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Candida species and inflammation mediators in denture stomatitis: detection in biological samples

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Mémoire présenté à la Faculté des études supérieures et postdoctorales

en vue de l'obtention du grade de

Maîtrise és Sciences (M.Sc.)

en microbiologie, infectiologie et immunologie

Janvier 2017

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Résumé

Introduction: La stomatite prothétique (SP) est une des conditions inflammatoires communes affectant la muqeuse du palais dur en contact avec une prothèse partielle ou complète. Les étiologiques les plus probable de la SP sont l'infection fongique, la présence de biofilm sur la prothèse, et les traumatismes.

Objectifs de notre étude: 1) détection des cytokines dans les échantillons congelés provenant de patients sains et affectés par la SP; 2) verification de la capacité de *Candida albicans* de croître sur des milieux sélectifs après une congelation prolongée; 3) évaluation de la relation entre *Candida* sp. et la SP; 4) evaluation de la prévalence de *Streptococcus mutans* chez les patients atteints ar la SP.

Méthode: Un total de 115 échantillons cliniques, conservés à -80°C depuis 2008 ont été utilisés pour cette étude. La présence de *C. albicans* et *C. tropicalis*, et *S. mutans* a été évaluée par PCR et par culture. Un ELISA en sandwich a été utilisé pour la détection et le dosage de l'IL-1β, IL-6 et le TNF. La présence de *Candida sp.* a été testé à l'aide de milieux de culture sélectifs pour tous les échantillons (n = 115) dans les 24 heures suivant le prélèvement et avant et après congélation 7 ans plus tard.

Résultats: La culture microbiologique sur les échantillons frais de 2008 a démontré que *C. albicans* était présent dans 21.7% de tous les échantillons. Cependant, après décongélation, la détection de *C. albicans* ne peut être considérée comme fiable (4.35%). L'utilisation de la PCR nous a permi de détecter la presence de *C. albicans, C. tropicalis, S. mutans* dans SP, sauf chez les patients sains. *C. tropicalis* est toutefois plus frequents que *C. albicans*, un résultat qui contraste avec la culture lorsque l'échantillon était frais.

Conclusion: Ces résultats montrent que la conservation à long terme à -80°C altère la capacité de détecter les microorganismes par culture (80%) et par PCR. En outre, nous ne pouvions détecter les cytokines dans les échantillons. Cependant, une association entre *Candida* sp, *S. mutans* et SP est probable.

Mots-clés: La stomatite prothétique, *Candida* sp. , Inflammation médiateur

Abstract

Introduction: Denture stomatitis (DS) is a common inflammatory condition affecting the hard palate of mucosal tissue in contact with complete or partial denture. The most likely etiological factors of DS are fungal infection, denture biofilm, and trauma.

Objectives: 1) To investigate the ability of *Candida* sp. to grow in specific media after long term storage 2) To investigate the relationship of *Candida* sp. and denture stomatitis 3) To test the prevalence of *S. mutans* in DS patients 4) To detect the value of IL1-β ,IFN-γ ,IL-6, and TNF-α in samples after long term storage

Method: A total of 115 clinical sonicate samples, taken from individuals over 63 years old, kept frozen at -80°C from 2008 were used for this study. The presence of *C. albicans* and *C. tropicalis*, and *S. mutans* was evaluated by PCR and culture. A sandwich ELISA was used for the detection and assay of IL-1β, IL6 and TNFα. Presence of *Candida* sp was tested by using specific media for all samples (n=115) within 24 hours of collection and before and after freezing 7 years later.

Results: Microbiological cultures on fresh samples in 2008 showed that *C. albicans* was present in 21.74% of all samples. However, after thawing, detection of *C. albicans* cannot be considered reliable (4.35%). The use of PCR allowed us to detect the presence of *C. albicans*, *C. tropicalis, S. mutans* in DS, except in healthy patients. However, *C. tropicalis* is more frequent than *C. albicans*, a result that contrasts with the culture when the sample was fresh

Conclusion: Our results show that the capacity to detect microbial cells by culture after long term storage at -80°C was decreased about 80%. In addition cytokines might be degraded, rinsed off or below our detection level. However, association between *Candida* sp., *S. mutans* and DS was found.

Keywords: Denture stomatitis, *Candida* sp.*,* Inflammation mediator

Table of contents

Résumé i

Abstract ii

Table of contents iii

List of tables vi

List of figures vii

List of acronyms and abbreviations viii

Acknowledgments x

Introduction 1

1 Literature review 3

1.1 Denture stomatitis 3

1.2 Classification of denture stomatitis 3

1.3 Prevalence and epidemiology 7

1.4 Symptoms 10

1.5 Histological finding 10

1.6 Etiological factors 10

1.6.1 Fungal infection (*Candida* species) 12

1.6.2 Denture biofilm 17

1.6.3 Trauma 18

1.7 Treatment of denture stomatitis 22

1.8 Methods and biological samples used for detection of microbial pathogens 22

1.9 Rationale behind our work 23

1.10 Hypotheses 24

1.11 Objectives 24

2 Material and Methods 25
2.1 Sample collection 25
2.2 Mycological investigation 25
2.3 Microbiological detection by PCR 26
2.3.1 Extraction of DNA 26
2.3.2 PCR reaction 28
2.3.3 Electrophoresis on agarose gel 29
2.3.4 Quantitative evaluation 29
2.4 Cytokine detection 31
2.5 Statistical analysis 32
3 Results 33
3.1 Demographics 33
3.2 Microbiological culture before and after long term storage 34
3.3 PCR detection level 36
3.4 PCR analysis 38
3.5 Association between *S. mutans* and *Candida* species 40
3.6 Comparing microbial culture and PCR for detecting *Candida* species 41
3.7 Cytokine detection in healthy and denture stomatitis subjects 45
4 Discussion 48
4.1 Prevalence of Candidiasis 49
4.2 Comparing results of 2008 and 2015 50
4.3 Association of *S. mutans* with *Candida* sp. and DS 52
4.4 Inflammation mediators and their role in DS 53
4.5 Limitation of study 54
4.6 Future study 55

5 Conclusion 56

6 Recommendation 56

Bibliography 57

List of tables

Table 1-1 Prevalence reported for denture stomatitis in denture wearers in the last decade (2006-2016)........ 9

Table 2-1 Primer sequences for selected bacterial and fungal species........ 30

Table 3-1 The classification of study subject according to denture stomatitis diagnosis........ 33

Table 3-2 The comparison of the analyzed sonicate samples (total n=115) according to the presence of *candida* species before and after 7years of freeze........ 35

Table 3-3 Comparison of sonicates samples according to the percentage of detected *Candida* species by PCR. 39

Table 3-4 Number of samples including both *S .mutans* and *Candida* species........ 40

Table 3-5 Comparison of the collected results from microbial culture (2008) and PCR (2016) for detection of *C. tropicalis* in denture stomatitis and non-denture stomatitis for 7-year freeze samples........ 43

Table 3-6 Comparison of the collected results from microbial culture (2008) and PCR (2016) for detection of *C. albicans* in denture stomatitis and non-denture stomatitis for 7-year freeze samples........ 44

Table 3-7 The Cytokines range of detection levels in sonicates samples (n=115) for IL-1β INF-γ and n=40 for TNF-α , IL-6 46

List of figures

Figure 1-1 Classification of denture stomatitis according to Newton 6

Figure 3-1 The comparison of the analyzed sonicate samples (total n= 39) according to the presence of *candida* species before and after 7 years of freeze. 35

Figure 3-2 Example of quantitative PCR assessment of *C. tropicalis* 37

Figure 3-3 Number of samples *C. albicans* found in denture plaque with microbial culture (2008) and PCR (2015) in DS and non-denture stomatitis group 42

Figure 3-4 Standard curves for INF-γ (1), and IL-1β (2) 47

List of acronyms and abbreviations

°	Degree
mM	Millimeter
µl	Microliter
mg	Milligram
G	Gram
kg	Kilogram
PCR	Polymerase chain reaction
OR	Odds ratio
SD	Standard deviation
PCR	Polymerase chain reaction
dNTP	Deoxyribonucleotide triphosphate
OD	Optical density
CFUs	Colony-Forming Units
%	Percentage
CI	Confidence Interval
IL	Interleukin
C.	*Candida*
ELISA	Enzyme-linked immunosorbent assay
IFN	Interferon
TNF	Tumor necrosis factor
SP.	Species

DS	Denture stomatitis
AMP	Ampere
Hz	Hertz
nm	Nanometer
pg	Picogram
ng	Nanogram

Acknowledgments

I would like to use this chance here firstly to appreciate outstanding support and assistance I received from my kind supervisor, Dr. Jean Barbeau. Without his support and encouragement none of the achievements released in this dissertation would be possible.

I would also like to thank to Dr. Elham Emami, my co-supervisor, who was my first point of contact at the University of Montreal. She has been a great help in the review process for improving this thesis.

I must here express my gratitude to Annie Leduc who was always beside me during my study and helped me. Special thanks to her for supporting me in all my questions, and providing significant guidance in critical points throughout the period of research.

I would also like to acknowledge Dr. Stephane Roy, and Mr. Pierre Rompre who helped me in different aspects during my research period and thank all my laboratory colleagues who participated directly or indirectly to my research.

Finally, special thanks to my family for all encouragements and their unconditional love.

Introduction

Denture stomatitis (DS), a common inflammatory condition, affects the mucosal tissue of the hard palate in contact with complete or partial denture and it is characterized by a chronic inflammation of the palatal mucosa. This condition affects 10% to 75% of all denture wearers. DS is considered the most common oral lesion among denture wearers (Barbeau, Séguin et al. 2003, Emami, Seguin et al. 2007, Emami, de Grandmont et al. 2008, Pereira-Cenci, Del Bel Cury et al. 2008, Gendreau and Loewy 2011, Tay 2014, Zwiri 2016).

Various etiological factors have been reported in the literature however, a consensus concerning the etiological factors of denture stomatitis have not yet been fully demonstrated (dos Santos, Hilgert et al. 2010). These disagreements could be related to differences in patient cohorts and DS classification and the various sampling and analysis methods used in the study of denture stomatitis.

The main etiological factors of DS have been reported to be *Candida* sp. infections, presence of denture biofilm, and trauma (Altarawneh, Bencharit et al. 2013, Marinoski, Bokor-Bratic et al. 2014). Although the direct role of yeasts is not completely known, denture biofilm composed of *Candida albicans* and oral bacteria is considered as an important factor in the development of denture stomatitis lesions (Marinoski, Bokor-Bratic et al. 2014, Gleiznys, Zdanaviciene et al. 2015). In addition, new evidence proposes that trauma may induce the inflammatory reaction in denture stomatitis (Emami, de Grandmont et al. 2008).

Different samples including whole saliva (stimulated or unstimulated saliva) (Simunovic-Soskic, Pezelj-Ribaric et al. 2010, Šimunović-Šoškić, Pezelj-Ribarić et al. 2010, Gasparoto, Sipert et al. 2012), palatal secretion (Wilson, Wilton et al. 2007), blood sample (Pietruski, Pietruska et al. 2000) and serum(Kantardjiev and Popova 2002) were used for study of denture stomatitis. Numerous studies used fresh samples (Emami, Seguin et al. 2007, Altarawneh, Bencharit et al. 2013), while analysis of frozen samples is also common for the study of inflammatory biomarkers (Barros, Al-Tarawneh et al. 2012, Gasparoto, de Oliveira et al. 2012). This is due to the need to analyse samples for future studies as samples may not always be processed immediately.

Different methods were also used to find a potential association between denture stomatitis and the presence of *Candida* sp. such as microbial culture analysis (Zomorodian, Haghighi et al. 2011, Altarawneh, Bencharit et al. 2013, Sanitá, de Oliveira Mima et al. 2013), standard PCR (Tay 2014), and Real-Time PCR (White, Williams et al. 2004) .

This research project aims to investigate the relationship of *Candida* sp. and *Streptococcus mutans*, with denture stomatitis by two different methods (PCR and microbial culture), and to find the effect of long term storage on the microbiological and biochemical quality of denture sonicate samples.

1 Literature review

1.1 Denture stomatitis

Denture stomatitis (DS) also known as denture sore mouth and prosthetic stomatitis is a common inflammatory condition, which affects the mucosa of the hard palate in contact with complete or partial dentures (Naik and Pai 2011).

1.2 Classification of denture stomatitis

Classification of DS may be useful as it allows practitioners to follow the evolution of the condition after initiating treatment or providing oral hygiene instructions to the patient.
In 1958 the first classification of denture stomatitis was described by Östlund (Östlund 1958). He classified denture stomatitis by three distinct pathological stages: localized inflammation; diffuse erythema limited to the denture's contact area, and granular inflammation (Östlund 1958).

In 1962, Newton described a new classification of DS based on Östlund (Östlund 1958) which has been commonly used in research and clinical practice since then (Newton 1962). Newton classified denture stomatitis in three types (Figure 1.1). Type I DS is a localized simple inflammation, and is often described as pinpoint hyperemia. Newton type II DS is represented by a diffused hyperemia of the supporting tissues located under the denture. Newton type III DS , also known as a granular type, is represented by a nodular hyperemia sometimes

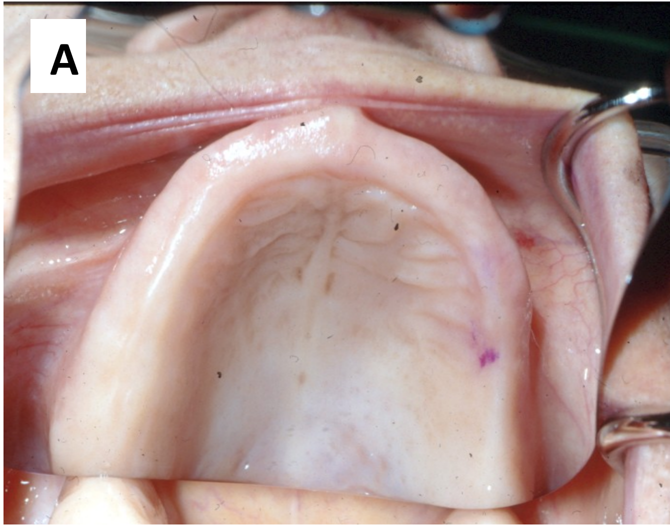
involving the entire palatal region but more commonly restricted to central areas (Newton 1962).

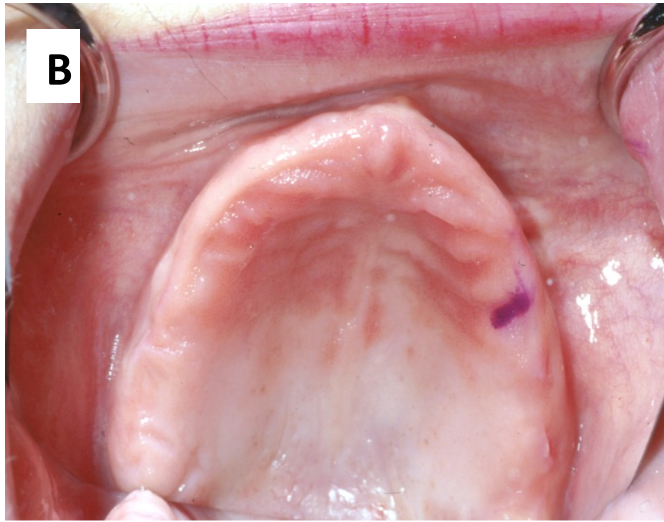
In 1970, Budtz-Jørgensen (Budtz-Jörgensen and Bertram 1970) used the same classification as Newton with a different terminology: simple localized inflammation, simple diffuse inflammation, and granular inflammation. Ten years later, Bergendal (Bergendal and Isacsson 1980) followed Östlund's classification and included only diffuse and papillary varieties. Schwartz et al (Schwartz, Young et al. 1988) classified DS according to the severity (intensity) of the inflammation and area (extent) of the disease . Severity index: 0: Normal pink mucosa, 1: Slight erythematous or mildly inflamed mucosa, 2: Moderately inflamed mucosa, 3: Severe or very pronounced inflamed mucosa. Area index: 0: No inflammation. 1: Inflammation extending up to 25% of denture-bearing tissue, 2: Inflammation extending between 25% and 50% of denture-bearing tissue, 3: Inflammation extending over 50% of the denture bearing mucosa.

Finally, in 2003, Barbeau et al (Barbeau, Séguin et al. 2003) modified the classification described by Newton to clarify the extent of inflammation in Newton type II and III DS. Letter "A" was used if less than 2 quadrants of the palate affected by stomatitis and letter "B" if it covers more than 2 quadrants. Newton's type I DS is considered as a healthy group in this classification.

In general, there are no useful differences in these classifications since all classifications are based on three critical conditions: distribution and location of inflammation (Budtz-Jörgensen

and Bertram 1970) , severity of inflammation (Bergendal and Isacsson 1980), and the extent of inflammation (Barbeau, Séguin et al. 2003) .





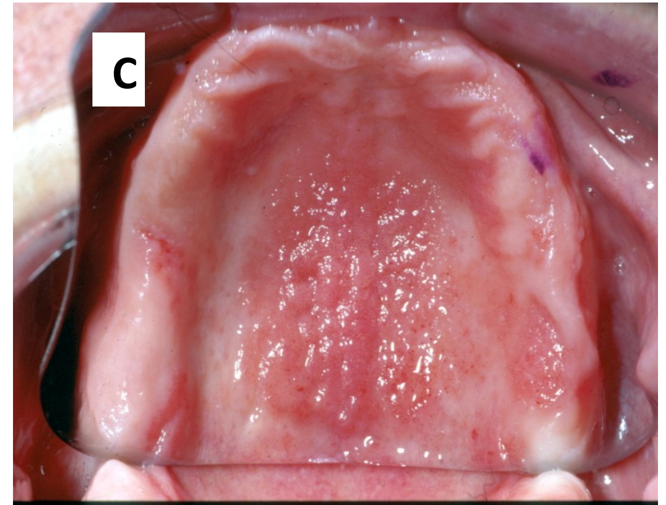


Figure 1-1 Classification of denture stomatitis according to Newton

A) Type 1 B) Type 2 C) Type 3

(Jean Barbeau laboratory)

1.3 Prevalence and epidemiology

Denture stomatitis is a frequent oral lesion with multiple etiological factors that affect a large number of patients wearing removable complete or partial dentures(Vasconcelos, Sampaio et al. 2010). The prevalence taken from the literature shows considerable variation mainly because of differences in research methodology, sample preparation and analysis, population demographics, and DS classification (Shulman, Rivera-Hidalgo et al. 2005, Gendreau and Loewy 2011, Kossioni 2011, Moosazadeh, Akbari et al. 2016)

The prevalence of denture stomatitis ranges from 10% to 75% of denture wearers (Barbeau, Séguin et al. 2003, Emami, Seguin et al. 2007, Garg, Singh et al. 2012). Table 1.1 shows these results from the last decade. The prevalence rate of denture stomatitis in province of Quebec with 14% edentulous (Emami, de Souza et al. 2013) has been reported 70.5% in average (Barbeau, Séguin et al. 2003, Emami, Seguin et al. 2007, Emami, de Grandmont et al. 2008).

Studies show that denture stomatitis is generally found in complete denture wearers (Shulman, Rivera-Hidalgo et al. 2005, Zissis, Yannikakis et al. 2006). Complete dentures, compared to partial dentures, cover a larger area of tissue therefore, it could increase the risk of denture stomatitis (Jainkittivong, Aneksuk et al. 2010). However, in partial denture wearers a prevalence of 1.1 % to 36 % denture stomatitis has been reported (Emami, Taraf et al. 2012).

Some authors found differences in the prevalence of denture stomatitis according to the gender and increasing the age of patients (Zissis, Yannikakis et al. 2006, Figueiral, Azul et al. 2007,

dos Santos, Hilgert et al. 2010, Tay 2014). However, other authors did not find any link between denture stomatitis and aging or gender (Zissis, Yannikakis et al. 2006, Emami, de Grandmont et al. 2008, Altarawneh, Bencharit et al. 2013, Mubarak, Hmud et al. 2015, Pesee and Arpornsuwan 2015).

Table 1-1 Prevalence reported for denture stomatitis in denture wearers in the last decade (2006-2016)

References	Country	Subjects	Prevalence
Emami et al. 2007	Canada	40	77,5%
Emami et al. 2008	Canada	175	63,6%
Baran et al. 2009	Turkey	30	38.5%
Divaris et al. 2010	Greece	873	6%
Ferreira et al. 2010	Brazil	335	15.2%
Gendreau et al.2010	Canada	173	64%
Mandali et al. 2011	Turkey	153	35.3%
Evren et al. 2011	Turkey	269	44%
Kossioni 2011	Greece	106	39.6%
Da Silva et al. 2011	Brazil	102	48.2%
Mozafari et al. 2012	Iran	202	54.6%
Cueto et al. 2012	Chile	126	37.1%
Sakar et al. 2013	Turkey	365	46.3%
Pesee et al. 2013	Thailand	128	52.3%
Lidia Y. Tay et al. 2014	Brazil	204	54.4%
Abhishek Gaur et al. 2015	India	608	59.25%
Abdalwhab Zwiri et al. 2016	Saudi Arabia	344	48.8%

1.4 Symptoms

Denture stomatitis is usually symptomless. However, some patients with this condition may complain of pain, mucosal bleeding, itching, burning, unpleasant taste, and dryness in their mouth (Gendreau and Loewy 2011, Tay, Jorge et al. 2014).

1.5 Histological finding

Histological differences of the palatal mucosa have been reported between healthy patients and patients with denture stomatitis.

Epithelial changes include a reduction in the number of epithelial cells and in the thickness of the epithelial layer observed in patients with denture stomatitis (Le Bars, Piloquet et al. 2001). Smears from palatal mucosa also demonstrated inflammation of the tissue with an increase in the number of inflammatory cells including polymorphonuclear leukocytes and lymphocytes in patients with denture stomatitis compare to patients with healthy mucosa (Le Bars, Piloquet et al. 2001, Altarawneh, Bencharit et al. 2013) .

1.6 Etiological factors

Many factors seem to be linked to the origin of denture stomatitis. These factors should be grouped in two Groups: predisposing and etiologic factors. Unfortunately, the etiology of this condition has not reached any consensus.

Several possible predisposing causes have been investigated including:

a. Smoking, which is considered predisposing factor (Barbeau, Séguin et al. 2003, Shulman, Rivera-Hidalgo et al. 2005, dos Santos, Hilgert et al. 2010, Navabi, Gholamhoseinian et al. 2013);
b. Poor denture hygiene which favours biofilm formation and proliferation of bacteria and yeasts (Cross, Williams et al. 2004, Marchini, Tamashiro et al. 2004, Dikbas, Koksal et al. 2006, dos Santos, Hilgert et al. 2010)
c. Wearing dentures overnight (Baran and Nalçacı 2009)
d. Allergic reaction to acrylic or other components of dentures(Rashid, Sheikh et al. 2015)
e. Prosthetic factors such as wearing a full denture rather than partial (Jainkittivong, Aneksuk et al. 2002, Emami, de Grandmont et al. 2008), instability of mandibular denture (Emami, de Grandmont et al. 2008), and wearing a partial removable denture (Emami, Taraf et al. 2012).
f. Impaired host immunity (Shulman, Rivera-Hidalgo et al. 2005, Webb, Thomas et al. 2005, Golecka, Oldakowska-Jedynak et al. 2006) as well as other type of systemic disorders including: Low levels of vitamin A (Shulman, Rivera-Hidalgo et al. 2005, Navabi, Gholamhoseinian et al. 2013), diabetes (Golecka, Oldakowska-Jedynak et al. 2006, Dorocka-Bobkowska, Zozulinska-Ziolkiewicz et al. 2010), hematological disorders (Shulman, Rivera-Hidalgo et al. 2005), and reduction of salivary flow (xerostomia) (Ramage, Tomsett et al. 2004)
g. Use of antibiotics (Pinke, Freitas et al. 2016)

Among the above mentioned factors, fungal infections (Poulopoulos, Belazi et al. 2007), denture biofilm (Campos, Marchini et al. 2008) , and oral trauma (Emami, de Grandmont et al. 2008) have been reported to play important roles in the etiology of denture stomatitis, and they will be the focus of our present work.

Several authors also suggest that DS is multifactorial as more than one etiological factor contributes to the presence of denture stomatitis (Shulman, Rivera-Hidalgo et al. 2005, Altarawneh, Bencharit et al. 2013, Syed, Chopra et al. 2015).

1.6.1 Fungal infection (*Candida* species)

For many years, *Candida* has been considered as one of the main etiological factors in denture stomatitis. *Candida* species which belongs to the normal flora of human mucosal oral cavity, gastrointestinal tract and vagina, are responsible for different clinical conditions from chronic mucocutaneous-candidiasis to invasive infections (Sardi, Scorzoni et al. 2013). These yeasts are considered as part of the normal microbiota in healthy humans but in immunocompromised situations they may cause systemic infection(Leigh, Steele et al. 2002). *Candida* containing over 150 species (Silva, Negri et al. 2012), but only a minority of that have been associated in human candidiasis. Among all *Candida* species, more than 17 are known to be etiological agents of human infections. However, more than 90 % of invasive infections are caused by *C. albicans, C. tropicalis, C. glabrata, C. krusei,* and *C. parapsilosis* (Sardi, Scorzoni et al. 2013).

C. albicans is considered as a commensal organism, because it is found in the mucosal membranes in the mouth, gastrointestinal tract, skin and vagina of healthy subjects. This

microorganism is thus considered as a part of microflora of healthy individual (Golecka, Oldakowska-Jedynak et al. 2006, Dalle, Wachtler et al. 2010). *C. albicans* plays a role in the development of polymicrobial biofilms on soft tissues and dentures. In a susceptible host, this opportunistic yeast can cause infections (Salerno, Pascale et al. 2011). *C. tropicalis* is identified as the most virulent of the *Candida*-non-*albicans* yeast species. It causes systemic infections (Butler, Rasmussen et al. 2009, Silva, Hooper et al. 2011, Silva, Negri et al. 2012) and has ability to adhere to epithelial cells (Meurman, Siikala et al. 2007).

C. glabrata is considered as the third most common yeast in systemic infections after *C. albicans* and *C. tropicalis*. These infections may be due to increasing usage of immunosuppressive therapy and antimycotic therapy (Meurman, Siikala et al. 2007). It is also common in the oral cavity of HIV patients(Meurman, Siikala et al. 2007).

C. krusei and *C. parapsilosis* are mainly present in patients who have critical illness including: patients with severe neutropenia, ill neonates and surgical intensive care unit patients (Meurman, Siikala et al. 2007).

I. Types of Candidiasis and risk factors

There are three main types of candidiasis: invasive candidiasis, thrush or oropharyngeal candidiasis, and genital candidiasis (Pappas, Rex et al. 2004) . The invasive or systemic candidiasis occurs mainly in severely compromised patients, for example in patients suffering from AIDS, undergoing organ transplantation, and chemotherapy for cancer (Spellberg,

Edwards et al. 2005, Richardson and Lass-Flörl 2008). Invasive candidiasis can affect the blood, heart, brain, eyes, bones, or other parts of the body (Pappas 2006). Since 1980s, the frequency of invasive fungal infections with serious diseases has increased specially among immunocompromised or hospitalized patients (Arendrup, Fuursted et al. 2005, Espinel-Ingroff, Canton et al. 2009)

The oropharyngeal candidiasis which is found in infants, the elderly and patients with weakened immune system, affects the oral cavity of patients. Oral thrush symptoms begin with congestive redness (erythema) of the oral mucosa, which is quickly covered with a white coating (Leigh, Steele et al. 2002, Kim and Sudbery 2011). Genital candidiasis is mainly observed as vaginitis (Kourkoumpetis, Manolakaki et al. 2010).

Different factors could promote different type of candidiasis including the age of the person, use of wide-spectrum antibiotics or corticosteroids, physiological conditions such as pregnancy, and medical conditions such as diabetes and HIV (Golecka, Oldakowska-Jedynak et al. 2006, Anwar, Malik et al. 2012, Yapar 2014). Wearing internal prosthetic devices (voice prosthesis and denture) is also a contributing factor for oropharyngeal candidiasis (Yapar 2014). Finally, other factors such as serious burns or surgery can be the cause of invasive candidiasis (Ha, Italiano et al. 2011). Therefore, studies show that candidiasis has a vast impact on the population.

II. Morphology and virulence of *Candida* sp.

Candida sp. can exist in two main morphological forms: the blastospore and filamentous forms (pseudohyphae and hyphae) (Kim, Kim et al. 2002). This dimorphism is under the control of several parameters: pH, temperature, and presence or absence of CO_2, carbon, or nitrogen (Brandt 2002). One of the main factors of *Candida's* virulence is the ability to switch between its morphology (Yang 2003). This characteristic, dimorphism, is an important feature allowing *Candida* to escape from the immune system of host defenses and increase its virulence (Henriques, Martins et al. 2007). In general there are three main stages thought to be important in *Candida* virulence: adherence to host tissue, response to changing environments by morphological plasticity, and secretion of enzymes to break down barriers and provide nutrients for growth (Haynes 2001).

Ramage et al, in 2005, demonstrated that blastospores mainly adhere to the biomaterial surface by interaction of non-specific factors such as surface hydrophobicity and electrostatic forces (Ramage, Saville et al. 2005). When *C. albicans* settles, it can adapt to changing environments by changing its morphology to hyphae or pseudohyphea and thus initiates colonization. This ability of switching between yeast, hyphal and pseudohyphal morphologies is important for virulence by promoting tissue penetration (Sudbery, Gow et al. 2004). Penetration can disrupt epithelial cells and provides more opportunity for *Candida* to adhere to the mucosal surfaces as well as providing a favorable environment for other microorganisms (Nasution 2013). Epithelial cells damaged by invasion of *Candida* respond to the infection by secretion of pro-inflammatory cytokines and inhibitory growth factor indicating that inflammation appears at the site of infection (Zhu and Filler 2010) .

Hyphae could play an important role in the virulence of this organism because it is larger than the blastospore which may impede phagocytosis by macrophages and polymorphonuclear leukocytes (PMNs) (Thompson, Carlisle et al. 2011, Nasution 2013). It has been found that only filamentous forms of *C. tropicalis* were able to invade the oral epithelium (Silva, Hooper et al. 2011).

III. Role of *Candida* sp. in denture stomatitis

Many studies have established a link between *Candida* sp. and stomatitis (Poulopoulos, Belazi et al. 2007, Tay 2014). *Candida*-associated denture stomatitis consider as the most frequent form of oral candidiasis (25-65%) (Salerno, Pascale et al. 2011). However, some studies did not confirm the link between presence of denture stomatitis and the number of *Candida* Colony-Forming Units (CFUs) isolated from the denture and palate of patients (Emami, de Grandmont et al. 2008).

Studies have shown that in the case of denture stomatitis, *C. albicans* was the most frequent species among patients (Shirtliff, Peters et al. 2009, Harriott and Noverr 2011, Tay 2014). Some studies have shown the ability of *Candida* to adhere and proliferate on different surfaces including: enamel surface, epithelial cells, acrylic surfaces, and surfaces created by microorganisms (biofilm) (O'Sullivan, Jenkinson et al. 2000, Darwazeh, Al-Refai et al. 2001). Various factors may influence the adhesion of *Candida* sp. on dentures such as salivary proteins and glycoproteins, low salivary pH, decrease in salivation, and oral bacteria (Chughtai, Naseer et al. 2013, Nasution 2013).

Although *Candida*-associated denture stomatitis is asymptomatic, this condition can act as reservoir for infections. Therefore the more effective treatment is a best way to control of the microbial plaque(Salerno, Pascale et al. 2011).

In addition, it has been shown that *Candida* sp. co-adheres with different species of oral bacteria, such as Streptococci (e.g., *S. gordonii*, *S. oralis, S. mutans*), thus increasing its potential colonization of dentures (Jenkinson HF 2002, Diaz, Xie et al. 2012, Nasution 2013).

1.6.2 Denture biofilm

Denture biofilm is a complex layer of microbial communities and their metabolites, which are organised in an extracellular polysaccharide matrix (Stoodley, Sauer et al. 2002). It has been reported that denture biofilm contains more than 10^{11} microorganisms per gram (Neppelenbroek 2015), including aerobic and anaerobic bacteria, yeasts, and amoebae (Glass, Conrad et al. 2010). Poor oral hygiene and denture cleaning increase the formation and accumulation of denture biofilm (Marchini, Tamashiro et al. 2004). Studies showed that oral biofilm formation is not only related to natural teeth but also the presence of a denture in the mouth provides conditions for the establishment and accumulation of microorganisms (Rocha, Luvizuto et al. 2008). This biofilm contains some pathogenic microorganisms which can produce toxins and metabolic waste. These toxins could promote the inflammatory process in denture stomatitis (Shulman, Rivera-Hidalgo et al. 2005, dos Santos, Hilgert et al. 2010, Glass, Conrad et al. 2010). Therefore, bacterial flora is suspected of being responsible for the development of denture stomatitis.

S. mutans plays an important role in the biofilm formation by providing binding sites for bacterial colonization on natural teeth and denture surfaces (Rocha, Luvizuto et al. 2008, Cho, Kim et al. 2015). This bacterium is considered the predominant species of denture plaque (Teles, Teles et al. 2012) and re-stablish on denture of edentulous patients after placement of denture (Mantzourani, Gilbert et al. 2010).

S. mutans, which is a common inhabitant of natural teeth and acrylic denture surfaces plays an important role in biofilm formation by providing binding sites for bacterial colonization and promotes adhesion of *Candida* sp. Studies show that *S. mutans* and *C. albicans* can promote binding to each other and the presence of sucrose favours this interaction through extracellular polysaccharides formed by *S. mutans* (Rocha, Luvizuto et al. 2008, Vasconcelos, Sampaio et al. 2010, Cho, Kim et al. 2015).

The association between *S. mutans* and *C. albicans* increases the effect of biofilm virulence by increasing biomass and production of exopolysaccharides (EPS) with *Candida*, and inducing the expression of virulence genes in *S. mutans*(Falsetta, Klein et al. 2014).

1.6.3 Trauma

Although this hypothesis seems to be recent, some authors in the past had already suspected that there was a link between mucosal trauma and stomatitis (Wright , Budtz-Jörgensen and Bertram 1970). Studies showed a relationship between instability of denture and denture stomatitis. They suggest that a lack of denture stability promotes the development of denture

stomatitis by application of strong forces in the mucosa and promoting localized ischemia and trauma (Webb, Thomas et al. 2005, Emami, de Grandmont et al. 2008, Pesee and Arpornsuwan 2015). A randomized controlled trial by Emami et al suggest that the risk of denture stomatitis was 4.5 fold in patients with lack of stability of lower denture compared to those with more stable dentures (Emami, de Grandmont et al. 2008). In this case, trauma could act as a co-factor that provides a situation for yeast to adhere and penetrate the tissue and induce inflammation and a pro-inflammatory response (Falgier, Kegley et al. 2011, Salerno, Pascale et al. 2011). Inflammation due to trauma could provide a favorable environment for bacterial and fungal colonization and induce denture stomatitis (Emami, de Grandmont et al. 2008).

I. Oral inflammation in DS

Oral inflammatory process is an interplay between inflammatory mediators and immune cells (Gurenlian 2009). Inflammatory mediators are messenger molecules that can positively or negatively impact the inflammatory process (Juul 2012). These mediators include adhesion molecules, growth factors, interferons (IFNs), and tumor necrosis factor (TNF). These mediators are released from different types of immune cells including mast cells, natural killer cells (NK), T cells, neutrophils and monocytes (Juul 2012).

An inflammatory response is used to protect body from injuries or infections (Gurenlian 2009) where the main signs of inflammation include redness, swelling, heat, and pain. These signs are indicative of underlying vasoactive and tissue injury processes. This protective mechanism

may induce tissue damage and results in chronic inflammation (e.g.) periodontitis, pulpitis, mucositis and stomatitis (McManus and Pinckard 2000).

The oral inflammatory response can be classified into two different types: acute or chronic inflammation and both probably play a role in DS and trauma (Li, Kolltveit et al. 2000, Hasturk, Kantarci et al. 2012).
An acute inflammation is a rapid process which responds to the site of injury by secretion of mediators, migration of neutrophils, and phagocytosis via monocytes-macrophages and PMNs (Hasturk, Kantarci et al. 2012, Buckley, Gilroy et al. 2014). A chronic inflammatory reaction is characterized by the presence of lymphocytes, macrophages, and fibrosis.

Neutrophils which are considered as the main effector cells of mucosal innate immunity in saliva of patients with *Candida*-related denture stomatitis, responds to fungal pathogens after activation by release of epithelial inflammatory mediators (cytokines) (Gasparoto, Vieira et al. 2009). Epithelial cells also recruit dendritic cells to present the processed antigens (fungal) to the T cells (Naglik, Moyes et al. 2011). Th1 and Th17 are considered to be the major cells for clearance of mucosal candidiasis by promoting the pro-inflammatory immune responses (Maciag, Mikolajczyk et al. 2016).

II. Inflammatory mediators in denture stomatitis

Many researchers have studied different inflammatory mediators in saliva and their role in denture stomatitis patients, however there is no consensus in their findings (Leigh, Steele et al. 2002, Barros, Al-Tarawneh et al. 2012, Pesee and Arpornsuwan 2015). Different sample types

and different methods used for study of cytokines in denture stomatitis patients may account for this lack of consensus. Samples include whole saliva (stimulated or unstimulated saliva) (Simunovic-Soskic, Pezelj-Ribaric et al. 2010, Šimunović-Šoškić, Pezelj-Ribarić et al. 2010, Gasparoto, Sipert et al. 2012), palatal secretion (Wilson, Wilton et al. 2007), blood (Pietruski, Pietruska et al. 2000), and serum(Kantardjiev and Popova 2002) . To make matters more complicated, different assay methods were used: direct (Šimunović-Šoškić, Pezelj-Ribarić et al. 2010) or sandwich (Pesee and Arpornsuwan 2015) Elisa, Quantikine Elisa kit (Pietruski, Pietruska et al. 2000), Fluorokine Map cytokine multiplex kits, and Bio-plex200 (Barros, Al-Tarawneh et al. 2012).

Since pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukins (IL) are important in the inflammation process many studies focused on these cytokines (Šimunović-Šoškić, Pezelj-Ribarić et al. 2010). Barros et al (Barros, Al-Tarawneh et al. 2012), studied different pro-inflammatory factors and they found that salivary levels of cytokines such as IL-1β and IL-8 increased in patients with Newton type II and III denture stomatitis. Since T lymphocytes are considered the major cells for clearance of mucosal candidiasis many studies focused on Th1, Th2, and Th17 cytokines (Maciag, Mikolajczyk et al. 2016). Some studies found no significant differences of Th1, Th2 (IFN-γ, IL-12, IL-4, IL-10), and Th17 (TNF-α, IL-6) between patients with and without denture stomatitis (Barros, Al-Tarawneh et al. 2012, Pesee and Arpornsuwan 2015). However, many studies found significant differences in the levels of Th17 cytokines (TNF-α, IL-6) in denture stomatitis patients (Pietruski, Pietruska et al. 2000, Šimunović-Šoškić, Pezelj-Ribarić et al. 2010, Gasparoto, Sipert et al.

2012). IL-2 which has a direct effect on T cell proliferation, showed no significant differences between denture stomatitis patients and healthy controls (Leigh, Steele et al. 2002)

1.7 Treatment of denture stomatitis

Many factors seem to be related to the origin of denture stomatitis and the etiology of this condition has not reached any consensus. Therefore, various treatments are used for the treatment of denture stomatitis including: antifungal, antibacterial, antiseptic agents, sterile denture by microwave exposure, use new denture, and treatment of tissues by laser (Maver-Biscanin, Mravak-Stipetic et al. 2004, Emami, de Grandmont et al. 2008, Neppelenbroek, Pavarina et al. 2008, Ribeiro, Pavarina et al. 2009, Abaci and Haliki-Uztan 2011, Glass, Conrad et al. 2011, Machado de Andrade, Cruz et al. 2012).

1.8 Methods and biological samples used for detection of microbial pathogens

Different methods and biological samples have been used to detect microbial pathogens in samples collected from patients with and without denture stomatitis.

These methods include: microbial culture analysis (Zomorodian, Haghighi et al. 2011, Altarawneh, Bencharit et al. 2013, Sanitá, de Oliveira Mima et al. 2013), basic PCR (Tay 2014) , and Real-Time PCR (White, Williams et al. 2004) .

Different biological samples were used for the study of DS include whole saliva (stimulated or unstimulated saliva) (Simunovic-Soskic, Pezelj-Ribaric et al. 2010, Šimunović-Šoškić,

Pezelj-Ribarić et al. 2010, Gasparoto, Sipert et al. 2012), palatal secretion (Wilson, Wilton et al. 2007), blood (Pietruski, Pietruska et al. 2000) , and serum(Kantardjiev and Popova 2002).

Many studies used fresh samples (Emami, Seguin et al. 2007, Altarawneh, Bencharit et al. 2013) and microbial culture analysis for the study of DS (Zomorodian, Haghighi et al. 2011, Altarawneh, Bencharit et al. 2013, Sanitá, de Oliveira Mima et al. 2013). However, analysis of frozen samples is also common for the study of inflammatory biomarkers (Barros, Al-Tarawneh et al. 2012, Gasparoto, de Oliveira et al. 2012). This is due to the necessity of saving biological samples for future analysis as biological samples may not always be processed immediately.

1.9 Rationale behind our work

There is no consensus concerning the etiological factors of DS. Although different possibilities have been proposed in the literature, strong links between suggested factors and denture stomatitis have not yet been fully demonstrated (dos Santos, Hilgert et al. 2010). However, the main etiological factors of DS have been reported to be fungal infections, denture biofilm, and trauma (Webb, Thomas et al. 1998). *C. albicans* has been suggested to be associated with denture-related stomatitis, however presence of this yeast in the development of this condition is not well understood and varies according to the different authors (Barbeau, Séguin et al. 2003, Emami, de Grandmont et al. 2008). The methodology of human sample collection is an important issue to decide what could be studied. Thus, in the literature, different samples used for the study of denture stomatitis include whole saliva (stimulated or

unstimulated saliva) (Simunovic-Soskic, Pezelj-Ribaric et al. 2010, Šimunović-Šoškić, Pezelj-Ribarić et al. 2010, Gasparoto, Sipert et al. 2012), palatal secretion (Wilson, Wilton et al. 2007), blood sample (Pietruski, Pietruska et al. 2000) , and serum(Kantardjiev and Popova 2002). As samples may not always be processed immediately, freezing for future analysis is common. Sometimes these samples can be kept in the freezer for years. Our main objective is to verify if long-term frozen samples from patients can still be used for microbiological analysis.

1.10 Hypotheses

Our hypothesis is as follows:

1. Frozen samples of denture sonicates can be used for cytokine detection and microbial analysis by PCR and culture.
2. There is a relationship between the presence of *Candida* sp. and *S. mutans* and denture stomatitis.

1.11 Objectives

This master's research project aims to:

a) To investigate the ability of *Candida* sp. to grow in specific media after long term storage

b) To investigate the relationship of *Candida* sp. and denture stomatitis

c) To test the prevalence of *S. mutans* in DS patients

d) To detect the value of IL1-β ,IFN-γ ,IL-6, and TNF-α in samples after long term storage

2 Material and Methods

2.1 Sample collection

Samples used for this study were collected by Katia Savignac (Savignac 2011) in 2008. A total number of one hundred fifteen sonicate samples were taken from 115 participants (males and females) over 64 years old and they were edentulous for a minimum of 5 years with complete dentures.

Samples we used for our investigation called denture biofilm sonicate and were prepared by Katia Savignac (Savignac 2011) in 2008 according to the protocol written by Emami (Emami, Seguin et al. 2007). After collecting denture from patients, dentures were rinsed off under running tap water and placed in two plastic bags (Ziploc®). First bag containing 30 ml sterile saline (0.85% NaCl) was inserted in a second bag and was sonicated for 5 minutes at room temperature in ultrasonic bath containing distilled water (Cole Parmer 26373, 50/60 Hz, 1.3 Amp) to remove microbial flora present on the denture. Denture sonicates were then transferred to a 50 ml sterile tube. A portion of collected samples was used for the clinical study of denture stomatitis in 2008 and the rest was kept frozen at -80° C until 2015.

2.2 Mycological investigation

Mycological investigation was performed twice: in 2008 and 2015. One hundred microliters of sonicates was transferred to BIGGY medium for the isolation of *Candida* sp. All cultures were

incubated at 37°C for 48 hours. When *Candida* sp. growth was observed, colonies were transferred to a specific growth medium (CHROMagar Candida, Paris, France) using filter paper and incubated for 24 hours at 37°C. In this medium different species of *Candida* develop a specific color.

2.3 Microbiological detection by PCR

C. albicans, *C. tropicalis*, and *S. mutans* were selected for evaluation of denture sonicates by polymerase chain reaction (PCR) techniques. For positive and negative controls pure cultures of each species and sterile DNase/RNase-free distilled water were used respectively. All bacterial and fungal strains and sequences of the PCR primers used in this study are listed with their respective references in Table 2.1.

2.3.1 Extraction of DNA

To extract DNA, 5ml of broth cultures and sonicate samples were used. Pure cultures of *C. albicans*, *C. tropicalis*, and *S. mutans* were used as controls. After centrifugation for 10 min at 16000 × *g*, the cell pellet was mixed with 200 µl lysis buffer (10mM Tris-HCl [PH8], 1mM EDTA, 100 mM NaCl, 1% sodium dodecyl sulfate, 2% Triton X-100), and proteinase K (Sigma-Aldrich, St. Louis, MO) to a final concentration of 20 mg/ml.

This mixture was incubated for 20 min at 56°C. Then, 200 µl DNase/RNase free distilled water, 0.3 g Ottawa Sand (Thermo Fisher Scientific), and 200 µl phenol-chloroform-isoamyl alcohol (25:24:1) (Invitrogen™, Life Technologies Co.) were added and the mixture was

vortexed for 3 min and centrifuged for 5 min. After centrifugation for 5 min at 16000 × *g*, 1 µl of 10 µg/ml RNase A (Sigma-Aldrich) was added to the supernatant and incubated for 15 min at 56°C. This followed by 3 times phenol-chloroform-isoamyl alcohol (25:24:1) (Invitrogen™, Life Technologies Co.) extraction procedure and 5 minutes centrifugation at 16000× *g* each time.

One millilitre of ethanol (100%) (Thermo Fisher Scientific), and 3 µl Glycogen (Invitrogen™, Life Technologies Co.) were added to the aqueous phase followed by an overnight incubation at -20 ºC to precipitate DNA.

Following incubation, centrifugation was performed for 20 minutes at 16 000 × *g*. The pellet was washed 2 times with ethanol (70%) (Thermo Fisher Scientific), and recentrifuged 15 minutes at the same speed used previously. Finally, the pellet was dried under a laminar flow hood and dissolved in 25 µl TE buffer (10 mM Tris-HCl [pH8], 1 mM EDTA). The DNA was quantified by using the ratio 260/280 nm with a spectrophotometer (Thermo Fisher Scientific, NanoDrop 2000/2000c, Wilmington, USA). To ensure that the DNA was not degraded, a 1% agarose gel electrophoresis migration (Sigma-Aldrich) containing Tris-acetate -EDTA buffer (TAE) (Sigma-Aldrich) and ethidium bromide (Invitrogen™, Life Technologies Co.) was performed.

2.3.2 PCR reaction

The PCR amplification procedures were accomplished as described in the related articles (Bu, Sathiapalan et al. 2005, Psoter, Ge et al. 2011) used for primer sequences (Table 2-1) with some modification by gradient PCR. The PCR reagents for all the targeted microorganisms included a Taq DNA Polymerase with 1x standard Taq buffer (New England Biolabs Inc.) containing $MgCl_2$ (10mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, pH of 8.3 at 25°C), and 0.2 mM dNTPs (Invitrogen™, Life Technologies Co.) were used. All PCR amplification procedures were performed in a thermal cycler (Bio-Rad Laboratories, Inc.) For each bacterial and fungal strain 3 µl (100 ng) of purified DNA of each sample was added to the PCR mixture.

The final volume of each PCR reaction mix of 25 µl for all three microorganisms contained 2.5 U of Taq DNA polymerase, 1x buffer containing 1.5 mM of $MgCl_2$, 0.2 mM of each dNTP, and 0.4 µM concentration of each primer.

The PCR conditions for *C. albicans* included the initial denaturation process at 95°C (5 min), followed by 30 cycles (95°C for 1 min, 55°C for 1 min, 72°C for 1 min and 29 s, 72°C for 7 min). The PCR amplification conditions for *C. tropicalis* were 95°C (5 min), followed by 30 cycles (95°C for 1 min, 61 °C for 1 min , 72°C for 1 min and 29 s, 72°C for 7 min). The conditions for *S. mutans* were 95°C (5 min), and then 30 cycles (95°C for 15 s, 56°C for 30 s, 72°C for 1 min, 72°C extension for 7 min). All PCR reactions were kept at 6°C before undertaking the PCR reaction.

2.3.3 Electrophoresis on agarose gel

PCR amplified products were analyzed by 1.5% agarose gel electrophoresis (Sigma-Aldrich, St.) stained with ethidium bromide (Invitrogen™, Life Technologies Co.) using TAE (Tris Acetate-EDTA buffer) electrophoresis buffer (Sigma-Aldrich)

Ten microliters of PCR amplified material was loaded on each gel with Orange G DNA loading buffer (Sigma-Aldrich) The DNA ladder 100 bp (Invitrogen™, Life Technologies Co.) was used for agarose gel electrophoresis. The gels were electrophoresed at 90 V for 45-minutes and visualized under UV light with an Alpha Imager HP System™ (ProteinSimple Inc., Santa Clara, USA). The gel images were recorded by a Fluorchem 8900™ (Imager ProteinSimple Inc., Santa Clara, USA).

2.3.4 Quantitative evaluation

To determine the minimum number of cells detected by PCR for each microorganism, a quantitative method was used. To find the minimum detection level, ten serial dilutions were prepared from pure cultures of each species diluted in distilled water. After extracting DNA from 5 ml of each dilution, the concentration of extracted DNA was read by spectrophotometer (Thermo Fisher Scientific-NanoDrop 2000/2000c, Wilmington, USA) at OD 260 nm. The extracted DNA of each dilution was amplified by PCR at conditions explained above for each species. Finally, we established a standard curve for each species to find the minimum detectable concentration by PCR.

To estimate the minimum number of cells that could be detected by PCR, colony counting of serial dilutions used for each species. To do this, 100 µl of each prepared dilution were transferred to TYE and TSA media for *S. mutans* and *Candida* sp. respectively, and only plates that had between 30 to 300 colonies were counted. According to the intensity of the specific band on an agarose gel achieved by PCR amplification, samples were categorized into two groups: positive samples with a visible PCR band and negative samples with no detectable PCR band.

Table 2-1 Primer sequences for selected bacterial and fungal species

BACTERIAL AND FUNGAL SPECIES	DNA PROBE STRAINS	PRIMER SEQUENCES (5′→3′)	AMPLICON SIZE (BP)
Candida albicans **(Bu, Sathiapalan et al. 2005)**	SC5314	Forward: TTTATCAACTTGTCACACCAGA Reverse: ATCCCGCCTTACCACTACCG	273
Candida tropicalis **(Bu, Sathiapalan et al. 2005)**	LSPQ	Forward: CAATCCTACCGCCAGAGGTTAT Reverse: TGGCCACTAGCAAAATAAGCGT	357
Streptococcus mutans **(Psoter, Ge et al. 2011)**	NCTC10449	Forward: TCG CGA AAA AGA TAA ACA AAC A Reverse: GCC CCT TCA CAG TTG GTT AG	479

2.4 Cytokine detection

The presence of IL-1β, IFN-γ, IL-6, and TNF-α in denture sonicate samples were assessed by enzyme-linked immunosorbent assay (ELISA). All tests were completed in duplicates and repeated two times.

IFN-γ (ProSci Incorporate, California, USA) was assayed using a biotinylated detection antibody (Thermo Fisher Scientific, Wilmington, USA), and IL-1β (Sigma-Aldrich, St. Louis, MO) was assayed using a commercial capture and detection antibodies (Sigma-Aldrich, St. Louis, MO). Human recombinant cytokines (IFN-γ and IL-1β) diluted in phosphate-buffered saline (PBS) was used as standard. Assays were performed in (EIA)/A2 96-well plates (Costar, Cambridge, MA) according to manufacturer's instructions and results were expressed in nanogram per milliliter.

Plates were incubated with a capture antibody (1 μg/ml) in a coating buffer (NaCl, Na_2HPO_4, NaH_2PO_4, H_2O) overnight at 4°C. The plates were washed 3 times with washing buffer (0.05% Tween-20, PBS) and coated with a blocking buffer containing 0.05% Tween 20, PBS and 1% bovine serum albumin (BSA) for 2 h at 37°C. The plates were then washed again 3 times with washing buffer and the human recombinant standard was diluted in PBS and 100 μl samples were added to each well in duplicate and incubated for 2 h at 37°C.

Following this incubation, plates were washed 3 times with washing buffer, and a biotinylated detection antibody (0.5 mg/ml) was added to each well and incubated for 2 h at 37°C. The

plates were washed 3 times after each step, and following these several washes, streptavidin-peroxidase diluted to 1:50000 (Sigma-Aldrich) was added to each well. After 30 min at 37°C the plates were emptied without washing and 200 µl of the OPD (o-phenylenediamine-dihydrochloride) peroxidase substrate (Sigma-Aldrich) was added to each well and incubated in the dark for 30 minutes at room temperature. Finally, the optical densities (OD) were read with an AD 340 plate reader (Beckman Coulter, Inc. California, USA) at 450 nm. Cytokine concentrations of each sample were quantified with the standard curve obtained from human recombinant standard diluted in PBS.

Forty sonicated samples (500 µl) including 21 healthy and 19 denture stomatitis including Newton type 2 and 3 were sent to the BioLegend company (BioLegend, San Jose, CA, USA) for detection of IL-6 and TNF-α.This company used bead-based sandwich assays (LEGENDplex Human Th Cytokine kit) and the tests were performed in duplicate.

2.5 Statistical analysis

Pearson Chi - square and Fisher's exact test (two- sided) were used to analyze the frequency of *C. abicans*, *C. tropicalis*, and *S. mutans* and their relation to denture stomatitis. McNemar tests were used to compare the number of *Candida* sp. able to grow before and after freezing, and to compare results collected by PCR (2015) and microbial culture (2008) for *Candida* species. Logistic regression analysis was performed to identify association between *S. mutans* and *Candida* species. The statistical significance level was established at $P \leq 0.05$. All analyses were performed with SPSS version 24 (SPSS Inc., Chicago, IL, USA).

3 Results

3.1 Demographics

A total number of one hundred fifteen clinical samples including both men and women participants were used for this study. These samples were collected by Katia Savignac (Savignac 2011) in 2008. The mean age of the sample population was 72 (SD ± 4) years. In this study DS Newton type I and healthy subjects with no signs or symptoms of denture stomatitis were put in the same group (n=72) and DS Newton type II and III in the DS group (n=42). Table 3.1 shows the distribution of each different type of sample.

Table 3-1 The classification of study subject according to denture stomatitis diagnosis

Denture stomatitis n=42		Non-denture stomatitis n=72	
Newton type II	Newton type III	Healthy	Newton type I
38	4	14	58

3.2 Microbiological culture before and after long term storage

The results from microbiological analysis of denture sonicates in 2008 (Savignac 2011) showed that 39 denture sonicate samples including DS and healthy were *Candida* carriers for *C. albicans*, *C. tropicalis*, and/or *C. krusei*. The cultures were negative in 76 samples including healthy and denture stomatitis subjects (Table 3.2). *C. albicans* was the most frequent species isolated by culture from 39 dentures sonicate samples (64%). Frequencies of *C. tropicalis* and *C. krusei* from denture sonicate samples were 10% and 33% respectively (Savignac 2011).

In 2015 the microbial culture was repeated on 39 denture sonicate samples in which *Candida* sp. was detected in 2008. *C. albicans* was able to grow only in 5 of these samples (12%). The number of *C. albicans* able to grow before freezing was statistically significant compared to the number grew after freezing (p=0.004). *C. tropicalis* and *C. kreusei* were unable to be cultivated after thawing samples (Figure 3.1).

Table 3-2 The comparison of the analyzed sonicate samples (total n=115) according to the presence of *candida* species before and after 7years of freeze

	Before freezing (2008) n =115		After freezing (2015) n=115	
	DS n= 43	Non-DS n= 72	DS n= 43	Non-DS n= 72
C. albicans	13	12	3	2
C. tropicalis	2	2	0	0
C. krusei	4	9	0	0

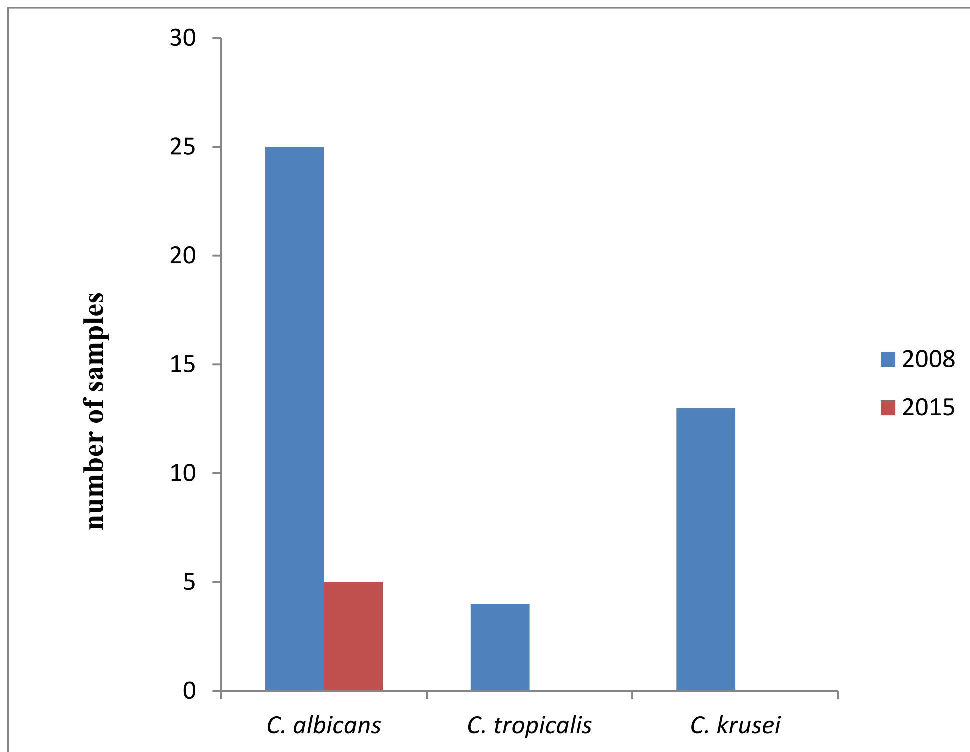


Figure 3-1 The comparison of the analyzed sonicate samples (total n= 39) according to the presence of *candida* species before and after 7 years of freeze.

3.3 PCR detection level

C. albicans, *C. tropicalis*, and *S. mutans* were evaluated by PCR in all sonicates. Analysis was performed twice for each sample and was repeated when differences in collected results were observed.

The sensitivity of PCR was tested by using a known number of cells of *C. albicans*, *C. tropicalis* and *S. mutans*. The DNA extracted from serially diluted cell suspensions of pure microorganisms (3 µl each) was used as a control for each species. The minimum detection level was found to be 337 fg fungal genomic DNA, corresponding to approximately 16 cells for *C. albicans*, and 97 fg fungal genomic DNA, around 11 cells, for *C. tropicalis.* Sensitivity of PCR for *S. mutans* was 117 fg bacterial genomic DNA, about 2000 cells.

Figure 3.2 demonstrates the example of PCR product for *C. tropicalis* where a band was detected down to approximately 10 cells (line 8).

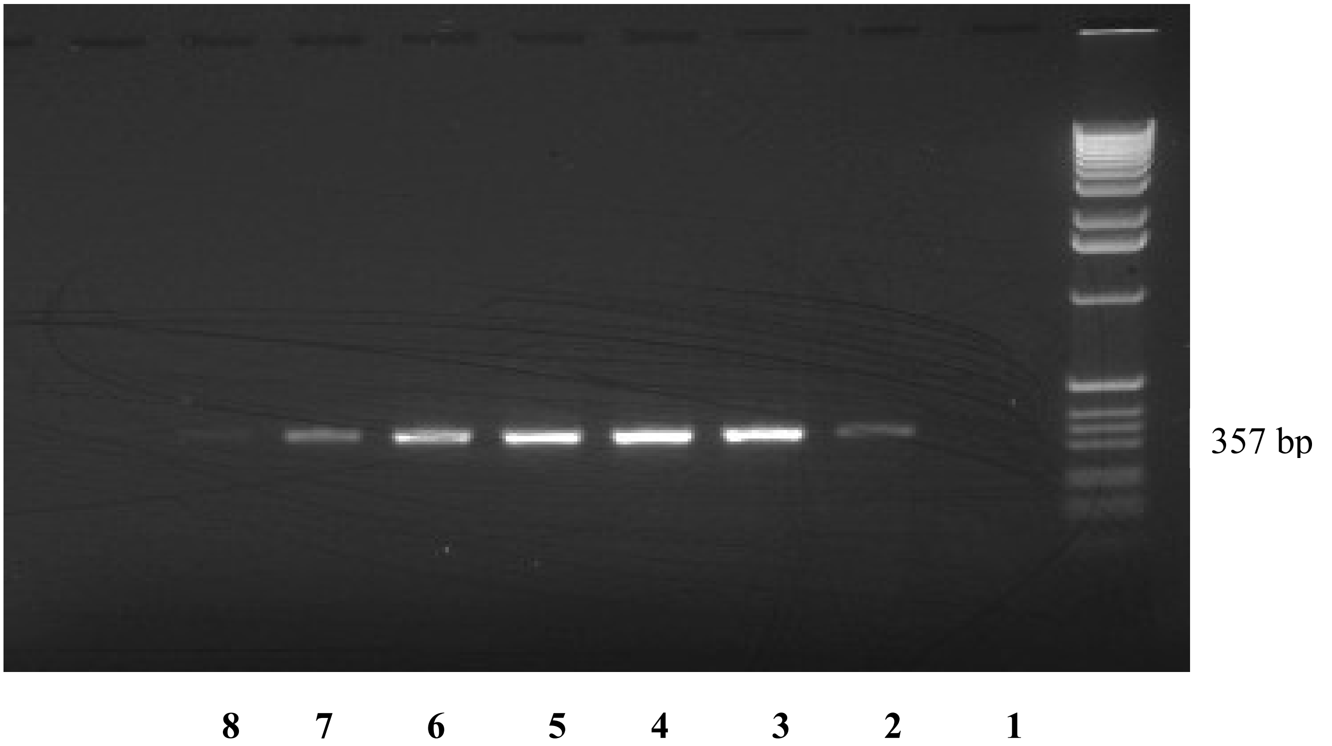


Figure 3-2 Example of quantitative PCR assessment of *C. tropicalis*

Line 3 to 8 show the number of cells
1=Negative control, **2**= 91fg DNA of *C. tropicalis*
3= 94×10^4 fg, **4**=94×10^3 fg, **5**=94×10^2 fg, **6**= 94×10^1 fg, **7**=94 fg, **8**=9.4 fg

3.4 PCR analysis

As mentioned above we considered healthy and denture stomatitis Newton's type I patients as the non-stomatitis or healthy group and DS Newton's type II and III as the denture stomatitis group. Our results showed that the prevalence of *C. albicans* (C. a.), and *C. tropicalis* (C. t.), was statistically significant in denture stomatitis (30.2% and 51.1% respectively) compared to healthy subjects (C. a. P = 0.001, and C. t.: P = 0.008). The result also showed that the prevalence of *S. mutans* was significantly higher in subjects with denture stomatitis compared with healthy samples (P=0.019). In denture stomatitis samples *S. mutans* was found in 18.6% but in non-denture stomatitis it was detected in only 4.1% of samples (Table 3.3).

Table 3-3 Comparison of sonicates samples according to the percentage of detected *Candida* species by PCR.

	Non-denture stomatitis (n=72)	**Denture stomatitis (n=43)**	**P-value***
C. albicans	6.9%	30.2%	0.001
C. tropicalis	25%	51.1%	0.008
S. mutans	4.1%	18.6%	0.019

* All P values calculated using Fisher's exact tests.

3.5 Association between *S. mutans* and *Candida* species

Statistical analysis was done for results collected by PCR to find any association between presence of *S. mutans* and *Candida* species and their effect on denture stomatitis (Table3.4).

The statistical analysis showed that there is no association between *S. mutans* and *C. tropicalis* (P=0.27) or *C. albicans* (P=0.815).

Table 3-4 Number of samples including both *S .mutans* and *Candida* species

	Non-denture stomatitis (n=72)	Denture stomatitis (n=43)	P-value*
Samples including both *C. albicans* and *S. mutans*	1	5	0.815
Samples including both *C. tropicalis* and *S. mutans*	3	7	0.27

* All P values calculated using Fisher's exact tests.

3.6 Comparing microbial culture and PCR for detecting *Candida* species

Comparison was done between results of microbial culture collected in fresh samples in 2008 (Savignac 2011) and PCR (2015) for *C. tropicalis* and *C. albicans*. In both DS and non-denture stomatitis samples (n=115), *C. tropicalis* was detected in only 4 samples with microbial culture (Savignac 2011) , while it was detected in 40 samples with PCR. Statistical analysis shows that there was a statistically significant difference between the PCR and microbial culture for *C. tropicalis* (P=0.001). Therefore, the number of samples positive for *C. tropicalis* with PCR was significantly higher when compared to microbial culture results (Table 3.5).

The results show no statistical differences (P>0.05) between the results collected by PCR and those from culture method for detection of *C. albicans*. *C. albicans* was detected in 13 denture stomatitis samples with both methods. However, the number of non-DS samples in which *C. albicans* was detected with PCR and microbial culture were 5 and 12 respectively (Figure3.3).

Fifteen samples were positive for *C. albicans* with culture while those samples were negative with PCR. However, eight samples were positive only with PCR (Table 3.6).

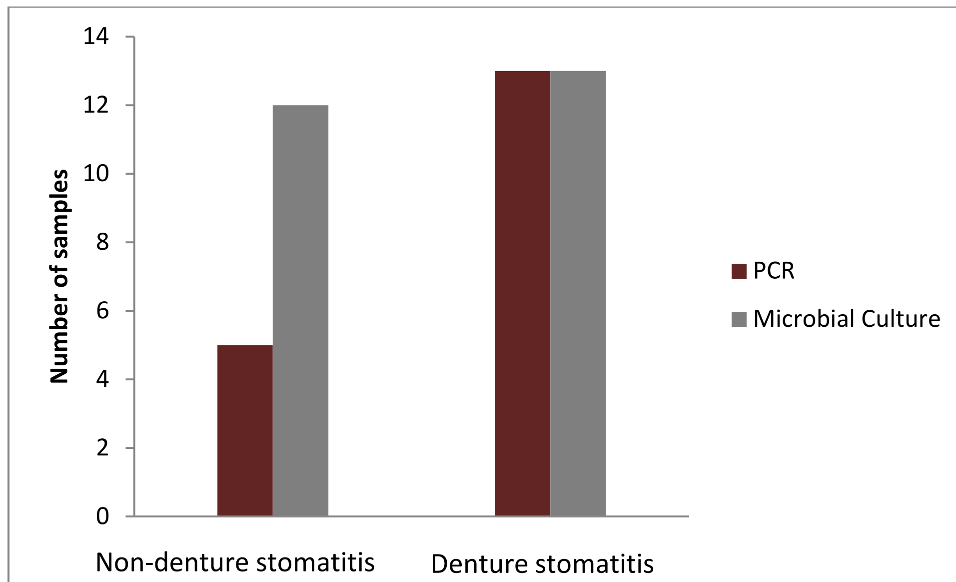


Figure 3-3 Number of samples *C. albicans* found in denture plaque with microbial culture (2008) and PCR (2015) in DS and non-denture stomatitis group

Table 3-5 Comparison of the collected results from microbial culture (2008) and PCR (2016) for detection of *C. tropicalis* in denture stomatitis and non-denture stomatitis for 7-year freeze samples

	Denture stomatitis N= 43	Non-denture stomatitis N= 72
Number of samples positive for *C. tropicalis* with PCR (2015)	22	18
Number of samples positive for *C. tropicalis* with microbial culture (2008)(Savignac 2011)	2	2

Table 3-6 Comparison of the collected results from microbial culture (2008) and PCR (2016) for detection of *C. albicans* in denture stomatitis and non-denture stomatitis for 7-year freeze samples

	Denture Stomatitis n=43	Non-denture stomatitis n=72
Number of samples positive for *C. albicans* with PCR (2015)	13	5
Number of samples positive for *C. albicans* with microbial culture (2008)(Savignac 2011)	13	12
Number of samples positive for *C. albicans* with both methods	7	3

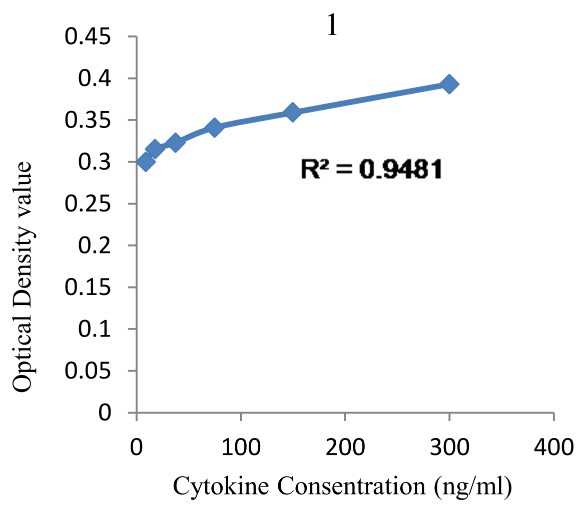
3.7 Cytokine detection in healthy and denture stomatitis subjects

We tested the hypothesis that frozen denture sonicate samples can be used for detection of selected cytokines such as INF-γ, IL-1β, IL-6, and TNFα. Analysis was done in duplicate, repeated 2 times, and a standard curve was prepared as illustrated in figure 3.4. Detection of INF-γ and IL1-β in all samples including healthy and denture stomatitis was done in our laboratory using Sandwich ELISA. The minimum detection level was ≥ 2 ng/ml and ≥ 20 ng/ml for INF-γ and IL-1β respectively (Table 3.7). Concentration of both cytokines was under minimum detection levels in all samples (n=115).

Forty samples including 21 non-denture stomatitis and 19 denture stomatitis were sent to BioLegend for analysis of TNF-α and IL-6 using bead-based sandwich assays. Concentrations were expressed in pg/ml protein and the minimum detection levels of TNF-α and IL-6 was ≥ 1.63 pg/ml and ≥ 0.59 pg/ml respectively (Table 3.7). Results showed that in all 40 samples the concentration of TNF-α and IL-6 was under minimum detection levels.

Table 3-7 The Cytokines range of detection levels in sonicates samples (n=115) for IL-1β INF-γ and n=40 for TNF-α , IL-6

Cytokine	Minimum and maximum Detactable Concentration
TNF-α n =40	1.63- 17587 pg/ml
IL-6 n =40	0.59- 36329 pg/ml
IL-1β n =115	20- 200 ng/ml
INF-γ n=115	2- 300 ng/ml



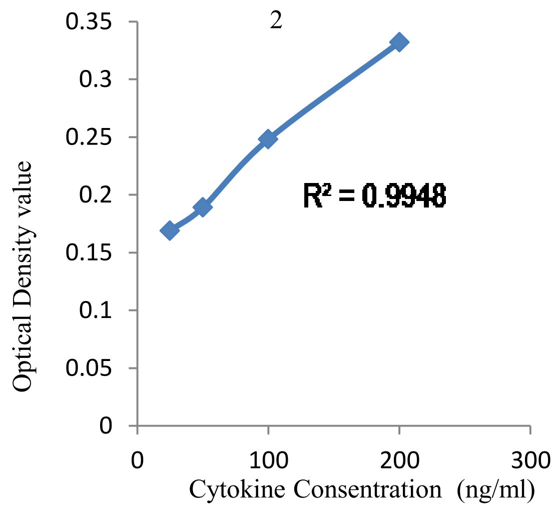


Figure 3-4 Standard curves for INF-γ (1), and IL-1β (2)

R^2 (coefficient of determination)

4 Discussion

Denture stomatitis is an inflammatory condition of the oral mucosa which affects the palate of edentulous patients wearing partial or complete dentures. Many factors have been reported to be associated with this disease. Despite numerous studies, there is no consensus on the etiology of this condition. The source of inflammation in denture stomatitis is still a matter of debate. The main cause of this condition has been linked to the presence of the *C. albicans.* However, trauma due to unstable dentures was found to have a significant role (Barbeau, Séguin et al. 2003, Emami, de Grandmont et al. 2008). In addition, microbial biofilms present on the denture can activate an immune response and cytokine secretion that causes inflammation (Nett 2016). Denture biofilms are mixed microbial communities where indigenous oral bacteria and yeast probably interact (Cavalcanti, Morse et al. 2015, Allison, Willems et al. 2016). There is considerable evidence supporting a role of *S. mutans* in increasing *C. albicans* virulence (Jenkinson and Douglas 2002, Altarawneh, Bencharit et al. 2013, Brusca, Irastorza et al. 2013, Falsetta, Klein et al. 2014).

We compared the results collected in 2008 in a clinical study (Savignac 2011) with our results in order to test our hypothesis that frozen samples of denture sonicate could be used for microbial analysis by PCR and culture. We further examined the potential association of *C. albicans*, *C. tropicalis*, *S. mutans*, and inflammation mediators with denture stomatitis in clinical samples collected in 2008 and stored at -80° C until 2015.

4.1 Prevalence of Candidiasis

The prevalence of *C. albicans* and *C. tropicalis* was evaluated in samples with and without denture stomatitis. We found that *C. tropicalis* (51.1%) was more prevalent than *C. albicans* (30.2%). However, many studies suggest that *C. albicans* is the major *Candida* isolated from the mucosa of patients with denture stomatitis compared to other types of *Candida* (Ramage, Tomsett et al. 2004, Daniluk, Tokajuk et al. 2006, Abaci, Haliki-Uztan et al. 2010, Altarawneh, Bencharit et al. 2013, Tay 2014).

The difference between our results and other studies could be explained by several reasons. The most important are differences between sample types and methods used for the study of DS. Many studies used stimulated or unstimulated whole saliva (Simunovic-Soskic, Pezelj-Ribaric et al. 2010, Šimunović-Šoškić, Pezelj-Ribarić et al. 2010, Gasparoto, Sipert et al. 2012), blood (Pietruski, Pietruska et al. 2000), serum(Kantardjiev and Popova 2002), swab from denture or mucosal surface (Altarawneh, Bencharit et al. 2013), and palatal secretion (Wilson, Wilton et al. 2007) while we used denture sonicate for the current study. Also, different detection methods were used such as microbial culture analysis (Zomorodian, Haghighi et al. 2011, Altarawneh, Bencharit et al. 2013, Sanitá, de Oliveira Mima et al. 2013), basic PCR (Tay 2014) , and Real-Time PCR(White, Williams et al. 2004) to detect *Candida* sp. In addition, different dietary habits and composition of saliva could affect the sustainability and ability of *Candida* sp. to adhere to an acrylic surface (Jin, Samaranayake et al. 2004, Pereira-Cenci, Cury et al. 2007, Pereira-Cenci, Del Bel Cury et al. 2008).

Surface roughness is also another factor that could affect the adherence of *Candida* to the surface of dentures. Studies dealing with the influence of surface roughness on *Candida* species found variable adherence levels to denture surfaces (Moura, da Silva et al. 2006, Pereira-Cenci, Cury et al. 2007). The rough surface compared to smooth surface of denture shows greater adhesion of *Candida* sp.(Pereira-Cenci, Cury et al. 2007).

4.2 Comparing results of 2008 and 2015

Collecting samples in a clinical study is not an easy process owing to the ethical committee requirement and patient recruitment. Thus, one must optimize the clinical samples not only for short term but also potentially for long term studies where new hypotheses can be generated and tested. Therefore, we compared results collected in 2008 (Savignac 2011) with our result in 2015 on the same samples. Clinical samples were collected in 2008 by Katia Savignac (Savignac 2011) from 115 patients with and without denture stomatitis. In 2008, the microbial analysis was done using culture media to detect *Candida* species and the remaining denture sonicates were kept frozen at -80 °C.

Collected results of microbial cultures in 2008 and 2015 on the same samples have shown that there is a major loss of viability of *Candida* species after long term freezing at -80°C. This was not entirely unexpected as our samples stored only in sterile saline. It has been reported that viability of *Candida* sp. drops significantly in buffer and water during freezing (Espinel-Ingroff, Montero et al. 2004).

Studies report addition of 10% glycerol before freezing the samples, minimize the formation of ice crystals in cells and increasing their recovery after freezing (Crespo, Abarca et al. 2000, Mariano, Gonçalves et al. 2007).

We used PCR in our study to compare with the results of fresh culture obtained in 2008. Our results showed striking differences between PCR and the culture method (2008) for the presence of *C. albicans* and more specifically *C. tropicalis.* The number of samples in which *C. tropicalis* was detected by PCR (34.7%) was significantly higher than what was found in 2008 with fresh culture (3.4%). As for *C. albicans*, the results obtained between PCR and fresh (2008) culture did not show a significant difference for DS samples. However, the number of samples of non-DS stomatitis (DS I and healthy patients) was found to be higher with microbial culture. *C. albicans* was detected positively only in 10 samples with both PCR and the culture method. However, it was positive in 8 samples only with PCR and in 15 samples only with culture method.

The PCR reaction is more sensitive and accurate than culture: PCR can detect both live and dead cells, as well as DNA released in the environment (Wahyuningsih, Freisleben et al. 2000, Pan and Breidt 2007, Àlvarez, González et al. 2013). Comparing PCR and culture, other authors also found sometimes weak similarity between culture and PCR (Mardh, Novikova et al. 2003, Levy, Fournier et al. 2013).

For those samples where *Candida* was not detected with PCR it is possible that the DNA was degraded or remaining cells were less than the minimum detection level. In addition, any potential PCR inhibitors in clinical samples could interfere with detection.

The higher presence of *C. tropicalis* in our analysis is interesting. Although *C. albicans* is considered as main cause of *Candida* related disease, recently a shift was observed toward *non-albicans* species (Serrano-Granger, Cerero-Lapiedra et al. 2005, Pereira-Cenci, Del Bel Cury et al. 2008). There is evidence that *Candida* is able to attach to the surface of acrylic dentures with the presence of different factors including surface charge, surface free energy, hydrophobicity, roughness, salivary composition and secretion rates, and antibody titers (Serrano-Granger, Cerero-Lapiedra et al. 2005, Moura, da Silva et al. 2006, Pereira-Cenci, Cury et al. 2007, Pereira-Cenci, Del Bel Cury et al. 2008). *C. tropicalis* compared to *C. albicans* has higher tendency to attach to the denture surface which might be due to its surface free energy values and hydrophobicity(Serrano-Granger, Cerero-Lapiedra et al. 2005).

4.3 Association of *S. mutans* with *Candida* sp. and DS

Recently many studies focused on fungal–bacterial interactions and how this relationship can modulate fungal virulence, pathogenesis, and resistance to anti-fungals.

Candida co-adhered with many oral bacteria including the viridans *Streptococci* group (Jenkinson HF 2002, Baena-Monroy, Moreno-Maldonado et al. 2004, Peters, Jabra-Rizk et al. 2010, Diaz, Xie et al. 2012). Our PCR reaction is suitable for the detection of *S. mutans* in frozen samples.

S. mutans was detected more often in DS samples as compared to non-DS, a finding found in the literature (Baena-Monroy, Moreno-Maldonado et al. 2005). However, our study did not find any association between *S. mutans* and *Candida* species. This can be due to reduced sensitivity of the PCR, sample type, and long term storage of samples (7 years).
Our results are in agreement with the hypotheses that appear to be association between *S. mutans* and denture stomatitis. However the sample size of our study is too small to draw any strong conclusions.

4.4 Inflammation mediators and their role in DS

According to the literature IL-1β, IL-6, IFN-γ, and TNF-α have been detected in patients with denture stomatitis (Pietruski, Pietruska et al. 2000, Simunovic-Soskic, Pezelj-Ribaric et al. 2010, Barros, Al-Tarawneh et al. 2012) . We wanted to test the hypothesis that our frozen samples could be used to detect and quantify selected cytokines. Detection was done with sandwich Elisa in our laboratory and with the Biolegend Company. None of our samples tested positive for the selected cytokines.

Different factors can affect the reliability of cytokine level measurements in clinical samples collected including the timing of sampling, sample handling, storage, the choice of stimulated or unstimulated whole saliva, plasma or serum, and techniques used for the detection of cytokines (Pietruski, Pietruska et al. 2000, de Jager, Bourcier et al. 2009, Simunovic-Soskic, Pezelj-Ribaric et al. 2010, Barros, Al-Tarawneh et al. 2012, Pesee and Arpornsuwan 2015, Pinke, Freitas et al. 2016).

Cytokines should be stored at -80 °C for the long term although these biomarkers start to degrade even at this temperature after 2 years (de Jager, Bourcier et al. 2009, Zhou, Fragala et al. 2010, Wang, Zhu et al. 2015). Therefore, we suggest that cytokines should be measured as soon as possible (within 1 hour) and avoid freezing and thawing cycles because many cytokines have short half-lives and start to degrade after collection (de Jager, Bourcier et al. 2009, Zhou, Fragala et al. 2010).

4.5 Limitation of study

Several limitation factors exist in this study. First, samples were collected and stored 7 years before our study and long-time storage may affect the quality and reliability of results. Secondly, a real-time quantitative PCR technique (qPCR) is more accurate and sensitive to detect the selected pathogens compare to standard PCR (Psoter, Ge et al. 2011). Thirdly, our samples were collected in saline without glycerol, a condition that must have reduced the possibility to detect live bacteria and cytokines (Crespo, Abarca et al. 2000, Mariano, Gonçalves et al. 2007). Fourth, during sample collection dentures were rinsed off under running tap water, which raised the possibility that the cytokines may have rinsed off.

4.6 Future study

For future studies glycerol and distilled water should be added to samples to maintain the viability of microorganisms. Also, DNA should be extracted first, and then stored in TE buffer at -80°C for long term storage. For study of cytokines, stimulated or unstimulated whole saliva must be used and it is better to do the analysis as soon as possible and should not be stored for more than 2 years.

5 Conclusion

In this study we set out to investigate the possibility of using clinical frozen samples of denture sonicate for detection of *Candida* species and its association with denture stomatitis by using PCR and culture methods after 7 years of storage. We also examined the possibility of detecting of cytokines in frozen samples.

Within the limitation of this study, the result shows that *Candida* species lose their capability to grow in specific media after long term of storage. There is an association between denture stomatitis and detection of *C. albicans*, *C. tropicalis,* and *S. mutans.* However, no correlation was found between *S. mutans* and *Candida* species. The results also show that the prevalence of *C. tropicalis* is more common in DS compare to *C. albicans.* Cytokines was not detected in all samples.

6 Recommendation

Concluding from the data we present here in this study, we suggest storing samples in distilled water and glycerol at -80°C for longer storage to increase cell viability. Furthermore, for DNA analysis we recommend extracting DNA and storing it in TE buffer before transferring samples at -80°C for long term storage. Also, we suggest to use stimulated or unstimulated whole saliva instead of denture sonicate for cytokines analysis, process samples as soon as possible (within an hour), and avoid freezing and thawing cycles.

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