and distribution of fungi. *Acta Odontol Scand*. 1974;32(5):329-333.

26. Lyon, DG, Chick, AO. Denture sore mouth and angular chelitis: A preliminary investigation into their possible association with Candida infection. *Dent Practit*. 1957;7:212-212-217.

27. Avon SL, Goulet JP, Deslauriers N. Removable acrylic resin disk as a sampling system for the study of denture biofilms in vivo. *J Prosthet Dent*. 2007;97(1):32-38.

28. Radford DR, Sweet SP, Challacombe SJ, Walter JD. Adherence of Candida albicans to denture-base materials with different surface finishes. *J Dent*. 1998;26(7):577-583.

29. Taylor RL, Verran J, Lees GC, Ward AJP. The influence of substratum topography on bacterial adhesion to polymethylmethacrylate. *J Mater Sci Mater M*. 1998;9:17-17-22.

30. Verran J., Taylor R.L., Lees G.C. Bacterial adhesion to inert thermoplastic surfaces. *Journal of Materials Science: Materials in Medicine*. 1996;7(10):597-597-601.

31. Teughels W, Van Assche N, Sliepen I, Quirynen M. Effect of material characteristics and/or surface topography on biofilm development. *Clin Oral Implants Res*. 2006;17(Suppl 2):68-81.

32. Levine MJ, Reddy MS, Tabak LA, et al. Structural aspects of salivary glycoproteins. *J Dent Res*. 1987;66(2):436-441.

33. Gocke R, Gerath F, von Schwanewede H. Quantitative determination of salivary components in the pellicle on PMMA denture base material. *Clin Oral Investig*. 2002;6(4):227-235.

34. Pereira T., Del Bel Cury AA., Cenci MS., Rodrigues-Garcia RC. In vitro *Candida* colonization on acrylic resins and denture liners: Influence of surface free energy, roughness, saliva and adhering bacteria. *The International Journal of Prosthodontics*. 2007;20(3):308-308-310.

35. Nikawa H, Hamada T, Yamamoto T, Kumagai H. Effects of salivary or serum pellicles on the Candida albicans growth and biofilm formation on soft lining materials in vitro. *J Oral Rehabil*. 1997;24(8):594-604.

36. Vasilas A, Molina L, Hoffman M, Haidaris CG. The influence of morphological variation on Candida albicans adhesion to denture acrylic in vitro. *Arch Oral Biol*. 1992;37(8):613-622.

37. Chandra J, Kuhn DM, Mukherjee PK, Hoyer LL, McCormick T, Ghannoum MA. Biofilm formation by the fungal pathogen Candida albicans: development, architecture, and drug resistance. *J Bacteriol*. 2001;183(18):5385-5394.

38. Edgerton M, Raj PA, Levine MJ. Surface-modified poly(methyl methacrylate) enhances adsorption and retains anticandidal activities of salivary histatin 5. *J Biomed Mater Res*. 1995;29(10):1277-1286.

39. Weintraub JA, Burt BA. Oral health status in the United States: tooth loss and edentulism. *J Dent Educ*. 1985;49(6):368-378.

40. Niessen LC JJ. Geriatric dentistry: aging and oral health. In: Papas AS, Niessen LC, Chauncey HH, ed. *Facing the challenge: the graying of America.* St. Louis: Mosby-Year Book; 1991:3-3-13.

41. Thomas-Weintraub A. Dental needs and dental service use patterns of an elderly edentulous population. *J Prosthet Dent*. 1985;54(4):526-532.

42. Shulman JD, Beach MM, Rivera-Hidalgo F. The prevalence of oral mucosal lesions in U.S. adults: data from the Third National Health and Nutrition Examination Survey, 1988-1994. *J Am Dent Assoc*. 2004;135(9):1279-1286.

43. Statistical Abstract of the US. . 1996.

44. Hunt RJ, Beck JD, Lemke JH, Kohout FJ, Wallace RB. Edentulism and oral health problems among elderly rural Iowans: the Iowa 65+ rural health study *Am J Public Health*. 1985;75(10):1177-1177-1181.

45. Atwood DA. The future of prosthodontics. *J Prosthet Dent*. 1984;51(2):262-267.

46. Douglass CW, Gammon MD, Atwood DA. Need and effective demand for prosthodontic treatment. *J Prosthet Dent*. 1988;59(1):94-104.

47. Narhi TO, Ainamo A, Meurman JH. Salivary yeasts, saliva, and oral mucosa in the elderly. *J Dent Res*. 1993;72(6):1009-1014.

48. Silverglade LB, Stablein MJ. Diagnostic survey of 9,000 biopsies from three age groups: under 60 years, 60-69 and over 70. *Gerodontics*. 1988;4(6):285-288.

49. Bergman JD, Wright FA, Hammond RH. The oral health of the elderly in Melbourne. *Aust Dent J*. 1991;36(4):280-285.

50. Budtz-Jorgensen E. The significance of Candida albicans in denture stomatitis. *Scand J Dent Res*. 1974;82(2):151-190.

51. Sumi Y, Miura H, Michiwaki Y, Nagaosa S, Nagaya M. Colonization of dental plaque by respiratory pathogens in dependent elderly. *Arch Gerontol Geriatr*. 2007;44(2):119-124.

52. Hugo FN, Hilgert JB, Medeiros LRF. Interventions for treating denture stomatitis. *Cochrane Database Syst Rev*. 2009.

53. Lombardi T, Budtz-Jorgensen E. Treatment of denture-induced stomatitis: a review. *Eur J Prosthodont Restor Dent*. 1993;2(1):17-22.

54. MacFarlane TW SL. Clinical Oral Microbiology. In: Wright, ed. *Fungal Infections*. London: ; 1989:122-122-139.

55. Newton AV. Denture sore mouth: A possible aetiology. *Brit Dental J*. 1962;112:357-357-360.

56. Arendorf TM, Walker DM. Denture stomatitis: a review. *J Oral Rehabil*. 1987;14(3):217-227.

57. Nyquist G. The influence of denture hygiene and the bacterial flora on the condition of the oral mucosa in full denture cases. *Acta Odontol Scand*. 1953;11(1):24-60.

58. van Reenen JF. Microbiologic studies on denture stomatitis. *J Prosthet Dent*. 1973;30(4):493-505.

59. Barbeau J, Seguin J, Goulet JP, et al. Reassessing the presence of Candida albicans in denture-related stomatitis. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*. 2003;95(1):51-59.

60. Webb BC, Thomas CJ, Willcox MD, Harty DW, Knox KW. Candida-associated denture stomatitis. Aetiology and management: a review. Part 2. Oral diseases caused by Candida species. *Aust Dent J*. 1998;43(3):160-166.

61. Berdicevsky I, Ben-Aryeh H, Szargel R, Gutman D. Oral candida of asymptomatic denture wearers. *Int J Oral Surg*. 1980;9(2):113-115.

62. Jorge Junior J, de Almeida OP, Bozzo L, Scully C, Graner E. Oral mucosal health and disease in institutionalized elderly in Brazil. *Community Dent Oral Epidemiol*. 1991;19(3):173-175.

63. Iacopino AM, Wathen WF. Oral candidal infection and denture stomatitis: a comprehensive review. *J Am Dent Assoc*. 1992;123(1):46-51.

64. Espinoza I, Rojas R, Aranda W, Gamonal J. Prevalence of oral mucosal lesions in elderly people in Santiago, Chile. *J Oral Pathol Med*. 2003;32(10):571-575.

65. Nevalainen MJ, Narhi TO, Ainamo A. Oral mucosal lesions and oral hygiene habits in the home-living elderly. *J Oral Rehabil*. 1997;24(5):332-337.

66. Eliasson L, Dahlen G, Heyden G, Moller A. The predominant microflora of the palatal mucosa in an elderly island population. *Acta Odontol Scand*. 1992;50(3):163-169.

67. Costerton JW, Lewandowski Z, Caldwell DE, Korber DR, Lappin-Scott HM. Microbial biofilms. *Annu Rev Microbiol*. 1995;49:711-745.

68. Blankenship JR, Mitchell AP. How to build a biofilm: a fungal perspective. *Curr Opin Microbiol*. 2006;9(6):588-594.

69. ten Cate JM, Klis FM, Pereira-Cenci T, Crielaard W, de Groot PW. Molecular and cellular mechanisms that lead to Candida biofilm formation. *J Dent Res*. 2009;88(2):105-115.

70. Samaranayake YH, Wu PC, Samaranayake LP, So M. Relationship between the cell surface hydrophobicity and adherence of Candida krusei and Candida albicans to epithelial and denture acrylic surfaces. *APMIS*. 1995;103(10):707-713.

71. Darwazeh AM, Lamey PJ, Samaranayake LP, et al. The relationship between colonisation, secretor status and in-vitro adhesion of Candida albicans to buccal epithelial cells from diabetics. *J Med Microbiol*. 1990;33(1):43-49.

72. Donlan RM, Costerton JW. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev*. 2002;15(2):167-193.

73. Seneviratne CJ, Jin L, Samaranayake LP. Biofilm lifestyle of Candida: a mini review. *Oral Dis*. 2008;14(7):582-590.

74. Ramage G, Vande Walle K, Wickes BL, Lopez-Ribot JL. Standardized method for in vitro antifungal susceptibility testing of Candida albicans biofilms. *Antimicrob Agents Chemother*. 2001;45(9):2475-2479.

75. Ramage G, VandeWalle K, Bachmann SP, Wickes BL, Lopez-Ribot JL. In vitro pharmacodynamic properties of three antifungal agents against preformed Candida albicans biofilms determined by time-kill studies. *Antimicrob Agents Chemother*. 2002;46(11):3634-3636.

76. Mukherjee PK, Chandra J, Kuhn DM, Ghannoum MA. Mechanism of fluconazole resistance in Candida albicans biofilms: phase-specific role of efflux pumps and membrane sterols. *Infect Immun*. 2003;71(8):4333-4340.

77. Jeganathan S, Lin CC. Denture stomatitis--a review of the aetiology, diagnosis and management. *Aust Dent J*. 1992;37(2):107-114.

78. Budtz-Jorgensen E. Etiology, pathogenesis, therapy, and prophylaxis of oral yeast infections. *Acta Odontol Scand*. 1990;48(1):61-69.

79. Ellepola AN, Samaranayake LP. Oral candidal infections and antimycotics. *Crit Rev Oral Biol Med*. 2000;11(2):172-198.

80. Mirth DB, Bartkiewicz A, Shern RJ. Development and in vitro evaluation of an intra-oral controlled-release delivery system for chlorhexidine. *Journal of Dental Research*. 1989;68(8):1285-1285-1288.

81. Konsberg R, Axell T. Treatment of Candida-infected denture stomatitis with a miconazole lacquer. *Oral Surg Oral Med Oral Pathol*. 1994;78(3):306-311.

82. Budtz-Jorgensen E, Carlino P. A miconazole lacquer in the treatment of Candida-associated denture stomatitis. *Mycoses*. 1994;37(3-4):131-135.

83. Kulak Y, Arikan A, Delibalta N. Comparison of three different treatment methods for generalized denture stomatitis. *J Prosthet Dent*. 1994;72(3):283-288.

84. Chandra J, Patel JD, Li J, et al. Modification of surface properties of biomaterials influences the ability of Candida albicans to form biofilms. *Appl Environ Microbiol*. 2005;71(12):8795-8801.

85. Minagi S, Miyake Y, Inagaki K, Tsuru H, Suginaka H. Hydrophobic interaction in Candida albicans and Candida tropicalis adherence to various denture base resin materials. *Infect Immun*. 1985;47(1):11-14.

86. Samaranayake LP, MacFarlane TW. An in-vitro study of the adherence of Candida albicans to acrylic surfaces. *Arch Oral Biol*. 1980;25(8-9):603-609.

87. McCourtie J, Douglas LJ. Relationship between cell surface composition of Candida albicans and adherence to acrylic after growth on different carbon sources. *Infect Immun*. 1981;32(3):1234-1241.

88. Quirynen M, Bollen CM. The influence of surface roughness and surface-free energy on supra- and subgingival plaque formation in man. A review of the literature. *J Clin Periodontol*. 1995;22(1):1-14.

89. Verran J, Maryan CJ. Retention of Candida albicans on acrylic resin and silicone of different surface topography. *J Prosthet Dent*. 1997;77(5):535-539.

90. Bollen CM, Lambrechts P, Quirynen M. Comparison of surface roughness of oral hard materials to the threshold surface roughness for bacterial plaque retention: a review of the literature. *Dent Mater*. 1997;13(4):258-269.

91. Yamauchi M, Yamamoto K, Wakabayashi M, Kawano J. In vitro adherence of microorganisms to denture base resin with different surface texture. *Dent Mater J*. 1990;9(1):19-24.

92. Kuhar M, Funduk N. Effects of polishing techniques on the surface roughness of acrylic denture base resins. *J Prosthet Dent*. 2005;93(1):76-85.

93. Quirynen M, Marechal M, Busscher HJ, Weerkamp AH, Darius PL, van Steenberghe D. The influence of surface free energy and surface roughness on early plaque formation. An in vivo study in man. *J Clin Periodontol*. 1990;17(3):138-144.

94. Davenport JC. The denture surface. *Br Dent J*. 1972;133(3):101-105.

95. Cannon RD, Chaffin WL. Colonization is a crucial factor in oral candidiasis. *J Dent Educ*. 2001;65(8):785-787.

96. Cannon RD, Holmes AR, Mason AB, Monk BC. Oral Candida: clearance, colonization, or candidiasis? *J Dent Res*. 1995;74(5):1152-1161.

97. Allison RT, Douglas WH. Micro-colonization of the denture-fitting surface by Candida albicans. *J Dent*. 1973;1(5):198-201.

98. Miyake Y, Fujita Y, Minagi S, Suginaka H. Surface hydrophobicity and adherence of Candida to acrylic surfaces. *Microbios*. 1986;46(186):7-14.

99. Seko S, Nakano H, Miyake Y, Suginaka H, Nihira H. Adherence of Candida albicans to urinary catheters. *Hiroshima J Med Sci*. 1986;35(4):363-371.

100. Verran J, Lees GC, Shakespeare, AP. The effect of surface roughness on the adhesion of Candida albicans to acrylic. *Biofouling*. 1991;3:183-183-191.

101. Newman HN. Adsorption of microorganisms to surfaces. In: Bitton G, Marshall KC, eds. *Retention of bacteria on oral surfaces.* New York: John Wiley & Sons; 1980:207-207-251.

102. Busscher H.J., Van Pelt A.W.J., De Boer P., De Jong H.P., Arends J. Effect of surface roughening of polymers on measured contact angles of liquids. *Colloids and Surfaces*. 1984;9(4):319-319-331.

103. Loney RW, Moulding MB, Hacker CH, Ritsco RG. Finishing and polishing of a poly (fluoroalkoxyphosphazene) resilient denture liner. *Int J Prosthodont*. 1994;7(4):362-367.

104. Anon P. "Which?" Consumers' Association. In: *Denture cleansers and fixatives.* January ed. London: ; 1983:29-29-33.

105. Puri G, Berzins DW, Dhuru VB, et al. Effect of phosphate group addition on the properties of denture base resins. *J Prosthet Dent*. 2008;100(4):302-308.

106. McCourtie J, Douglas LJ. Extracellular polymer of Candida albicans: isolation, analysis and role in adhesion. *J Gen Microbiol*. 1985;131(3):495-503.

107. Nikawa H, Hamada T. Binding of salivary or serum proteins to Candida albicans in vitro. *Arch Oral Biol*. 1990;35(7):571-573.

108. McCourtie J, MacFarlane TW, Samaranayake LP. Effect of saliva and serum on the adherence of Candida species to chlorhexidine-treated denture acrylic. *J Med Microbiol*. 1986;21(3):209-213.

109. Kraus FW, Orstavik D, Hurst DC, Cook CH. The acquired pellicle: variability and subject-dependence of specific proteins. *J Oral Pathol*. 1973;2(3):165-173.

110. Baier RE, Glantz PO. Characterization of oral in vivo films formed on different types of solid surfaces. *Acta Odontol Scand*. 1978;36(5):289-301.

111. Sipahi C, Anil N, Bayramli E. The effect of acquired salivary pellicle on the surface free energy and wettability of different denture base materials. *J Dent*. 2001;29(3):197-204.

112. Oppenheim FG, Xu T, McMillian FM, et al. Histatins, a novel family of histidine-rich proteins in human parotid secretion. Isolation, characterization, primary structure, and fungistatic effects on Candida albicans. *J Biol Chem*. 1988;263(16):7472-7477.

113. Waltimo T, Vallittu P, Haapasalo M. Adherence of Candida species to newly polymerized and water-stored denture base polymers. *Int J Prosthodont*. 2001;14(5):457-460.

114. Dexter SC, Sullivan JD, Williams J, Watson SW. Influence of Substrate Wettability on the Attachment of Marine Bacteria to Various Surfaces. *Appl Microbiol*. 1975;30(2):298-308.

115. Olsen I, Birkeland JM. Initiation and aggravation of denture stomatitis by sucrose rinses. *Scand J Dent Res*. 1976;84(2):94-97.

116. Samaranayake LP, McCourtie J, MacFarlane TW. Factors affecting th in-vitro adherence of Candida albicans to acrylic surfaces. *Arch Oral Biol*. 1980;25(8-9):611-615.

117. Millsap KW, Bos R, Van Der Mei HC, Busscher HJ. Dot assay for determining adhesive interactions between yeasts and bacteria under controlled hydrodynamic conditions. *J Microbiol Methods*. 2000;40(3):225-232.

118. Bos R, Van der Mei HC, Busscher HJ. A quantitative method to study co-adhesion of microorganisms in a parallel plate flow chamer. II: Analysis of kinetics of co-adhesion. *J Microbiol Meth*. 1995;23:169-169-182.

119. Dhir G, Berzins DW, Dhuru VB, Periathamby AR, Dentino A. Physical properties of denture base resins potentially resistant to Candida adhesion. *J Prosthodont*. 2007;16(6):465-472.

120. Leung KP, Crowe TD, Abercrombie JJ, et al. Control of oral biofilm formation by an antimicrobial decapeptide. *J Dent Res*. 2005;84(12):1172-1177.

121. Ritchie GM, Fletcher AM, Main DM, Prophet AS. The etiology, exfoliative cytology, and treatment of denture stomatitis. *J Prosthet Dent*. 1969;22(2):185-200.

122. Yildirim MS, Hasanreisoglu U, Hasirci N, Sultan N. Adherence of Candida albicans to glow-discharge modified acrylic denture base polymers. *J Oral Rehabil*. 2005;32(7):518-525.

123. Ethylenediaminetetraacetic Acid and Related Chelating Agents *Ullmann's Encyclopedia of Industrial Chemistry*. ;A10.

124. Uyen HM, van der Mei HC, Weerkamp AH, Busscher HJ. Zeta potential and the adhesion of oral streptococci to polymethylmethacrylate. *Biomater Artif Cells Artif Organs*. 1989;17(4):385-391.