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A comparison between DNA-DNA checkerboard hybridization and culture techniques for the detection of *Candida* species in denture stomatitis

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Ce mémoire intitulé:

A comparison between DNA-DNA checkerboard hybridization and culture techniques for the detection of *Candida* species in denture stomatitis

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A été évalué par un jury composé des personnes suivantes:

Dre Gisèle Mainville, présidente-rapporteur
Dre Elham Emami, directrice de recherche
Dr Jean Barbeau, co-directeur de recherche
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RÉSUMÉ

Introduction

Selon la littérature, les évidences sur l'utilisation et l'application potentielles de la technique d'hybridation à damier d'ADN-ADN dans le diagnostic de la stomatite prothétique associée à la *Candida* (DS) sont limitées. En outre, la littérature suggère que les biomarqueurs inflammatoires de la salive pourraient offrir une nouvelle avenue pour le diagnostic précoce de cette maladie.

Objectifs

Les objectifs de ce projet de recherche de maîtrise étaient les suivants: 1) Fournir des informations sur la précision diagnostique de la culture conventionnelle et de la technique d'hybridation à damier d'ADN-ADN pour la détection d'espèces de *Candida* dans DS et d'étudier son impact sur le diagnostic clinique de cette maladie, et 2) Examiner systématiquement les données disponibles sur les biomarqueurs salivaires présents dans DS.

Méthodes

Objectif 1): Le biofilm palatin de 26 participants diagnostiqués avec DS a été analysé pour détecter et quantifier les espèces de *Candida* en utilisant des techniques d'hybridation à damier d'ADN et d'ADN-ADN. En utilisant chaque technique comme référence standard pour l'autre, la précision diagnostique des deux techniques a été examinée et comparée à l'aide des tests Kappa et McNemar. Le test de Spearman a été utilisé pour examiner l'association entre la quantité totale de *Candida* et les scores d'inflammation totale.

Objectif 2): La revue systématique a suivi les lignes directrices relatives aux rapports systématiques et aux méta-analyses (PRISMA). Le niveau de preuve a été évalué à l'aide de

l'échelle 2011 du centre d'Oxford pour la médecine fondée sur des preuves (OCEBM). La qualité méthodologique a été évaluée à l'aide de la déclaration du renforcement des rapports d'études observationnelles en épidémiologie (STROBE) et classée selon l'échelle d'Olmos.

Résultats

Objectif 1): Pour toutes les espèces de *Candida*, la spécificité de la technique de culture variait entre 52% et 88,5% et entre 92,9% à 100% pour le damier. Il y avait un désaccord entre les deux techniques. La sensitivité pour les deux techniques a été observée comme nulle pour toutes les espèces. La corrélation entre *Candida* et les scores d'inflammation n'a pas été statistiquement significative pour la culture, mais une corrélation statistiquement significative a été observée avec la technique du damier ($p = 0,05$).

Objectif 2): La majorité des études incluses dans la revue systématique ont montré que les niveaux d'IL-6, CCL3 et TGF-β, GM-CSF et TNF-α étaient plus élevés chez les personnes âgées atteintes de DS, comparativement aux plus jeunes ou individus sains ($p <0,05$). Quelques études ont toutefois observé une différence non statistiquement significative dans les niveaux de la plupart des cytokines salivaires (IL2, IL12, IFN-Ɣ, IL-4, IL-8, IL-10, IL-17, TNF-α et ICAM -1) entre DS et les porteurs sains de prothèses dentaires.

Conclusion

Les résultats des études menées dans le cadre de ce projet de recherche de maîtrise suggèrent que l'hybridation à damier d'ADN-ADN a une meilleure précision diagnostique par rapport à la culture pour la détection d'espèces de *Candida* dans la DS. En outre, les taux de certaines cytokines salivaires spécifiques peuvent être associés à l'inflammation palatine observée dans la DS. Une recherche plus poussée est nécessaire pour confirmer ces résultats.

Mots-clés

Stomatite prothétique, hybridation à damier ADN-ADN, *Candida*, biofilm oral, biomarqueurs, revue systématique.

ABSTRACT

Introduction

According to the literature, evidence on the potential use and application of DNA-DNA checkerboard hybridization technique in the diagnosis of *Candida*-associated Denture Stomatitis (DS) is scarce. Furthermore, the literature suggests that the inflammatory biomarkers in saliva could offer a new venue for the early diagnosis of this disease.

Objectives

The objectives of this master's research projects were to: 1) Provide evidence on the diagnostic accuracy of conventional culture and DNA-DNA checkerboard hybridization techniques for the detection of *Candida* species in DS, and to investigate its impact on the clinical diagnosis of this disease, and 2) To systematically examine the available evidence on the salivary biomarkers present in DS.

Methods

Objective 1): Palatal biofilm of 26 participants diagnosed with DS was analyzed to detect and quantify *Candida* species using culture and DNA-DNA checkerboard hybridization techniques. Using each technique as the standard reference for the other, the diagnostic accuracy of both techniques was examined, and compared using Kappa and McNemar tests. Spearman's rank test was used to examine the association between total *Candida* and total inflammation scores.

Objective 2): The systematic review followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. The level of evidence of the included studies was graded using the Oxford Center for Evidence-Based Medicine (OCEBM) 2011

scale. The methodological quality was assessed using Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement, and graded according to the Olmos scale.

Results

Objective 1): For all *Candida* species, the specificity of the culture technique ranged from 52% to 88.5%, and between 92.9% to 100% for the DNA-DNA checkerboard hybridization technique. There was a lack of agreement between the two techniques. The sensitivity for both the techniques was observed to be zero for all species. The correlation between *Candida* and inflammation scores was not statistically significant for the culture method, however a statistically significant and positive correlation was observed for the DNA-DNA checkerboard hybridization technique ($p=0.05$).

Objective 2): The majority of studies included in the systematic review, showed that the levels of IL-6, CCL-3, TGF-β, GM-CSF, and TNF-α were higher in older individuals with DS, as compared to younger individuals with DS, or healthy individuals ($p<0.05$). In contrast, a few studies also observed a non-statistically significant difference in the levels of most salivary cytokines (IL-2, IL-12, IFN-Ɣ, IL-4, IL-8, IL-10, IL-17, TNF-α, and ICAM-1) between DS and healthy denture wearers.

Conclusion

The results of the studies undertaken during this master's research project suggest that DNA-DNA checkerboard hybridization shows greater diagnostic accuracy for the detection of *Candida* species in DS, as compared to the culture technique. Furthermore, the levels of some specific salivary cytokines may be associated with the palatal inflammation observed in DS. Further research is needed to confirm these results.

Keywords

Denture stomatitis, DNA-DNA checkerboard hybridization, *Candida*, oral biofilm, biomarkers, systematic review.

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LIST OF SYMBOLS AND ABBREVIATIONS

%: Percentage

AIDS: Acquired immune deficiency syndrome

ALS: Agglutinin-like sequence

ATCC: American type culture technique

BCR: Biofilm and cell wall regulator

BHIYE: Brain heart infusion broth supplemented with yeast extract

CAH: Carbonic anhydrase

CCH: Calcium channel homolog

CFU: Colony forming units

CMA: Corn meal agar

CRC: Concentrated rinse cultures

CYTN: Cystatin

DES: Denture-associated erythematous stomatitis

DNA: Deoxyribonucleic acid

DS: Denture stomatitis

EFG: Enhanced filamentous growth protein

ELA: Elastase activity

ELISA: Enzyme-linked immunoabsorbent assay

FIG: Factor-induced gene

GM-CSF: Granulocyte-macrophage colony-stimulating factor

GPI: Glycosylphosphatidylinositol

GTT: Germ tube test

HAE: Haemophilus aegyptius endonuclease

HWP: Hyphal wall protein

ICAM: Intercellular adhesion molecule

IFN: Interferon

Ig: Immunoglobulins

IL: Interleukin

LC-MS/MS: Liquid chromatography-mass spectrometry/liquid chromatography-tandem mass spectrometry

LIP: Lipase precursor

m/z: Mass-to-charge ratio

MID: Mating pheromone-induced death

MeSH: Medical subject heading

MSP: Mitochondrial sorting of proteins

NaCl: Sodium chloride

NaOH: Sodium hydroxide

NLA: Neisseria lactamica

NO: Nitric oxide

NRC: Neat rinse cultures

OCEBM: Oxford centre for evidence-based medicine

PBS: Phosphate buffered saline

PCR: Polymerase chain reaction

PDA: Potato dextrose agar

PFGE: Pulsed field gel electrophoresis

PFU: Pyrococcus Furiosus

PM: Percent method

PPIA: Peptidyl-prolyl *cis-trans* isomerase

PRISMA: preferred reporting items for systematic reviews and meta-analyses

PWB: Primary wash buffer

RFLP: Restriction fragment length polymorphism

rRNA: Ribosomal ribonucleic acid

SAP: Secreted aspartic proteases

SCM: Standard curve method

SDA: Sabouraud dextrose agar

SDB: Sabouraud dextrose broth

SELDI-TOF/MS: Surface enhanced laser desorption/ionization time of flight mass spectrometry

sIg: Secretory immunoglobulins

SPO: Salivary peroxidase

spp: Species

SSA: Stress-seventy subfamily A

STAT: Statherin

SWB: Secondary wash buffer

TAQ: Thermus aquaticus

TE: Tris-Ethylenediaminetetraacetic acid

TEC: Transposon enhancement control

TGF: Transforming growth factor

TNF: Tumor necrosis factor

VEGF: Vascular endothelial growth factor

VSM: Visual scoring method

VSV: Vesicular stomatitis virus

DEDICATION

Dedicated to my parents, wife, and my three beautiful children

This was only possible with your prayers, encouragement, support, and positivity all of you bring to my life

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CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

Denture Stomatitis (DS) or Denture-associated Erythematous Stomatitis (DES) is an oral biofilm-associated chronic inflammatory disease, which affects the oral mucosa covered by a removable prosthesis [1-7]. It is the most prevalent oral disease among edentate individuals [7-10], remaining asymptomatic or often presenting with symptoms like mucosal tenderness, bleeding, halitosis, burning sensation, xerostomia and dysphagia [4, 11-14]. Additionally, it has been linked to various systemic diseases, especially among hospitalized patients, individuals with a compromised immune system and elders with cognitive impairments and dementia [15-18].

While multiple risk factors have been investigated for their role in the etiology of this disease, the role of *Candida* continues to be frequently highlighted in the literature [5, 19, 20]. Consequently, current practice primarily focuses on antifungal prescriptions in addition to oral hygiene improvement [2, 21-23]. Following laboratory culture, a diagnosis may be made if the *Candida* count is found to be >400 CFU/ml of saliva, in an otherwise healthy individual [24-26]. However, in the current literature, there continues to be a lack of good quality reports to determine a direct cause-and-effect relationship between *Candida* and DS. We argue that the association of any microorganism to a disease is limited to the ability of the technology or method used to detect the microorganism. Therefore, clearly, the presence of *Candida* and its potential association with DS is dependent on the diagnostic test applied and its accuracy to

detect *Candida* spp. within the samples. The relationship between these two variables of interest should be investigated utilizing recently developed molecular methods such as the DNA-DNA checkerboard hybridization technique [27]. Since such techniques drastically differ from previous non-genetic tests such as the conventional laboratory culture, it is therefore imperative to determine the diagnostic accuracy of such methods in comparison to culture, to improve our understanding of the role of *Candida* in DS.

In the following sections of this chapter, we focus on the literature encompassing the risk factors of DS, the observed salivary immune response, and the methods available for the identification and quantification of *Candida*.

1.2 DENTURE STOMATITIS

1.2.1 Epidemiology

DS, DES or *Candida*-associated denture stomatitis is a chronic, erythematous oral inflammatory condition, observed on the oral mucosa covered by a dental prosthesis [1, 3-7, 10]. It affects both, complete and partial denture wearers and is most commonly observed on the denture bearing palatal mucosa, with a lower incidence in the mandibular mucosa [1, 9, 13, 21, 28]. DS is the most prevalent form of oral disease reported among completely edentate individuals and serves as the main indicator of poor oral health in this population [29, 30].

Despite the fact that people now tend to retain their natural teeth well into old age, the increase in the average life expectancy coupled with poorer socioeconomic status results in tooth loss and hence, the use of complete or partial prostheses becomes inevitable [7, 30-32]. Since complete tooth loss and denture use are most prevalent among disadvantaged individuals with

a low socioeconomic status, it is expected that this disease is more prevalent in this population group [29, 33].

DS affects a significant number of denture-wearing individuals. It is reported that 1 in every 3 [7-9], or 2 in every 3 individuals wearing dentures [34, 35], may present with some severity of this disease. The global prevalence of DS is reported to range between 15% and 77% [7, 30, 36-39]. University-based studies in the province of Quebec in Canada, have reported a prevalence up to 77.5% in a sample of complete denture wearers who visited university dental clinics [7, 40, 41]. While wearing complete dentures has been frequently reported to have a statistically significant correlation between DS [7, 9, 38, 42-44], some studies have also observed DS within study samples wearing partial dentures [8, 26, 33, 45].

Several studies have also shown that children and adults wearing acrylic partial dentures, obturators and ortho-appliances can be affected by DS [13, 33, 46, 47]. Furthermore, a systematic review by Emami et al. [21] reported that up to 36% individuals wearing chrome-cobalt or acrylic partial dentures may also present varying degrees of DS. However, it must be considered that the wide variations in the reported global prevalence of denture stomatitis may be attributed to the differences in the diagnosis, methods of data collection, choice of the study population; and associated geographic, socio-demographic and lifestyle characteristics [7, 21, 40, 41].

A higher prevalence of DS is observed in elders due to long-term denture use, lack of dexterity in performing oral hygiene, polymedication and decreased host immunity [38, 48-52]. It has also been reported to affect female denture wearers more often than males [12, 13, 38]. A possible explanation of a higher incidence among women observed by other authors may be due to the possibility that more women may sleep with their dentures due to aesthetic concerns

as previously reported by Coelho et al. [53]. In contrast, a recent study by Iosif et al. [54] in 2016 with a small sample size of 56 participants concluded that there were no statistically significant age and sex differences between subjects with and without *Candida*-associated DS. However, such difference in studies' results could be related to type II error and underpowered studies.

1.2.2 Classification

Various classifications have been presented in the literature over the last few decades, aiding in the clinical diagnosis and staging of DS. The most commonly used classification continues to be the one presented by Newton [55], which is as follows:

Type I: Pinpoint hyperaemic lesions, particularly around the orifices of the ducts of the palatal mucous glands (localized inflammation).

Type II: Diffuse erythema observed on the denture bearing mucosa (generalized inflammation).

Type III: Inflammatory papillary hyperplasia (granular appearance).

A modified version of Newton's classification was presented by Barbeau et al. [28]. This modified version considers not only the type or intensity of inflammation but also identifies the extent or the spread of inflammation, by dividing the denture bearing mucosa into quadrants.

Type I: Pinpoint hyperaemic lesions, particularly around the orifices of the ducts of the palatal mucous glands (localized inflammation).

Type II: Diffuse erythema observed on the denture bearing mucosa (generalized inflammation).

Subclass A: Inflammation limited to 1 or 2 quadrants

Subclass B: Inflammation extending to 3 or 4 quadrants

Type III: Inflammatory papillary hyperplasia (granular appearance).

Subclass A: Inflammation limited to 1 or 2 quadrants

Subclass B: Inflammation extending to 3 or 4 quadrants

While both, the original and the modified Newton classification are regularly used, a more comprehensive classification presented by Schwartz et al. [56] provides a better representation of the severity (intensity) and area (extent) of the disease, making it easier to apply in a clinical setting:

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 tissue

The score obtained on the severity and area index are then summed up to obtain a final inflammation score which may vary between 0 and 6 [56].

1.3 ETIOLOGY AND RISK FACTORS FOR DENTURE STOMATITIS

The etiology of DS is considered to be multifactorial in nature and continues to be poorly understood [7, 25, 54, 57, 58]. As the name of this pathological condition suggests, the presence of mucosal inflammation associated with DS is dependent upon the introduction of a denture into the oral cavity [59]. The mere presence of a partial or complete denture will initiate contact and promote microbial adhesion to the oral mucosa [45, 60]. Several underlying factors may predispose individuals and increase their susceptibility to DS [61, 62]. These risk factors may, therefore, be divided into "local" or prosthesis-associated factors, and "general" or systemic risk factors [9, 58, 63, 64].

Local or prosthesis-associated modifiable risk factors of DS are trauma from unadjusted or ill-fitting dentures, the age of the prosthesis, denture hygiene related factors which include denture cleaning/brushing and denture wearing habits i.e., interrupted, continuous and/or nocturnal wear [2, 5, 7, 9, 25, 28, 38, 41, 43, 65, 66]. As the dentures age, they lose retention and stability due to the pathological changes in the edentulous oral cavity such as the development of mobile ridges, and reduction in the vertical dimension of occlusion, thus inducing trauma to the oral mucosa [25, 41, 44, 67-71]. It has also been suggested that an important cause of denture instability is an improper inter-occlusal relationship altering the patterns of occlusal load transmission to the tissues under the denture bases, resulting in DS [67, 72, 73].

The continuous and nocturnal denture wear is considered to inflict uninterrupted pressure on the denture bearing tissues, inhibit the oxygenation of the oral mucosa, and impede the

cleaning effect of the tongue and saliva, thus making the oral mucosa more sensitive to cell injury and prone to inflammation [9, 28, 41, 74-76]. Furthermore, mucosal coverage by the denture base creates an acidic and an anaerobic local microenvironment that promotes pathogens like *Candida* spp. and other microorganisms to proliferate within the biofilm, producing toxins and metabolic waste responsible for cell injury and resultant inflammation, the main clinical feature of DS [66, 77-80].

General or systemic risk factors for DS reported in the literature include old age, smoking, obesity, sugar consumption [7, 9, 10, 14, 28, 38, 45, 63, 81, 82], xerostomia, diabetes mellitus and immunosuppressive conditions such as AIDS [14, 45, 63, 83, 84], the use of antibiotics, corticosteroids, hormones and other xerogenic agents [45, 82, 85-88], and lastly, malnutrition including deficiencies in proteins, iron, vitamin A and B [9]. Martori et al. [80] conducted a cross-sectional study involving 84 geriatric denture wearers and examined the correlation between various local and systemic risk factors and DS. Using multiple logistic regression models with the observed inflammation as the dependent variable, an association was observed between DS and low salivary pH (OR 0.057; 95% CI 0.01-0.48), smoking (OR 152.8; 95% CI 2.28 to >999) and sugar consumption (OR 6.917; 95% CI 1.17-40.9).

In general, it is difficult to ascertain a direct cause-and-effect relationship between the factors nominated as etiological factors in the literature because of the studies' design and their cross-sectional nature [21, 28, 40, 41]. However, from the available evidence, three factors may play an important role in the occurrence of DS [7, 89, 90]. These include mucosal trauma [28, 36, 41, 91], oral biofilm and specific bacteria [76, 92], as well as pathogens such as *Candida* spp., and more specifically, *Candida albicans* [2, 36, 40, 76, 78, 93].

1.3.1 Mucosal trauma

The role of denture-induced trauma has been frequently reported as a risk factor for DS [25, 36, 41, 80, 91]. Historical studies conducted by Budtz-Jorgensen research group involving 58 DS patients using complete dentures for a very long period of time (mean age of denture 26.8 years), suggested that the inflammation observed in DS was increasingly linked with poor denture hygiene and continuous mucosal irritation resulting in mechanical trauma caused by ill-fitting dentures and unbalanced occlusion [4, 70].

The susceptibility of the palatal mucosa to trauma induced by a denture may also be dependent upon the presence or absence of natural teeth in the opposing jaw, as well as the type of prosthesis [67]. This is further supported by a study conducted by Emami et al. [41] which concluded that the risk of DS was 4.5 times greater in patients wearing mandibular conventional dentures than in those who were rehabilitated with more stable implant-assisted overdentures.

An animal model study on mucosal biomechanics showed a high correlation between histopathological changes in the palatal mucosa and occlusal forces transmitted due to instability and poor retention of the prosthesis [94]. These continuous forces compromise the circulation under the mucosa, thus resulting in swelling, edema, mucosal inflammation and eventually bone resorption [95-99]. It is therefore suggested that the mucosa covered by a denture base may not exhibit signs of inflammation in the absence of mechanical pressure or trauma caused by dentures [7, 28, 73, 94, 97].

1.3.2 Oral biofilm and bacterial species

The commensal microbiota of the oral cavity comprises a wide variety of microorganisms, including viruses, protozoa, fungi, and bacteria [100]. These microorganisms colonize different parts of the oral cavity like the teeth, gingiva, tongue, mucosa, throat and the palate [101], by adhering to a glycoprotein pellicle, and proliferating to form the dental plaque [102], and in the presence of a denture, forming the denture biofilm. This biofilm is a "*microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, embedded in a matrix of extracellular polymeric substances that they have produced*" [103-108].

The oral biofilm covering the mucosa and denture surfaces provides ideal conditions for the microorganisms within the biofilm to proliferate, and cause mucosal inflammation [109, 110]. These microorganisms continue to co-aggregate, further utilizing habitat-specific nutrients [111], thus forming a heterogeneous and highly diverse ecological environment in the oral cavity, upon which the health and disease status of the host is dependent [10, 112].

Comparing the microbiome of denture wearers, those with and without DS, it has been suggested that bacterial species such as α-hemolytic *Streptococci* and *Neisseria* may play an important role in the inflammation associated with DS [113]. It has also been observed that while *Candida* spp. were in higher quantities in DS patients, bacterial species such as *Streptococci*, *Lactobacilli,* and *Actinomyces* were also present in the denture biofilm of patients with DS [114]. Budtz-Jorgensen et al. [115] observed similar findings in their study utilizing 1239 isolates of denture biofilm samples taken from DS patients. They concluded that DS was associated with a high bacterial count, mainly gram-positive rods (median 45%) comprising of *Lactobacillus* spp. (median 19%) and *Actinomyces* spp. (median 9%).

Van Reenen [116] examined the changes in the counts of *Streptococci* and *Candida* spp. isolated from the denture biofilm of denture wearers with DS. They observed a reduction in inflammation after prescribing antibiotics, suggesting that bacteria may play an important role in the inflammation observed. This may be explained by the synergistic nature of *Streptococci*, which create a favorable environment for yeasts by producing lactate, providing carbon for the yeast to feed and thrive upon [117]. Investigating the oral microbiome associated with DS using a high-throughput 16S rRNA sequencing technology, O'Donnell et al. [10] showed that the denture biofilm in DS subjects had a higher proportion of *Bacteroidia* attributed to *Prevotella* and *Veillonella* ($p<0.05$). Additionally, the inflamed mucosa also had a high prevalence of *Actinobacteria* and *Bacteroidia*, suggesting similarities between the denture and mucosal biofilm [10].

The microbial diversity of the oral biofilm may consequently point towards a possible role of non-candidal microorganisms in addition to *Candida* spp. in the etiology of DS [93, 118].

1.3.3 *Candida* species and denture stomatitis

Candida spp. have received the most attention as the primary etiology of DS, as studies report a high prevalence of these microorganisms in DS patients [5, 19, 20]. *Candida* spp. exist as commensal but opportunistic microorganisms on the epithelial surfaces of the human body, including the oral cavity [109, 119-121]. It has been reported that about 75% to 100% of the population may demonstrate *Candida* specific immunity [121-123], suggesting previous exposure to the microorganism.

The most common site considered to harbor *Candida* is the mucosal surface of the denture base [4, 124]. Budtz-Jorgensen et al. [125] conducted an epidemiological study involving 560

individuals above the age of 65 years and compared mucosal and denture biofilm samples of those with and without DS. Their results showed that in yeast form, *Candida albicans* were the most common species grown in pure culture among both study groups. However, there was a statistically significant difference in the concentration of *Candida* hyphae between the two groups; 77% in individuals with DS and 47% in individuals without DS (x^2 test, $p < 0.001$) [125]. Additionally, the presence of inflammatory cells along with hyphae was higher in DS patients (65%), as compared to healthy participants (14%) [125]. The presence of yeast has also been reported in the unstimulated saliva in 90% of study subjects with DS [126].

Candida albicans have also been reported to be the most prevalent species isolated from healthy and immunocompromised patients suffering from DS [127-129]. Budtz-Jorgensen et al. [110] showed that *Candida* spp. count in DS subjects was 100 times higher than in healthy denture wearers, with *Candida albicans*, *Candida tropicalis* and *Candida glabarata* as the most commonly isolated species. Furthermore, MacFarlene et al. [1] reported that the most prevalent *Candida* spp. in DS lesions were the *albicans*, followed by *glabrata* and *tropicalis.*

1.3.3.1 *Candida* virulence factors

The ability of *Candida* to trigger a host immune response and cause inflammation is due to various virulence factors. These factors include:

I. Dimorphism

An important property of *Candida*, that plays a role in its virulence and pathogenicity is dimorphism, which is the ability that *Candida* exhibits to transition between yeast and hyphal forms, frequently observed in diseased conditions [130].

Dimorphism plays an important role in the formation of the biofilm and also aids in tissue invasion [131]. The biofilm formation involves two steps: Following the initial role of adherins, dimorphism ensures candidal adherence to the substrate in the hyphal state and later, dispersion from the biofilm in the yeast state following hyphal replication and extracellular matrix formation [132, 133]. While *Candida* in the hyphal form is observed to show a higher level of invasiveness, the yeast form exhibits increased virulence [134, 135]. Several *Candida* transcriptional factors, namely Bcr1, Tec1, and Efg1 are also considered to play an important role in the formation of the oral biofilm on mucosal and prosthetic surfaces [136].

Dimorphism also regulates the contact sensing ability of *Candida* through which it senses contact surfaces and switches from yeast form to hyphal growth, resulting in tissue invasion [131]. Furthermore, certain extracellular calcium channels; Cch1, Mid1, and Fig1, as well as the polarisome module; Ras-like GTPase Rsr1/Bud1, have shown to regulate the ability of hyphae to grow in a particular directional pattern, depending on the topology and surface characteristics of the substrate [137, 138].

II. Host recognition and cellular attachment

Of particular importance in the oral cavity, is the ability that *Candida* possesses to recognize and attach to host cells through various surface mannoproteins called adhesins [139]. Most notable of these are Agglutinin-like sequence (ALS) proteins Als1–7 and Als9, particularly Als3 which has been shown to be up-regulated in oral epithelial cells [140, 141]. Another protein, Hwp1 aids in forming covalent links between candidal hyphae and host cells [142]. Both ALS and Hwp1 proteins are glycosylphosphatidylinositol (GPI)-linked proteins and aid in candidal adhesion to host cells as well as biofilm formation [142, 143]. In addition to host cell adhesion, Als3 and Ssa1 have also shown to play a role in the cellular invasion by acting

as invasins that bind with host cell ligands resulting in induced endocytosis, whereby the fungal cell is engulfed into the host cell [144, 145]. In contrast to a passive endocytosis mechanism triggered by invasins, the viable *Candida* hyphae penetrate the host cells through an active invasion mechanism [146].

III. Tissue hydrolysis

Another important virulent characteristic of *Candida* is its ability to release hydrolytic enzymes such as proteases, phospholipases and lipases which aid in tissue penetration following cellular adhesion [147]. The proteases (Sap1-10) comprise the largest hydrolase family in *Candida albicans* and are considered to play a significantly virulent role in epithelium invasion [148, 149]. Phospholipases (A, B, C, and D), and lipases (LIP 1-10) are also considered to play an important role in the disruption of host cell membrane and pathogenicity of *Candida albicans* [150, 151].

IV. Withstanding pH

Candida have also shown great adaptability to the surrounding pH of the host environment, which can vary greatly depending upon the location; from very acidic (pH 2) in the stomach to slightly alkaline on the palatal mucosa (pH 7.34) [152, 153]. In addition, its ability to transform into hyphal form helps it to withstand an acidic environment [130]. Furthermore, it also inherits processes to regulate extracellular pH by uptaking amino acids and cleaving them intracellularly, thus producing ammonia to alkalinize the surrounding pH [154, 155].

More recently, literature has raised questions regarding the association between *Candida albicans* and DS. For instance, Emami et al. [40] investigated the relationship between myceliated colonies of *Candida* and DS and showed a non-statistically significant difference

in the counts of *Candida albicans*, among patients with DS and healthy subjects. Similarly, another study observed that the high *Candida* count in DS was associated with the area of inflammation (mucosal area coverage), and not the intensity or severity of inflammation [28]. This coupled with evidence of a high recurrence rate of DS after the cessation of antifungal therapy [156-158], may suggest a more complex role of other factors such as the host immune response to microbial insult.

1.4 HOST IMMUNE RESPONSE OBSERVED IN DENTURE STOMATITIS

Saliva plays a critical role in maintaining the integrity of the hard and soft tissues in the oral cavity by regulating the local immune response observed in oral inflammatory diseases, like DS [159]. The inflammatory cascade is triggered as a protective response to cell injury, exhibiting an interaction between cells and inflammatory mediators such as vasoactive amines (histamine, serotonin), phospholipids (platelet-activating factor), arachidonic acids (prostaglandins, leukotrienes) and cytokines (tumor necrosis factor, interleukins, interferons, and colony stimulating factors) [160-162]. This physiological process leads to vasodilatation, increased microvascular permeability, cellular activation, cellular adhesion and coagulation, which increases the available oxygen and nutrients at the site of injury, thus generating heat and provoking tissue edema [160, 162].

A notable immune response is observed within saliva in response to the pathogenic microorganisms causing DS [50, 163-165], thereby playing a protective role in the host defense mechanism [166]. Salivary and blood neutrophils serving as biomarkers mediate cytokine liberation through diapedesis, chemo-attraction, phagocytosis and activation events,

therefore responding in an acute manner to protect against the establishment of oral diseases [167].

Saliva also plays a role in the humoral and cell-mediated adaptive immunity, displayed by the high prevalence of immunoglobulins [168] and distinct cytokine profiles [163]. In fact, the predisposition towards *Candida* infections among the elderly denture-wearing population may be explained by salivary immunosenescence or the deterioration of immunity due to the advancement of age [169]. Furthermore, defense mechanisms such as phagocytosis (or uptake) have been shown to be impaired among the elderly [50], while induced neutropenia through IL-17 pathway blockade in a mice model also increased disease susceptibility [170-172].

Other components such as salivary proteins like secretory immunoglobulin A (IgA), lactoferrin, lysozyme, and histatins, also function as biomarkers and have antifungal effects [173, 174]. Among these, IgA acts as a barrier protecting against antigen invasion and is seen in a higher concentration in DS [175]. Most importantly, lactoferrin inhibits bacterial growth by sequestering essential iron and also exhibits non-iron-dependent antibacterial, antifungal, antiviral, antitumor, anti-inflammatory, and immunoregulatory activities [174]. Differential susceptibility to DS may be attributed to differential concentrations of the aforementioned salivary proteins, which are measured by proteomic profiling of saliva in DS patients [176]. For instance, levels of Vascular endothelial growth factor (VEGF), which are considered to be the underlying markers of chronic inflammatory or autoimmune conditions in the oral cavity, were observed to be decreased in type I DS and increased in type II DS, when comparing DS subjects with and without type 2 diabetes mellitus [71, 177, 178].

1.5 METHODS FOR THE ISOLATION AND DETECTION OF *CANDIDA* SPECIES

1.5.1 *Candida* isolation techniques

A variety of methods have been used for the isolation of microorganisms including *Candida* from the site of inflammation in the oral cavity. The decision to select a particular method is dependent on the objective of the research, the nature of the lesion observed and the kind of technique planned to be used for the quantification and identification of various *Candida* spp. The following methods are validated sampling methods used for *Candida* isolation from the oral cavity:

I. Smear

This process involves the collection of a superficial sample from the site of inflammation in the oral cavity by firmly wiping the area using a sterile wooden stick or blade [25, 179], or gently using a cytology brush [180]. Obtained smears are observed on a glass slide either in moist form fixed with ether/alcohol (1:1) [179], Cytofix/Cytoperm (Becton, Dickinson and Company, Franklin Lakes, NJ) [25], or as a dry smear following staining using either Gram-stain or Periodic acid-Schiff (PAS) techniques [179, 181]. Microscopic examination of an oral smear obtained from a suspected *Candida*-associated denture stomatitis lesion will reveal the presence of *Candida* spp., visible either as hyphae or blastophores [181, 182].

II. Swab

In addition to smears, mucosal swabs are also one the most widely used methods to screen and diagnose oral and systemic diseases [183]. Additionally, they are also frequently used to

isolate DNA for genomic and forensic research [183, 184]. In order to isolate microorganisms such as *Candida* spp. found in DS, a cotton swab sterilized in the laboratory, or a pre-packaged sterile swab may be used [185]. Similar to a smear collection, the sample is collected by gently running the cotton swab over the site of the inflammatory lesion on the palate, rugae area and the denture fitting surface [2, 25, 186]. In order to ensure microbial viability, the sample is stored on ice while being transported to the laboratory [186].

III. Imprint culture

Benefiting from the adherence properties of *Candida*, another technique termed as imprint culture is also routinely used for isolating and quantifying *Candida* from the oral cavity [20, 181, 186, 187]. This involves using a sterile foam-like pad dipped in saline or a liquid medium like Sabouraud dextrose broth (SDB), placing it at the specific site to be investigated i.e., inflamed oral mucosa or the denture fitting surface, and leaving it in place for 10 to 60 seconds [24, 188-190]. The imprint pad is then placed on Sabouraud dextrose agar (SDA) and left for 60 minutes to ensure that the sample has been adequately transferred onto the agar before incubation [189]. A variation of this method allows the foam to be left *in-situ* for the first eight hours of the 48-hour incubation cycle [179].

IV. Oral rinse

A swab or an imprint culture is often followed by an oral rinse for further analysis. This is done by asking the patient to rinse with 10ml of sterile phosphate buffered saline (PBS: 0.01 M, pH 7.2) for 60 seconds [179, 191]. The rinse is collected in a sterile container and transferred to the laboratory for analysis.

In the laboratory, the collected oral rinse may either be cultured on SDA as neat rinse cultures (NRC) or as concentrated rinse cultures (CRC) [191]. In order to concentrate the obtained rinse, the neat rinse is centrifuged at 1700g for 10 minutes [186, 191]. More recently, centrifuging the neat rinse at 2000g for 10 minutes [192] and at 2300g for 20 minutes [185] has also been reported. Following the removal of the supernatant, the pellet obtained from the centrifugation process is mixed in a predefined amount of the original solution (500 μL) and inoculated onto agar media in 100 μL aliquots using a spiral plating system [185, 188].

V. Saliva

Biomarkers within human saliva provide extensive information about the etiology, pathophysiology, and prognosis of various diseases [193-195]. Additionally, a collection of biofluids such as human saliva serves as a non-invasive method for the screening and diagnosis of oral and systemic diseases [183, 195]. Salivary culture serves as a reliable method for quantifying *Candida* spp. isolated from the oral cavity and assists in differentiating between the carrier and infectious states [196]. As a reference, Epstein et al. [197] demonstrated that a salivary *Candida* count of >400 CFU/ml is considered as an infected state, while <400 CFU/ml of saliva is considered as a carrier state [25, 26, 198].

Depending on the study objective, stimulated or whole unstimulated saliva may be collected utilizing various commercially available collection kits. Stimulated saliva can be collected using Salivette® with cotton swabs (Sarstedt, Nümbrecht, Germany) or using paraffin gum (Ivoclar Vivadent, Schaan, Lichtenstein) [199]. The patient is asked to swallow the saliva already present in the mouth, then chew on a paraffin gum or strip for 2 minutes, followed by spitting the saliva in a sterile container [25]. Provided that the patient has normal salivary production, unstimulated whole saliva may be used for quantifying *Candida* using culture

techniques [163, 169, 200, 201]. The standard protocol for the collection of unstimulated saliva requires the patient to sit upright with their head tilted slightly forward, and passively drool into a sterile 50ml falcon tube for 5 minutes [168, 202]. The total quantity of saliva produced over the predefined duration of time is recorded in order to calculate the average salivary flow (ml/min) [168, 202].

VI. Tissue biopsy

Candida-associated DS in its severe form may present with hyperplastic tissue or papillary hyperplasia on the palatal tissue region covered by the denture [55]. A tissue biopsy taken from the affected site is indicated for histopathological examination. The procedure involves anesthetizing the palatal mucosa, followed by a 2-4 mm full thickness punch biopsy from the keratinized epithelium to the periosteum [25]. The histopathological examination involves observing the biopsies for epithelial and connective tissue inflammatory reactions [203].

1.5.2 *Candida* detection and differentiation techniques

In a laboratory setting, the samples obtained from the above-mentioned methods may be processed using a variety of techniques. While some of these techniques are simply limited to the detection (absence or presence) and quantification of *Candida* using culture media, others may be used in the identification and differentiation of particular *Candida* spp. based on their morphology and genetics.

I. Laboratory culture

Culture media have been used for isolating and detecting microorganisms in a controlled laboratory environment for well over a century. The initial attempts to use solid media for growing microorganisms outside the human body can be traced back to the early 1830s when

Italian scientist Bartolomio Bizio successfully cultured a chromomeric bacterial species, *Serratia marcescens* [204]. However, it was not until 1881, when Robert Koch upon receiving advice from Fanny Hesse used agar to make the first stable solid culture media [205]. The French dermatologist Sabouraud later formulated a standardized method of developing agar media to culture fungi and bacteria [206].

Sabouraud dextrose agar (SDA) and Sabouroud dextrose broth (SDB) continue to be the most commonly used media for the isolation of *Candida* spp. [207]. The slightly acidic nature (5.6 pH) and high content of dextrose (4%) of this medium ensure rapid fermentation and acid production, thus inhibiting bacterial growth [207, 208]. The clinical samples are inoculated on the medium and incubated at 37^{o}C for 24-48 hours [25, 209] or 48-72 hours [210, 211]; following which convex, smooth, creamy colored *Candida* colonies can be observed [210, 211]. Certain antifungals like azoles may also be added to the culture medium for the selective growth of *Candida,* or to test drug susceptibility [212-215]. In addition to SDA and SDB, numerous other commercially available non-selective agar and broth media may also be used for growing *Candida*, including Potato dextrose agar, Nutrient agar, Brain heart infusion broth supplemented with yeast extract (BHIYE) and Corn meal agar (CMA) [216]. However none of these culture media permit the differentiation between various *Candida* spp., and therefore the use of differential media is employed [207, 208, 217].

Differential media such as Pagano-Levin agar, CHROMagar Candida (CHROMagar, Paris, France), Albicans ID (bioMerieux, Marcy l'Etoile, France) and Fluoroplate (Merck, Darmstadt, Germany) allow for the differentiation between various *Candida* spp. [188, 218, 219]. Based on the color of individual colonies observed on CHROMagar Candida following the incubation cycle, *Candida* spp. can be differentiated as *C. albicans* (green), *C. tropicalis*

(blue) and *C. krusei* (light pink / pale rose), with a sensitivity and specificity between 95% [220] and 99% [218]. Albicans ID media (bioMerieux, Marcy l'Etoile, France) and CHROMagar Candida, both have shown comparable sensitivity and specificity close to 100% for the differentiation between *albicans* and non-*albicans Candida* spp. [221], which appear as blue and green colored, smooth colonies on each medium, respectively [218, 219].

While the above-mentioned media rely on the chromogenic substrate in their composition, Fluoroplate (Merck, Darmstadt, Germany) contains a fluorogenic substrate which differentiates between *albicans* and non-*albicans Candida* spp. based on their fluorescence observed under a 365nm UV light, whereas all non-*albican* species appear pale white [219, 222, 223].

II. Morphologic test

The use of microbiological cultures is often followed by a morphologic test such as the Germ tube test (GTT), for the presumptive identification and differentiation between *albicans* and non-*albicans Candida* spp. [188, 210, 211]. The test utilizes the dimorphic nature of *Candida albicans* recognized as a virulence factor, which gives it the ability to switch between yeast and mycelial forms, and the formation of chlamydospores [224-229]. Candidal dimorphism or morphological switching can be induced under conditions such as the presence of an inducing substrate like human serum, optimal temperatures (>33^{o}C), an approximately neutral pH and starvation [227, 230].

Using a straight wire, a colony of yeast or a small inoculum grown on solid media is transferred into a tube containing either human or animal serum and incubated at 37^{o}C for 2-3 hours [231-233]. Other media used may include serum substitutes [229], 1% bactopeptone in 2% agar [234, 235], 0.1% glucose in 2% New Zealand agar [235], rice extract and

carbohydrate media [236], and bovine albumin [232, 233]. Following incubation, a small amount of the suspension is placed on a glass slide, covered with a coverslip and observed under a microscope for the formation of filaments or hyphae [211, 231, 232]. Microscopic examination reveals the formation of true hyphae which appear as cylindrical tubes extending from the body of the yeast with no constriction at their base [237], an appearance typical of *C. albicans* and *C. dubliniensis*, differentiating them from *C. glabrata* and *C. krusei* [210, 211]. Furthermore, when inoculated on solid agar containing Tween 80 and incubated for 72 hours at 22^{o}C, *C. albicans* and *C. dubliniensis* exhibit chlamydospore formation [188, 238].

Apart from *C. albicans* and *C. dubliniensis* other *Candida* spp. like *C. stellatoidea* and *C. tropicalis* may also exhibit germ tube formation [188, 239]. It is therefore essential to adhere to the 2-3 hour time limit for the incubation cycle, as species other than *albicans* and *C. dubliniensis* may also start to develop germ tubes as the incubation period increases [225, 240]. Since *C. albicans* and *C. dubliniensis* share morphological similarities, that is, both species develop germ tube and chlamydospores [224-226, 241]; further differentiation between the two is often required. Incubation of the inoculated media at 42^{o}C can be used as a confirmation of the presence of *C. albicans,* as *C. dubliniensis* do not form germ tube at an elevated temperature and test negative for germ tube formation [225].

III. Genetic tests

While the above mentioned conventional techniques are valid methods for *Candida* detection and differentiation, they are nonetheless limited to the presumptive identification of the microorganisms, based on either the color and appearance on culture media or their morphology following a GTT [237]. The following methods, however, are more sensitive and

specific, providing a definitive identification of *Candida* spp. based on the genetic variability between various strains [242].

i. Electrophoretic karyotyping & Restriction fragment length polymorphism

Electrophoretic karyotype analysis involves the separation of the *Candida* chromosomal DNA or other macromolecules on an electrophoretic gel such as the agarose gel matrix [243, 244]. The separation is done based on the size of the DNA molecule and involves two steps; preparing the DNA while ensuring minimal to no degradation, and separating the DNA molecules by applying an electrical current through a process called gel electrophoresis [243, 245, 246].

However, conventional electrophoresis techniques are often limited due to their inability to separate molecules over 25-50 kilobases (kb) [243, 244, 247]. Yeast DNA molecules, which may range over several hundred kilobases can, therefore, be separated using mechanisms such as Pulsed field gel electrophoresis (PFGE) [248, 249], thus enabling the detection and identification of fungi, including *Candida* spp. [182, 246, 247]. The original technique as described by Schwartz et al. separates the DNA molecules in agarose matrices by the alternate activation of electrical fields placed perpendicular to one another [243, 247]. A distinct pattern can be observed due to the relative number and size of the chromosomes of various microbial species using PFGE, therefore making electrophoretic karyotyping useful in differentiating between closely related microbial species [247].

Restriction fragment length polymorphism (RFLP) provides an alternate to karyotyping. This process involves the isolated DNA to be digested and cleaved, or fragmented using DNA restriction enzymes (*MspI, NlaIII, HaeIII, DdeI, EcoRI and BfaI*), prior to being subjected to electrophoresis in an agarose gel matrix [250-253]. DNA fragments of varying length then

hybridize with the specific DNA sequence used as an RFLP probe, following gel electrophoresis [250]. The resultant bands can be observed due to the luminescent dye used in the gel [188]. However the use of electrophoretic karyotyping and RFLP have several limitations, as they are expensive, require specialised equipment, and need 48 hours for DNA extraction and 72 hours for PFGE (which depending on the size of the molecules can take up to weeks) [247].

ii. Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) based techniques provide a rapid and cost-effective alternative to electrophoretic karyotyping and RFLP [254, 255]. In addition, they are highly sensitive and specific [256] for the detection of pathogenic microorganisms, including oral pathogens [249, 257, 258], as compared to conventional microbiological techniques [249]. They are based on the production of a large quantity of any specified DNA for analysis, by repeating the DNA extension reaction, bounded by primers [258, 259].

The original technique described by Saiki et al. [256, 260], was first employed for the enzymatic amplification of beta globulin genomic sequences for the prenatal diagnosis of sickle cell anemia. Since then, various PCR techniques developed over the course of years have been widely used for the detection and identification of yeast and *Candida* spp. [252, 261-267]. Furthermore, PCR is also regularly used for the definitive differentiation between various *Candida* spp., especially C. *albicans* and C. *dubliniensis* [253, 268-270].

The components of a conventional PCR typically include a DNA template which contains the target sequence, a DNA polymerase enzyme such as the *Taq* DNA polymerase or *Pfu* DNA polymerase [254, 270], which are short pieces of single stranded DNA responsible for producing DNA sequences complementary to the target DNA, and primers which are short

strands of complementary DNA that enable the DNA polymerase enzyme to add nucleotides to the primer DNA strand [271].

Each PCR cycle involves three stages: Denaturation of the template DNA by heating at 94^{o}C for 1 minute in order to break the hydrogen bonds between the strands, cooling down of the reaction to approximately 57^{o}C for 1 minute resulting in the primers forming bonds with the template DNA in a process termed as annealing; and the final process called extension where the reaction is reheated to 72^{o}C for 1 minute, allowing the polymerase enzymes to add nucleotides to the primers thus completing a single DNA replication cycle [126, 260, 267].

iii. DNA-DNA checkerboard hybridization

DNA-DNA checkerboard hybridization technique is a culture independent, molecular technique used for the identification and quantification of microorganisms, including those that are non-cultivable [188, 272, 273]. The technique was initially introduced by Socransky et al. [27] for the study of microorganisms isolated from periodontal lesions based on their genetic variability and has since been widely used in dentistry for studying microbiota in a variety of oral conditions [272-279]. Instead of focusing on a few microorganisms, this technique analyzes the samples for a large number of microorganisms, allowing for a more exploratory perspective and thus, may successfully highlight a microorganism in quantities that may be out of its normal range. That is to say, a microorganism previously neglected by researchers may show a pattern which may play an important role in the pathogenesis of the disease [280].

The DNA-DNA checkerboard hybridization technique allows 28 samples to be simultaneously analyzed for 40 microbial species on a single membrane, using whole genomic DNA probes as controls [27, 272, 273, 281]. As described by Socransky et.al [27], and modified by

Nascimento et al. [282], samples collected from the oral cavity or the site of the lesion are stored in tubes containing 0.15ml TE buffer, into which 0.15ml of 0.5M NaOH is added and boiled for 5 minutes. Following this denaturation process, the denatured DNA samples are deposited onto a 15x15 nylon membrane (Boehringer Mannheim® or Hybond N+®, GE Healthcare Life Sciences do Brazil, São Paulo-SP, Brazil) using a Minislot 30 ™ (Immunetics, Cambridge, MA, U.S.A) and affixed using a UV light (Stratalinker 1800, Stratagene, La Jolla, CA, U.S.A), followed by baking at 120°C for 20 minutes [27] or at 80°C for 2 hours [282]. The membrane with the fixed sample DNA and control DNA (10^5 and 10^6 microbial cells of each species), is prehybridized at 42°C for 1 hour [27] or 60°C for 2 hours (0.5 M NaCl; 0.4% w/v blocking reagent) [274, 282], and then placed in a Miniblotter 45™ (Immunetics; Cambridge, MA, U.S.A) in a perpendicular or cross-ways pattern and hybridized overnight at 42°C [27] or 60°C [274]. The membrane is then washed and visualized for hybridization signals using Storm Fluorimager™ (Molecular Dynamics, Sunnyvale, CA, U.S.A) which are then converted to absolute counts [27, 280].

CHAPTER 2

METHODOLOGY

2.1 PROBLEMATIC AND OBJECTIVES

Candida species have been considered the most important factor predisposing denture wearers to DS [2, 40, 118, 283]. The diagnosis of *Candida*-associated DS can only be made by analyzing the biological samples of patients with DS using a variety of microbiological techniques. Microbial culture is the most commonly employed laboratory technique, providing a semi-quantitative or quantitative estimate of the *Candida* count, expressed as Colony forming units (CFU) [207, 284]. A count of $\geq$400 CFU/ml is indicative of a moderate to high *Candida* load and may be considered for antifungal treatment [25]. In addition to non-specific media like SDA, selective media such as CHROMagar is also required to specifically differentiate between various *Candida* spp. [284].

Performing multiple procedures for the accurate detection of *Candida* spp. can be a laborious and cumbersome task that requires a significant amount of time and resources. DNA-DNA checkerboard hybridization technique developed by Socransky et al. [27] is a molecular diagnostic method, that provides an alternate method for *Candida* detection, and has been used in numerous studies to identify and quantify multiple microbial species including *Candida* in clinical samples [242, 272, 285, 286].

According to the literature, evidence on the potential uses and application of DNA-DNA checkerboard hybridization technique in the diagnosis of *Candida*-associated DS is scarce.

Furthermore, since the inflammatory biomarkers in saliva could offer a new venue for the early diagnosis of this disease, this two-part master's research project aims at providing new evidence and shed light on these topics.

Specific objectives

2.1.1 Checkerboard vs. culture

1. Primary objective: To compare the diagnostic accuracy of microbial culture and DNA-DNA checkerboard hybridization techniques for the detection of *Candida* spp. in the palatal biofilm of denture wearers with DS, using each technique as the reference for the other.
2. Secondary objective: To compare the relationship between *Candida* counts using the two methods, and the extent and severity of palatal inflammation, in denture wearers diagnosed with DS.

We hypothesize that there is no statistically significant difference between microbial culture and DNA-DNA checkerboard hybridization techniques in their diagnostic accuracy for the detection of *Candida* spp., and there is no association between palatal inflammation in denture wearers diagnosed with DS and *Candida* counts measured by the two methods.

2.1.2 Systematic review

1. To identify and evaluate the quality of literature examining the differences in salivary biomarker profiles of healthy denture wearers, and those with DS.

2.2 RESEARCH METHODOLOGY

2.2.1 Checkerboard vs. culture

2.2.1.1 Study design and study participants

The first part of this master's research project is a secondary analysis of the data, which was collected in our previous two-center (Canada, Brazil) trial entitled: "The effect of palatal brushing on denture stomatitis" (registered as NCT01643876 on Clinicaltrials.gov) [2].

The data analysis was conducted only on the data obtained from Brazil (University of São Paulo, Ribeirão Preto) since checkerboard hybridization was only conducted at this center. Therefore, this study included twenty-six participants (male, n = 4, female, n = 22) as previously detailed in the published article [2].

2.2.1.2 Data collection and measurement instruments

Data collection included a clinical examination for the diagnosis of DS and a microbiological investigation using microbial culture and DNA-DNA checkerboard hybridization.

Clinical investigation

Clinical diagnosis of DS was carried out by two trained dentists and was defined according to the Schwartz's area and severity index [56].

I. Schwartz index:

Inflammation area index:

0: No inflammation

1: Inflammation of the palate extending up to 25% of the palatal denture-bearing tissue

2: Inflammation of the palate extending between 25% and 50% of the palatal denture-bearing tissue

3: Inflammation covering more than 50% of the palatal denture-bearing tissue

Inflammation severity index:

0: Normal tissue

1: Mild inflammation (slight redness, no swelling or edema)

2: Moderate inflammation (redness with some edema)

3: Severe inflammation (acutely inflamed redness, edema)

A score between 0 and 6 for total inflammation was then given, which was the sum of the scores of area and intensity of inflammation [56].

Biological sample collection and microbiological investigation

I. *Candida* detection by culture

Palatal biofilm was collected from the center of the palatal mucosa using a sterile cotton swab. Following processing and 10-fold serial dilution with saline (10^0, 10^{-1} and 10^{-2}), the samples were plated in duplicates on SDA, incubated at 37^{o}C for 48 hours, and expressed as CFU/ml. The colonies were then transferred onto selective media and incubated at 37^{o}C for 48 hours, in order to identify *Candida* spp. The details have been provided in the previous publication [2], and in Chapter 3 (first manuscript).

II. *Candida* detection by DNA-DNA checkerboard hybridization

Palatal biofilm from the anterior maxillary ridge and rugae was collected using disposable brushes (Cavibrush, Dentscare Ltda; Joinville, SC, Brazil) and inserted in microtubes

containing 150 µL of TE buffer and 150 µL of 0.5 M NaOH. The modified version of the checkerboard DNA-DNA hybridization technique was followed [242, 282, 287, 288].

2.2.1.3 Data analysis

Prior to conducting analysis, data entry and data cleaning were conducted. The variables were re-coded and final dataset was constructed. The percentage of various *Candida* spp. detected using each technique were obtained. The agreement between the two techniques on the presence of *Candida* spp. was calculated using the Kappa coefficient (κ) [289]. As shown below, the coefficient values range from -1 to +1, where 0 represents the amount of agreement that can be expected from random chance, and 1 represents perfect agreement.

<0.0 – 0.20	No agreement
0.21 – 0.39	Minimal agreement
0.40 – 0.59	Weak agreement
0.60 – 0.79	Moderate agreement
0.80 – 0.90	Strong agreement
>0.90	Almost perfect agreement

In addition, McNemar test was used to compare paired nominal data (absence versus presence) in regard to *Candida* spp. The diagnostic accuracy, defined as the sensitivity and specificity of each *Candida* detection method was calculated according to the following formulae and presented as a percentage [290, 291].

Sensitivity = Number of true positives/ (number of true positives + number of false negatives)

Specificity= Number of true negatives/ (number of true negatives + number of false positives)

The sensitivity and specificity of each method were calculated with the data generated by the other method as the reference [287].

For the secondary objective, the total inflammation score was calculated as the sum of the Schwartz area and severity index scores [56]. For the checkerboard technique, the total *Candida* score was calculated by summing the scores of the five *Candida* species according to the coding index presented in Table 2. For the culture technique, the *Candida* counts were first converted to checkerboard scores using the coding index [287] presented in Table 3, and then summed to obtain the total *Candida* score. A Spearman's rank correlation analysis was performed to examine the association between the total *Candida* scores and the level of inflammation using each method.

2.2.1.4 Ethical considerations

The ethical approval for this study was obtained from the Université de Montréal in Canada (CERES, certificate #12-019-CERES-D), and the University of São Paulo (Ribeirão Preto) in Brazil (IRB certificate # 00625912.6000.5419).

2.2.2 Systematic review

Medline (via OvidSP), PubMed, EMBASE and Cochrane Central Register for Controlled Trials were searched for relevant literature up to April 2017, using a detailed search strategy. Inclusion criteria included: 1) experimental and observational studies reporting on the salivary biomarkers in DS, 2) adult human participants, 3) presence of a control group or subgroup analysis. Publications in a language other than in English or French as well as case reports/series or reviews were excluded.

The level of evidence was graded using the Oxford Center for Evidence-Based Medicine (OCEBM) 2011 scale, while the assessment of methodological quality was conducted using Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement and graded according to the Olmos et al. scale [21, 292]. The details have been provided in Chapter 3 (second manuscript).

2.3 Research Significance

To our knowledge, this master's research project presents two novel studies. Both studies are clinically relevant and important since they examine the diagnostic capacity of various techniques used in the diagnosis of a prevalent oral disease. Furthermore, the knowledge gap identified by these studies will serve to conduct future studies.

2.4 STUDENT'S ROLE IN THE PROJECT

The student did the literature review, data management and data analysis for both projects. The two manuscripts included in Chapter 3 were written completely by the student.

The candidate presented the work related to this research project during several scientific meetings and conferences:

1) Oral presentation: Research seminars, Faculty of Dental Medicine, Université de Montréal, 2016.
2) Poster presentations:
    I. Journée Scientifique, Faculty of Dental Medicine, Université de Montréal, 2016.

II. International Association of Dental Research, General session, Korea, June 2016.

CHAPTER 3

RESULTS

3.1 MANUSCRIPT 1

Comparison between DNA-DNA checkerboard hybridization and culture for identification of *Candida* species in *Candida*-associated denture stomatitis

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ABSTRACT

Statement of problem

The etiology of Denture stomatitis (DS) is multifactorial, with *Candida* species considered the most important risk factor. Culture technique has been used for diagnostic confirmation of *Candida*-associated DS. However, with molecular methods like DNA-DNA checkerboard hybridization gaining popularity, it is imperative to examine the diagnostic accuracy of such methods for the detection of *Candida* in DS.

Purpose

The objective of this study was to compare the diagnostic accuracy of microbial culture and DNA-DNA checkerboard hybridization for the detection of *Candida* and to examine its relationship with the extent and severity of inflammation observed clinically in patients with DS.

Material and methods

The palatal biofilm of 26 denture wearers with a diagnosis of DS was collected using sterile swabs for culture, and disposable brushes for DNA-DNA checkerboard hybridization. Each method was examined for its sensitivity and specificity for the detection of *Candida*, using the other as the reference. Kappa and McNemar tests were used to compare the agreement between the two methods. Additionally, a Spearman's rank test was used to examine the association between total *Candida* scores as measured by each method, and total inflammation scores.

Results

The specificity for the detection of all *Candida* species, using the culture technique ranged between 52% and 88.5%, and between 92.9% and 100% for the DNA-DNA checkerboard

hybridization technique. There was a lack of agreement between the two techniques. The correlation between *Candida* and inflammation scores was not statistically significant for the culture technique. However, a statistically significant positive correlation was observed with the DNA-DNA checkerboard hybridization technique ($p=0.05$).

Conclusion

The results of this study suggest that DNA-DNA checkerboard hybridization shows greater diagnostic accuracy as compared to culture for the detection of *Candida* in DS. Further research is needed to confirm these results.

INTRODUCTION

Candida species have been considered as an important risk factor for *Candida*-associated denture stomatitis (DS) [1-4]. The diagnosis of this disease can only be confirmed by analyzing the biological samples using a variety of microbiological techniques available for the detection of *Candida* growth [5-8].

To this end, conventional microbial culture using selective and non-selective media like Sabouraud dextrose agar (SDA) and CHROMagar (CHROMagar *Candida*, Paris, France) respectively, is the most commonly used laboratory technique, providing a semi-quantitative estimate of the *Candida* count expressed as Colony Forming Units (CFU) [9, 10]. A count of $\geq$400 CFU/ml is indicative of a moderate to high *Candida* load and may be considered for antifungal treatment [7].

Performing multiple procedures for the accurate detection of *Candida* spp. can be a laborious and cumbersome task that requires significant amounts of time and resources. DNA-DNA checkerboard hybridization technique developed by Socransky et al. [11] is a molecular diagnostic method that provides an alternative for microbial detection and has been used in numerous studies to provide simultaneous semi-quantitative estimates of multiple microbial species in clinical samples [12-17]. However, there appears to be a gap in the knowledge and our understanding of the potential uses and application of DNA-DNA hybridization technique, when compared to conventional microbiological techniques for the diagnosis of DS, and the *Candida* spp. profile associated with this disease.

Therefore, the primary objective of this study was to compare the diagnostic accuracy of microbial culture and DNA-DNA checkerboard hybridization techniques for the detection of *Candida* spp. in the palatal biofilm obtained from individuals with DS, using each technique

as a reference for the other. The secondary objective was to assess the difference between these two *Candida* detection methods in regard to the association between *Candida*, and the extent and severity of palatal inflammation observed clinically in these patients.

METHODOLOGY

Study design, setting, and participants

This manuscript presents the secondary data analysis of the previous clinical trial entitled: "The effect of palatal brushing on denture stomatitis" (Clinicaltrials.gov #NCT01643876) [2]. The trial was conducted at the Faculties of Dentistry of the Université de Montréal in Canada (CERES, certificate #12-019-CERES-D), and the University of São Paulo (Ribeirão Preto) in Brazil (Certificate # 00625912.6000.5419). All participants had provided informed consent for various data analyses. The main results of the trial have been previously published [2].

Briefly, the trial used a single group, pre-test / post-test design. Eligibility criteria for participation in the trial were: 1) Individuals 18 years old or older, 2) Wearing a complete upper denture, and 3) Clinically diagnosed for DS. Participants were excluded if they had uncontrolled diabetes, anemia, xerostomia or immunosuppressive conditions, or if they used antibiotics, antifungals or corticosteroids in the four weeks immediately preceding the study. In addition, patients undergoing chemotherapy or radiotherapy were also ineligible to participate. This secondary analysis was conducted on the data (n= 26; males n= 4, females n = 22) obtained from the Brazil center only since this center utilized both microbiological techniques for the detection of *Candida* spp.

Data collection and measurement instruments

Two trained and calibrated examiners performed a visual examination of the denture bearing palatal mucosa of each participant of the study for the diagnosis of DS. The observed palatal inflammation was graded according to the Schwartz's index [18]. The clinical examination was followed by microbiological investigation for the detection of *Candida*, comprising of microbial culture and DNA-DNA checkerboard hybridization techniques.

Sample collection and laboratory investigations

Microbial culture

A sterile cotton swab was used to collect the palatal biofilm from the inflamed mucosa at the center of the palate, covering an area of approximately 1cm². The swab was then transferred into a sterile tube containing 5ml of saline (0.85% sodium chloride) and sonicated in an ultrasonic bath containing distilled water (Cole Parmer 08890-21, 50/60 Hz, 1,3 Amp), for 2 minutes [1]. Each collected sample was vortexed for one minute, followed by 10-fold serial dilution with saline (dilution factors: 10^0, 10^{-1} and 10^{-2}) [2]. Diluted samples (100 μL) were plated in duplicate on 4% SDA (Difco™, Becton, Dickinson and Company, USA), and incubated at 37^o C for 48 hours. Following incubation, the yeast colonies observed were counted, corrected for volume and dilution, and expressed as CFU/ml [2]. In order to identify the *Candida* spp., a sterile filter paper was used to obtain an imprint of colonies that were then transferred to a chromogenic selective medium (CHROMagar Candida, Paris, France) and incubated at 37^o C for 48 hours.

DNA-DNA checkerboard hybridization

For the checkerboard analysis, disposable brushes (Cavibrush, Dentscare Ltd; Joinville, SC, Brazil) were used to collect the palatal biofilm from the anterior maxillary ridge and the

palatal rugae region. Each brush was inserted in microtubes containing 150 µL of TE buffer (10 Mm Tris-HCl, 1 Mm EDTA pH 7.6), and 150 µL of 0.5 M NaOH.

A modified version of the original checkerboard DNA-DNA hybridization technique was used for the qualitative quantification of the microbial colonization of the palatal biofilm [12]. The samples obtained were vortexed for four minutes, followed by boiling at 95ºC for five minutes and then cooling in ice. The contents were then neutralized by adding 800 µL of 5M ammonium acetate. A nylon membrane (Hybond N+®, GE Healthcare Life Sciences do Brazil, São Paulo-SP, Brazil) placed in a 30-slot apparatus (Minislot 30™; Immunetics, Cambridge, MA, U.S.A) was used to concentrate the contents of each tube individually. A defined amount of genomic DNA corresponding to either 10^5 or 10^6 cells of each species was used as control, as presented in Table 1. These were assembled, denatured, precipitated and applied to the membrane surface, then exposed to 80ºC for two hours for fixing microbial DNA, followed by pre-hybridization of the membrane at 60ºC for six hours in a hybridization solution (Buffer hybridization GE; NaCl 0.5 M; Blocking reagent 0.4% w/v). The membrane containing DNA from palatal biofilm was transferred to Miniblotter 45™ (Immunetics, Boston, MA, U.S.A) for applying whole genomic probes from target species inside channels positioned at 90º to the applied sample DNA. The device was wrapped and incubated at 60ºC for sixteen hours for hybridization under gentle agitation. The membranes were then washed twice, at 65°C for 30 minutes, in primary wash buffer (PWB) (Urea 2M; SDS 0.1%; NaH2PO4 50 mM pH 7.0; NaCl 150 mM; MgCl2 1mM; Blocking reagent 0.2) and twice in secondary wash buffer (SWB) (Tris base 1M; NaCl 2M, MgCl2 1M), at room temperature, for fifteen minutes. The detection of hybridization signals followed a chemiluminescent reaction obtained by applying 6.7ml of the CDP-Star reagent (GE Healthcare) on the membranes for five minutes. The

excess reagent solution was drained and each membrane was then sealed in a plastic bag. Chemiluminescent signals were detected by exposing the membrane to ECL Hyperfilm-MP (GE Healthcare) twice (using 1 and 3 hours of exposure). The film registers hybridization signals as dark spots in the intersection between collected specimens' DNA and probes, the intensity of which can be compared to spots corresponding to control samples corresponding to 10^5 or 10^6 cells, and categorized according to the visual scoring method (VSM) [13, 19], as presented in Table 2.

Data and statistical analysis

The percentage of total study samples that tested positive for the presence of each *Candida* spp. using the culture and DNA-DNA checkerboard hybridization techniques was calculated individually (Positive samples / Total number of samples). The agreement between the two techniques on the presence of *Candida* was calculated using the Cohen's Kappa [20]. The coefficient values (κ) ranges from -1 to +1, where 0 represents the amount of agreement that can be expected from random chance, and 1 represents perfect agreement [21]. Additionally, McNemar test was used to compare paired nominal data in regard to the presence of *Candida*. The diagnostic accuracy, defined as the sensitivity and specificity of each *Candida* detection method was calculated according to the following formulae and presented as a percentage [22, 23].

Sensitivity = Number of true positives/ (number of true positives + number of false negatives

Specificity= Number of true negatives/ (number of true negatives + number of false positives

The sensitivity and specificity of each method were calculated with the data generated by the other method as reference [13].

For the secondary objective, the total inflammation score was calculated as the sum of the Schwartz area and severity index scores [18]. For the checkerboard technique, the total *Candida* score was calculated by summing the scores of the five *Candida* species according to the coding index presented in Table 2. For the culture technique, the *Candida* counts were first converted to checkerboard scores using the coding index [13] presented in Table 3, and then summed to obtain the total *Candida* score. A Spearman's rank correlation analysis was performed to examine the association between the total *Candida* scores and the level of inflammation using each method.

RESULTS

The percentage of the samples testing positive for each *Candida* spp. using DNA-DNA checkerboard hybridization technique and culture technique is presented in Table 4. For DNA-DNA checkerboard hybridization technique these were: *Candida albicans* (11.5%), *Candida dubliniensis* (34.6%), *Candida glabrata* (38.5%), *Candida krusei* (30.8%), *and Candida tropicalis* (46.2%). *Candida tropicalis* was the only species that was detected with the culture technique, present in 3.8% of the total samples. The Kappa coefficients for all *Candida* spp. presented in Table 5, were <0, showing a "lack of agreement (<0 to 0.2)" between the two techniques in regard to the presence of *Candida* spp. The difference in the detection of *Candida* spp. between the two techniques was statistically significant for all species ($p<0.05$) except *Candida albicans* ($p=0.25$), also presented in Table 5 (Additional data: Appendix 1).

The specificity of the DNA-DNA checkerboard hybridization technique for each *Candida* spp. calculated by using culture as the reference method was, *Candida albicans* (88.5%), *Candida dubliniensis* (65.4%), *Candida glabrata* (61.5%), *Candida krusei* (69.2%), and *Candida*

tropicalis (52%). The specificity of culture calculated by using checkerboard as the reference method was 100% for all *Candida* spp., except for *Candida tropicalis* which was 92.9%. However, the sensitivity of DNA-DNA checkerboard hybridization and culture for the detection of *Candida* spp. using each technique as a reference standard for the other was observed to be zero for all species (Additional data: Appendix 2).

Figure 1 and 2 present a visual depiction of the distribution of the inflammation scores in relation to *Candida* scores using the two techniques. There was a non-significant correlation between *Candida* scores and the extent and severity of inflammation using the culture technique (Spearman's correlation coefficient $\rho = -0.3$; $p > 0.05$), and a statistically significant positive correlation (Spearman's correlation coefficient $\rho = 0.4$; $p=0.05$), using DNA-DNA checkerboard hybridization technique.

DISCUSSION

The aim of this study was to compare two laboratory-based techniques, namely: microbial culture and DNA-DNA checkerboard hybridization, for their diagnostic accuracy for the detection of *Candida* spp. in patients with denture stomatitis. The results from this study suggest that although both techniques had suboptimal sensitivity, checkerboard hybridization technique had better specificity for the detection of *Candida* spp., at least when using the other as a reference. Additionally, both techniques did not exhibit any agreement for the detection of *Candida* spp. Furthermore, when comparing the correlation between *Candida* count and inflammation scores, a statistically significant correlation was observed using DNA-DNA checkerboard hybridization. According to the extent of our search thus far, this study appears

to be the first comparing the diagnostic accuracy of conventional laboratory cultures and DNA-DNA checkerboard hybridization for the detection of *Candida* spp. in patients with DS.

While DNA-DNA checkerboard hybridization has been widely used for studying microbiota in a variety of oral conditions [11, 24-31], conventional culture techniques continue to be routinely used for the identification and quantification of *Candida* spp. Since checkerboard allows for the simultaneous analysis of samples for a large number of microorganisms it is, therefore, possible to identify microbial cells that may not have been previously detected by conventional culture techniques [11, 24, 30, 32, 33]. This may be due to the fact that while culture techniques are limited to quantifying *Candida* spp. on the basis of viability, checkerboard can detect non-viable *Candida* spp. as well, on the basis of their genetic variability [34, 35]. This may improve the understanding of a possible role of byproducts of non-viable *Candida* in the inflammation observed. It has been reported previously that non-viable *Candida* contributes to cellular invasion through induced endocytosis and therefore, may play an important role in the pathogenicity of *Candida* [36]. It should be considered that although the palatal mucosa is in close contact with the denture surface and may harbor a large quantity of *Candida* species, a significant quantity of these may be uncultivable. Therefore, the use of checkerboard in such a case may prove advantageous.

Furthermore, culture techniques can be significantly time consuming, requiring up to 24-72 hours for the final identification of species [7, 37-39]. In clinical practice where targeted therapy may be necessitated, an excessively time consuming laboratory procedure may have an impact on clinical and patient outcomes. Our results are in contrast with some studies where culture techniques demonstrated satisfactory sensitivity and specificity utilizing selective media. These studies showed the overall sensitivity and specificity to range between

92% and 99% for the detection of *Candida albicans, tropicalis,* and *krusei* [32, 40-43]. However, it should be noted that these studies were not specific to denture stomatitis, and a gap in this area of research was evident.

The diagnostic accuracy of laboratory techniques is also dependent on procedural errors that may occur during the sampling and storage process [44-46]. In this study, biofilm samples were collected from the inflamed parts of the palatal mucosa, transported on ice and processed within recommended time frames [2, 7, 12, 47, 48]. It was interesting to observe that most of the samples showed negative results for the presence of *Candida* spp. using culture. This may be due to the limitations of culture in comparison to molecular techniques [34, 35, 49]. However, it was also observed that both techniques yielded an overall low *Candida* count, which may highlight the importance of the site from where the oral samples were collected. It has been reported that samples taken from whole saliva, and fitting surfaces of dentures yield a higher *Candida* count as compared to swabs from denture bearing palatal mucosa in patients with DS [7, 50]. Similar observations were also made in our main clinical trial, where 18.8% and 77% of denture sonicate and mucosal swabs respectively, were negative for *Candida* species [2]. Additionally, the use of different apparatus for the collection of samples may also have an impact on the detection of target microorganisms [45]. Therefore, even though standardized, routinely used sampling methods were used for each technique in this study, the possibility that cotton swabs and brushes used for culture and checkerboard respectively may have played a role in the differences in *Candida* counts observed between the two techniques should be considered.

The main limitation of this study is its limited sample size. Therefore, caution is advised for the interpretation of these results. However, this pilot data will serve for the development of future studies using DNA-DNA checkerboard hybridization for the diagnosis of DS.

CONCLUSION

DNA-DNA checkerboard hybridization has the potential to be used as an alternative method for the detection and quantification of *Candida* species in denture stomatitis. Further studies are however needed to improve the quality of evidence available.

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Table 1: Microbial species assessed by DNA-DNA checkerboard hybridization

Species	*Reference
Candida albicans	90028
Candida dubliniensis	7987S
Candida glabrata	2011
Candida krusei	6258
Candida tropicalis	4563

*ATCC, except if other collection is mentioned

Table 2: Coding index for signals generated by DNA-DNA checkerboard hybridization

0	No signal
1	Signal weaker than the lowest control standard ($<10^5$)
2	Signal equal to the lowest control standard ($=10^5$)
3	Signal stronger than the lowest but weaker than the highest control standard ($>10^5$ and $<>10^6$)
4	Signal equal to the highest control standard ($=10^6$)
5	Signal stronger than the highest control standard ($>10^6$)

Table 3: Conversion index of culture based CFU count to DNA-DNA checkerboard hybridization scores

CFU count	Converted checkerboard score
0	0
<75,000	1
>75,000 and <125,000	2
>125,000 and <875,000	3
>875,000 and <1,125,000	4
>1,125,000	5

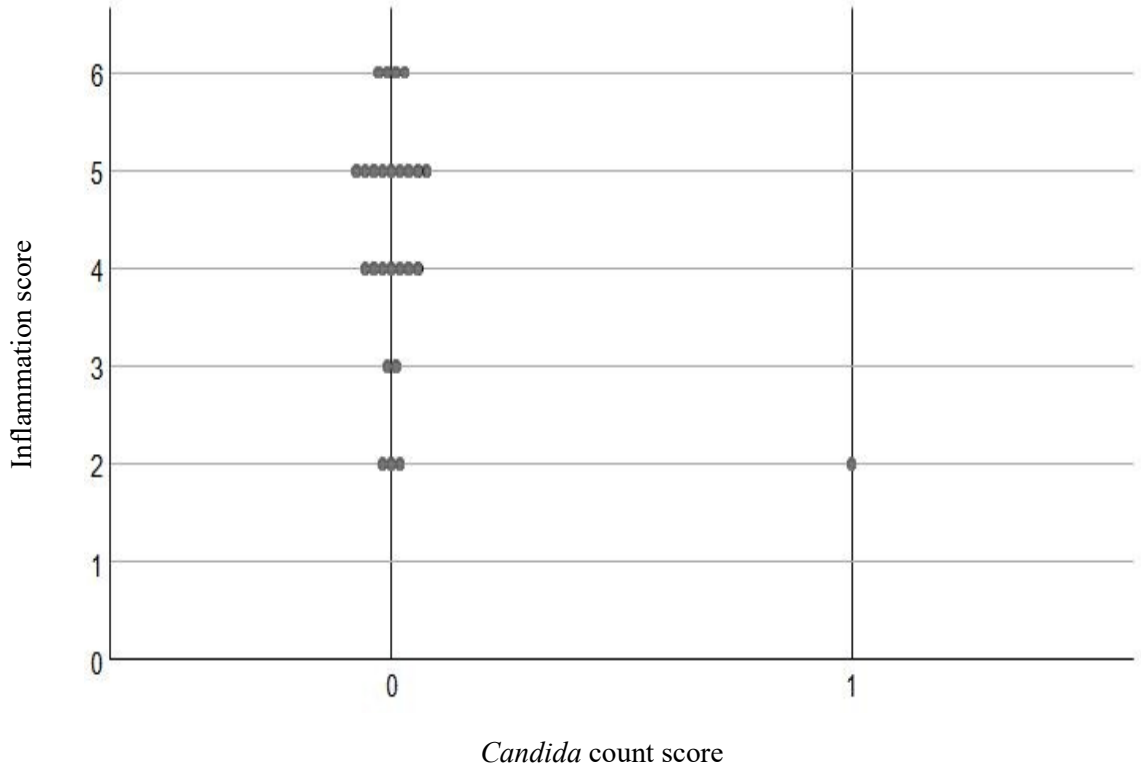
Table 4: Percentage of total samples positive for each *Candida* species using DNA-DNA checkerboard hybridization and culture techniques

Technique	*C. albicans*	*C. dubliniensis*	*C. glabrata*	*C. krusei*	*C. tropicalis*
Checkerboard	11.5	34.6	38.5	30.8	46.2
Culture	0	0	0	0	3.8

Table 5: Comparison between DNA-DNA checkerboard hybridization and culture techniques for the detection of *Candida* species

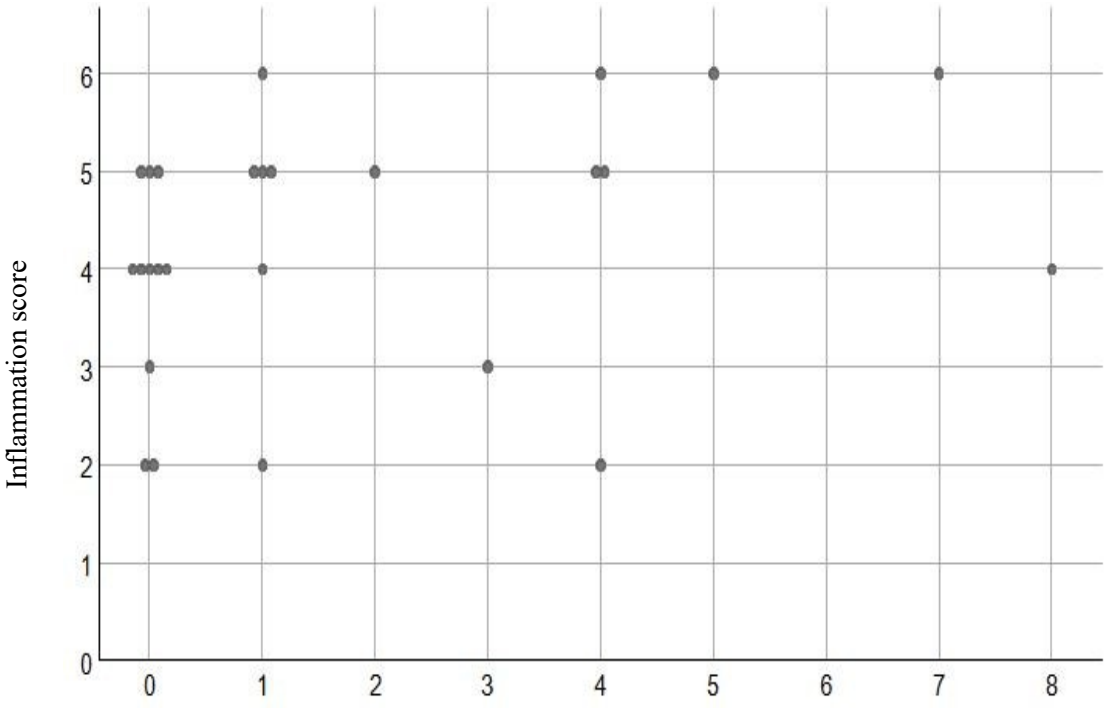
	C. albicans	*C. dubliniensis*	*C. glabrata*	*C. krusei*	*C. tropicalis*
Kappa	0	0	0	0	-0.07
P value[a]	.25	.004	.002	.008	.003

[a] McNemar Test



Spearman's correlation coefficient (p value) -.30 (.13)

Figure 1: Distribution of inflammation and total *Candida* score using culture technique



Spearman's correlation coefficient (p value) .380 (.05)

Figure 2: Distribution of inflammation and total *Candida* scores using checkerboard techniques

3.2 MANUSCRIPT 2

Salivary biomarkers in denture stomatitis: A systematic review

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ABSTRACT

Introduction

Denture Stomatitis (DS) is an oral biofilm-associated chronic inflammation of the palatal mucosa associated with denture use. A better understanding of the inflammatory salivary biomarkers involved in this disease may offer the opportunity to use these biomarkers for the early diagnosis of DS and monitor the palatal inflammation, along with understanding the association of DS with systematic diseases.

Objective

To systematically examine the literature on the salivary biomarker profile of individuals with DS compared to healthy individuals.

Methods

The systematic review followed the Preferred reporting items for systematic reviews and meta-analyses (PRISMA) guidelines. Medline (via OvidSP), PubMed, EMBASE and Cochrane central register for controlled trials were searched for eligible studies from the beginning of the archives until April 2017, and complemented by hand searching. Experimental and observational studies with adult participants, and including a control group or subgroup analysis, providing data on salivary biomarkers were included in this review. Publications in languages other than English or French were excluded. The level of evidence was graded using the Oxford center for evidence-based medicine (OCEBM) 2011 scale, while the assessment of methodological quality was conducted using Strengthening the reporting of observational studies in epidemiology (STROBE) statement, and graded according to the Olmos scale.

Results

From a total of 882 citations, 20 were selected for full-text review, and 9 were included in the systematic review (8 observational, 1 clinical trial). There were some contrasting observations between the studies included. A majority of the included studies suggested that there was a statistically significant difference in the levels of salivary cytokines (IL-6, CCL3, and TGF-β, CXCL8, GM-CSF and TNF-α) between DS and healthy controls ($p<0.05$). Only, two studies concluded that the difference in the levels of several salivary cytokines (IL2, IL12, IFN-Y, IL-4, IL-8, IL-10, IL-17, TNF-α, and ICAM-1), between DS and healthy groups was not statistically significant. The level of evidence for the majority of studies was 3 according to the OCEBM scale. Three studies were graded as C, four as grade B and one as grade A using the Olmos scale.

Conclusion

Current evidence suggests that the palatal inflammation observed in denture stomatitis is significantly associated with the levels of specific salivary cytokines. Future studies using more rigorous designs are recommended to further clarify the diagnostic capacities of salivary biomarkers in DS.

INTRODUCTION

Denture Stomatitis (DS) is an oral biofilm-associated chronic inflammatory disease that affects the oral mucosa covered by a removable prosthesis [1-6]. It is the most prevalent disease among completely edentate individuals, affecting one-third of all denture wearers [6-9]. In addition, it affects individuals wearing partial prosthesis or obturators, and individuals undergoing orthodontic treatment with intraoral removable appliances [10-14]. While DS is generally asymptomatic, some patients may experience mucosal swelling or bleeding, altered taste, burning sensation, halitosis, xerostomia, and dysphagia [3, 15-18].

The etiology of DS is considered to be multifactorial, and continues to be poorly understood, since various factors predispose individuals to this disease [6, 19-22]. These risk factors as identified in the literature include poor oral and denture hygiene habits, tobacco use, denture trauma, nocturnal denture wear [4, 6, 8, 21, 23-30]; the use of medication such as corticosteroids [31-36]; immunosuppressive conditions and xerostomia [18, 35, 37-39], which alter the immunological and physiological functions of saliva within the oropharynx [40, 41].

The oral and denture biofilm plays a critical role in the pathogenesis of DS, as it provides a favorable environment for the colonization of microorganisms such as *Candida* spp. [23, 42-45]. While defense mechanisms such as phagocytosis may be impaired in both young and elderly patients affected by DS [45], older population groups may be further predisposed to DS due to age-related deterioration in host immunity [46]. The inflammatory cascade observed in DS demonstrates an interaction between cells and inflammatory mediators such as vasoactive amines (histamine, serotonin), phospholipids (platelet-activating factor), arachidonic acids (prostaglandins, leukotrienes) and cytokines (tumor necrosis factor, interleukins, interferons, and colony stimulating factors) [47-49]. The release of cytokines is

mediated by the host innate immunity through local or recruited neutrophils and macrophages responding in an acute manner through diapedesis, chemo-attraction, phagocytosis and activation events [50, 51]. A higher prevalence of IgG immunoglobulins and distinct cytokine profiles in DS can suggest the involvement of saliva in the humoral and cell-mediated adaptive immunity [44, 52].

Salivary secretory immunoglobulin A (sIgA), lactoferrin, lysozyme, and histatins have demonstrated antifungal effects [53, 54]. While IgA protects against antigen invasion and is seen in higher concentrations in saliva obtained from DS subjects [13], lactoferrin inhibits microbial growth by sequestering essential iron and also demonstrates non-iron-dependent antibacterial, antifungal, antiviral, antitumor, anti-inflammatory, and immunoregulatory activities [54]. Similarly, a reduction in salivary neutrophils, induced through an IL-17 pathway blockade may increase the susceptibility to DS [55-57]. Therefore, examining salivary function and the concentration of various salivary biomarkers in DS may be useful in assessing susceptibility to this disease [58].

The objective of this systematic review was to identify and evaluate the quality of evidence on the reported association between salivary biomarkers and DS. The specific question addressed was: What changes are observed in levels of various salivary biomarkers among adult denture wearers with and without DS?

METHODOLOGY

Protocol and registration

This systematic review has an unpublished protocol. The reporting has been done according to the PRISMA guidelines [59].

Inclusion and exclusion criteria

All experimental and observational studies reporting on the salivary profile observed in DS were eligible for screening, irrespective of the demographic characteristics of the participants involved. Studies were only included in the review if: 1) They were conducted with adult human participants, 2) DS was the main focus of the study, 3) They involved the collection of saliva for analysis, 4) They had a control group or subgroup analysis, and 5) A full text was available in English or French. Studies that did not meet the inclusion criteria, or were based on animal models, case reports/series, or reviews were excluded.

Search strategy

Combinations of various MeSH (Medical subject heading) terms and keywords were used to develop a detailed search strategy with the assistance of an expert librarian at the health sciences library of the Université de Montréal presented in Table 6. Using the search strategy, a comprehensive literature search was conducted on various databases including Medline (via OvidSP), PubMed, EMBASE and Cochrane central register for controlled trials from the beginning of archives to April 2017. Additionally, grey literature was searched on Google scholar and System for information on grey literature in Europe. No age, language or publication year limits were applied during the execution of the search strategy.

Data Collection and Analysis

Data Screening and study selection

Two reviewers (MFK and MA) independently scanned titles and abstracts of all retrieved articles for relevance. Articles meeting the general inclusion criteria and relevance were selected for full-text review, and their bibliographies were further scanned manually to identify any other potential studies. Following the full-text review, studies not meeting the

inclusion criteria were subsequently excluded and the reason for exclusion was recorded. A schematic flowchart representing the various steps involved in the study selection process of the systematic review is shown in Figure 3. Disagreements were resolved through discussion and when required, the expertise of a third reviewer (EE) was sought. Inter-reviewer reliability was evaluated with Kappa statistic to ensure "good" agreement (κ value >0.7) [60].

Data Extraction

Following data screening and study identification, the selected studies were independently reviewed by the reviewers (MFK and MA) for data extraction. From each study, the following data were collected: authors, year of publication, country of the study, study type and design, characteristics of the sample population, type of saliva (stimulated/unstimulated) and clinical classification of DS.

Methodological quality and risk of bias assessment

The selected studies were independently analyzed by both reviewers. The level of evidence was established using the Oxford center for evidence-based medicine (OCEBM) 2011 scale [61]. The quality of observational studies was assessed using the Strengthening the reporting of observational studies in epidemiology (STROBE) checklist, and graded according to the Olmos scale as; “A”- the study is in agreement with more than 80% of the STROBE criteria, “B”- 50% to 80% criteria met, and “C”- less than 50% of criteria met [62, 63]. Clinical trials were assessed for risk of bias according to the Cochrane collaboration’s Risk of bias (RoB) assessment tool using Review Manager 5.3. It included selection, performance, attrition, detection and reporting bias.

Statistical analyses

A wide variety of outcomes were investigated by the studies included in this systematic review. Due to the lack to homogeneity among the studies included, a priori meta-analysis was not planned for this review.

RESULTS

Study characteristics

A total of 882 studies were identified by database searching, and 376 titles and abstracts were eligible for screening after duplicate removal. Based on their relevance, only 20 studies were selected for full-text review. Out of those selected for full-text assessment, 11 were excluded, and a total of 9 studies were included in the final pool of studies selected for the systematic review process.

Among the studies included, the oldest was conducted in 2002 [44] and the most recent in 2015 [22]. Three studies were conducted in USA [44, 52, 58], three in Brazil [45, 64-66], one in Croatia [67], one in Spain [68] and one in Thailand [22]. All studies had a cross-sectional design except one [67], which was an RCT. Participants in eight of the nine studies were recruited through university-based clinics or hospitals, while one study recruited through elderly nursing homes [68]. The sample sizes of the studies ranged from 17 to 128 participants, and the age ranged between 33 and 84 years. All studies comprised of an adult (over the age of 18 years) study sample.

The inclusion and exclusion criteria employed by the studies were similar i.e., participants with mucosal or palatal lesions other than DS were excluded. Similarly, participants with significant or serious systemic diseases were also excluded. One study [68] however, did not

exclude on the basis of local or systemic factors as the objective of this study was to identify various local and systemic risk factors of DS. Additionally, another study [67] did not explain their exclusion criteria clearly. The diagnosis of DS was primarily based on visual examination of the palatal mucosa and confirmed by microbiological tests. While eight of the included studies used the Newton's classification of DS [69], one study [44] did not mention any specific index for visual examination. All studies collected unstimulated whole saliva for analysis. The characteristics of the included studies and the relevant findings are summarized in Table 7.

Study outcomes and measurements

Six studies [22, 44, 45, 64, 66, 67] reported levels of various salivary cytokines in DS, while two [52, 58] reported the proteomic profiles and one reported salivary pH levels [68]. Most studies reported on at-least two saliva related outcomes.

In most studies salivary cytokines were quantified using the Enzyme-linked immunoabsorbent assay (ELISA) and presented as pg/ml protein [22, 44, 45, 64, 66, 67]. Proteomic profiling was done using the Surface enhanced laser desorption/ionization time of flight mass spectrometry (SELDI-TOF/MS) [52], and Liquid chromatography-mass spectrometry/liquid chromatography-tandem mass spectrometry (LC-MS/MS) [58]. The salivary neutrophil function was determined by measuring the neutrophil phagocytic activity expressed as the mean percentage of viable neutrophils [45].

Methodological quality and level of evidence

Following the OCEBM scale 2011 [70] for grading the level of evidence, six studies were graded as level 3 [22, 44, 45, 58, 64, 66], one as level 3b [52] and two as level 2 [67, 68]. Using the STROBE checklist, and the grading criteria by Olmos et al. [63] for quality

assessment, three [44, 45, 58] out of the 9 observational studies were graded C, four [22, 52, 64, 66] were graded B, and one [68] was graded A.

While most studies adequately presented the study objective, only one clearly stated the study hypothesis [68]. Similarly, while the study setting, location, inclusion or exclusion criteria were adequately presented along with the diagnostic criteria used, most studies failed to provide clear details about the sampling techniques, sample size calculation, allocation generation and concealment. Details about how the missing data were interpreted were also absent. Key study findings were presented in all reports, but discussion on limitations and generalizability of results were lacking.

All studies except one [58] briefly addressed potential sources of bias, methods of measurements, outcome variables and methods of analysis. However, only one study [68], discussed all these details adequately and was subsequently graded "A" for its methodological quality. The majority of the studies provided a source of funding. The risk of bias assessment performed for the only clinical trial included in the systematic review [67], showed either a "high" or "unclear" risk for all categories; presented in Figure 4.

Salivary cytokines and denture stomatitis

The six studies that examined the relationship between salivary cytokines and DS in people with, and without DS showed similar results, while the remaining two studies showed contrasting results [22, 44, 45, 64, 66, 67].

Examining cytokines (CXCL-8, GM-CSF and TNF-α) that are known to affect neutrophil function, one study [45] showed that the levels of CXCL-8 were higher in both young and older DS groups, than their age matched controls without DS ($p<0.01$). Additionally, both older groups (with and without DS) had higher levels of CXCL-8 than the young groups (with

and without DS) ($p<0.01$). Levels of GM-CSF were found to be higher in young DS group than age matched controls, and with both older groups ($p<0.01$). No difference was observed between older DS group and age matched controls ($p>0.01$). TNF-α was significantly higher in older DS subjects than their age matched controls without DS and with young DS group ($p<0.01$). No statistically significant difference was observed when comparing the young DS group with their age matched control group ($p>0.01$). The only clinical trial eligible for this systematic review [67] also showed that there was a statistically significant post-treatment reduction in the levels of TNF-α ($p<0.001$) and IL-6 ($p<0.001$) following laser phototherapy in the experimental group, but not in the control group.

Another study found that the levels of IL-6, CCL-3 and TGF-β were also higher in elderly DS patients than in elderly and young controls ($p<0.001$) [64]. Similarly, another study [66] showed that the salivary levels of IL-4 were higher in elders with DS ($p<0.05$), levels of IL-10 were higher in young DS group ($p<0.05$) and levels of IL-12 were lower in elders with DS ($p<0.001$), when compared with their respective age matched controls. Furthermore, levels of IL-4 were higher ($p<0.05$) while IFN-Ɣ were lower ($p<0.001$) in elders regardless of DS status.

However, one study comparing the Th-1 and Th-2 cytokine profiles in patients with and without DS, showed no significant difference in mean levels of IL-2, IL-12, IFN-Ɣ, IL-4 and IL-10 ($p>0.05$) [44]. Similarly, another study [22] also showed that the differences observed in levels of IL-6, IL-8, IL-10, IL-17, TNF-α and ICAM-1 in elders and adults were not statistically significant, regardless of the status of DS or the presence of *Candida* spp. ($p>0.05$).

Salivary neutrophil function

Various characteristics of neutrophil function in the saliva were examined in three different studies by the same research group [45, 64, 66]. The first study investigating age related changes in salivary neutrophils, showed that individuals with DS (both old and young DS groups) had a reduced salivary neutrophil count when compared to their respective age matched controls ($p<0.01$). Overall, the older age group (with and without DS) had a significantly higher neutrophil apoptosis rate ($p<0.01$), lower neutrophil count ($p=0.0015$), and lower phagocytic activity ($p<0.01$), than the two younger age groups [45].

Investigating expression of various proteins on salivary neutrophils in the second study [64], it was shown that there was an increased expression of TLR4 ($p<0.05$) and CD16 ($p<0.01$) in the young group than the older group, regardless of disease status. Similarly, a higher expression of CD32 and CD11b was observed in the younger group, with or without DS ($p>0.05$). The third study [66] concluded that there is a decreased expression of CD66b in elderly DS subjects ($p<0.01$) and decreased CD64+ levels in young DS subjects ($p<0.001$) compared to their respective age matched controls. Furthermore, an increased expression of CD69 was reported in both DS groups, regardless of age ($p<0.01$).

Pro-inflammatory mediator activity and salivary pH levels

Only one study [64] examined the pro-inflammatory activity of salivary peroxidase (SPO) and elastase in elderly patients with DS, and compared it with healthy elderly and young controls. SPO activity was observed to be lower in the elderly groups than in the younger controls, and lowest in elderly DS patients ($p<0.001$). Similarly, elastase activity (ELA) was also lower in both elderly groups, than in healthy young controls ($p<0.05$) regardless of disease status.

However, nitric oxide (NO) levels were found to be highest in elderly DS patients and lowest in healthy young controls ($p<0.001$).

Only one study [68] examined the role of salivary pH in the etiology of DS, and concluded that there was a positive correlation between salivary pH and any stage of DS ($p=0.018$).

Salivary proteomic profile

Only two studies examined the salivary proteomic profiles to observe the differences between DS and healthy individuals [52, 58]. Comparing the proteomic profiles of edentate individuals with and without DS using SELDI-TOF/MS [52], 61 protein masses were identified. Of these, 48 masses were small peptides ($m/z < 2300$) and were up regulated in DS type II. Using LC-MS/MS four salivary gland proteins were identified; statherin (STAT), cystatin-SN (CYTN), carbonic anhydrase 6 (CAH6) and peptidyl-prolyl *cis-trans* isomerase (PPIA). The levels of each were compared between DS and controls.

As compared to controls, the DS group had a three-fold increase in levels of STAT ($p=0.001$), a two-fold increase in KNG-1($p=0.04$), a four to six-fold increase in DSC-2 ($p=0.008$), and a two-fold increase in PPIAs ($p<0.01$), CYTN and CYTC ($p<0.05$). In addition, immunoglobulin (Ig) fragments were also found to be elevated in individuals with DS type II and to a lesser extent in DS type III. Similar results were also observed in the other study that presented proteomic profiles in DS type II, III and healthy controls [58], showing an increase in the differential expressions of CYTN, CYTC, CAH6 and several Ig fragment levels in DS patients ($p<0.01$) compared to controls.

DISCUSSION

Overall, this systematic review suggests that DS may be associated with an impaired salivary defense mechanism. The levels of various salivary components such as salivary cytokines and neutrophils may vary according to the disease severity and to a certain extent, the age of the host [22, 45, 46, 64, 65].

In fact, most studies included in this review suggest that a statistically significant difference in levels of various cytokines can be observed in both young and elderly patients with DS compared to their age-matched controls [22, 44, 45, 64, 66]. Similar results were found in the only clinical trial included, that reported a decrease in the levels of TNF-α and IL-6 in DS subjects, as the inflammation was reduced following laser phototherapy, irrespective of age [67].

Results from most of the included studies are also in agreement with other studies that have investigated the role of salivary biomarkers in DS in the presence of systemic diseases. For instance, vascular endothelial growth factor (VEGF) considered being the underlying biomarker of chronic inflammation and autoimmune conditions in the oral cavity is decreased in type I DS and increased in type II DS, among individuals with type 2 diabetes mellitus, thus making them more susceptible to DS [71-73]. Collectively, these findings highlight the importance of saliva and its components towards developing and maintaining local immunity to DS.

Studies included in this review did not vary significantly in their inclusion criteria, diagnostic indices, and study design. They recruited adult complete denture wearers, used valid indices for disease classification and collected unstimulated whole saliva for analyzing biomarkers. However, almost all studies had a cross-sectional design that is not ideally suited for inferring

causality and association, as it is not possible to determine the sequence of events [74]. Additionally, variations in standardized systems used for the processing of samples and weak methodological quality of most studies suggest that caution should be used when interpreting and generalizing the results. For instance, including non-edentate patients wearing partial dentures may lead to different results given that the presence of teeth may change the microbial environment, and hence the immune response and expression of biomarkers [9]. Further investigation of the host's immune reaction can help promote a better understanding, the role saliva and salivary biomarkers play in the initiation of the disease and of the underlying mechanisms governing its pathogenesis.

CONCLUSION

Current evidence suggests that the levels of specific salivary cytokines may be associated with palatal inflammation observed in DS. A clear understanding of the nature of salivary biomarkers in DS has numerous clinical implications and may help in efforts to improve patient care. Future studies using more rigorous designs are recommended to further clarify the association between DS and salivary biomarkers.

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Table 6: Medline and PubMed search strategy

1	exp Dentures/	43195
2	Space Maintenance, Orthodontic/	700
3	Palatal Obturators/	1774
4	("denture$" or "palatal obturator$" or "orthodontic space maintenance$").mp. [mp=title, abstract, original title, name of substance word, subject heading word, keyword heading word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]	49616
5	exp Stomatitis/	15532
6	Candidiasis, Oral/	4522
7	((Mouth Diseases.mp. or specific dental prosthesis/ae or Stomati$.mp. or mucositi$.mp. or oromucosititi$.mp. or denture.mp.) adj3 stomatitis.mp.) or oral candidias$.mp. or thrush.mp. or oral monilias$.mp. [mp=title, abstract, original title, name of substance word, subject heading word, keyword heading word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]	25734
8	Saliva/	37961
9	Biofilms/	23656
10	("Saliva$" or "biofilm$").mp. [mp=title, abstract, original title, name of substance word, subject heading word, keyword heading word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]	139751
11	1 or 2 or 3 or 4	50214
12	5 or 6 or 7	33117
13	8 or 9 or 10	139751
14	11 and 12 and 13	313
(((dentures[MeSH]) OR ("space maintenance, orthodontic"[MeSH]) OR (palatal obturators[MeSH:noexp]) OR (((denture*[tiab] OR palatal obturator*[tiab] OR orthodontic space maintenance*[tiab])))) AND (((Stomatitis[MeSH]) OR (Candidiasis Oral[MeSH:noexp]) OR (((Mouth Diseases[tiab] OR specific dental prosthesis/ae[tiab] OR Stomati*[tiab] OR mucositi*[tiab] OR denture stomatitis[tiab] OR oral candidias*[tiab] OR thrush[tiab] OR oral monilias*[tiab])))) AND (((Saliva[MeSH:noexp]) OR (Biofilms[MeSH:noexp]) OR (((Saliva*[tiab] OR biofilm*[tiab])))))		

Table 7: Summary of characteristics of included studies and their findings

Author, year (Country)	Design	STROBE Quality	Level of evidence	Sample size (gender)	Mean age (years) ± SD	Study population	Saliva collection	Saliva related outcomes & Result summary	
Leigh, 2002 (USA)	Cross-sectional	C	3	Controls: 9 (3♂, 6♀) Cases: 8 (3♂, 5♀)	63.6 ±59.1	Complete and partial denture wearers	Unstimulated	Th-1 and Th-2 type cytokine profile: Mixed Th-1 and Th-2 cytokine profile observed No significant difference in mean IL-2, IL-12, IFN-ϒ, IL-4, and IL-10 between DS and non-DS patients (p>0.05)	
Gasparoto, 2009 (Brazil)	Cross-sectional	C	3	Group 1: Controls: 14, Cases: 14 Group 2: Controls:14, Cases: 14	 68.6 ± 0.9 69.4 ± 3 38.1 ± 3.9 33.8 ± 2.3	Complete denture wearers	Unstimulated	Function of salivary neutrophils: ↓ neutrophil count in older DS group than matched controls (p<0.01) ↓ neutrophil count in older controls than young controls (p=0.01) ↑ salivary neutrophils apoptosis rate in older DS group (p<0.01) ↓ salivary neutrophil phagocytic activity in elderly DS (p<0.01)	Salivary cytokine levels: ↑ CXCL8 levels in older DS group than matched controls (p<0.01) ↑ CXCL8 levels in young DS patients than matched controls (p<0.01) ↑ CXCL8 levels in older groups than young groups (p<0.01) No diff. in GM-CSF between older DS and matched controls (p>0.01) ↑ GM-CSF in young DS than matched controls (p<0.01) ↓GM-CSF in older group than young group (p<0.01) ↑ TNF-α in older DS group than matched controls (p<0.01) No diff. in TNF-α between young DS and matched controls (p>0.01) ↑ TNF-α in older DS group than young DS (p<0.01) ↓TNF-α in older controls than young control (p=0.003)

Simunovic-Soskic, 2010 (Croatia)	RCT	N/A	2	Placebo: 20 (8♂,12♀) Intervention: 20 (7♂,13♀)	58.8 ± 5.8 60.5 ± 6.4	Complete denture wearers	Unstimulated	Salivary level of TNF-α and IL-6 before and after LPT: Significant difference observed; before and after in TNF-α and IL-6 levels in treatment group ($p<0.001$); and between treatment and control group ($p<0.001$)		
Gasparoto, 2012[a] (Brazil)	Cross-sectional	B	3	Controls: 12 Cases: Group 1: 12 Group 2: 12 (N/A)	36.7 ± 4.8 66.5 ± 1.6 69.3 ± 2.8	Complete denture wearers	Unstimulated	Salivary levels of SPO, ELA, and NO activity: SPO activity lowest in elderly DS group ($p<0.001$) ↓ in older control than young control ($p<0.001$) ELA activity ↓ in older groups than young ($p<0.05$). No diff. between older DS and matched controls ($p<0.05$) NO activity ↑ in older DS than matched controls ($p<0.005$). ↓ in younger group than elders ($p<0.001$) Highest in elderly DS group ($p<0.001$)	Salivary cytokine levels: ↑ TGF-β levels in elder control than young controls ($p<0.001$) No diff. in IL-6, CCL3 between elder controls and young controls ($p>0.05$)	Expression of TLR4, CD16, CD32, CD11B on salivary neutrophils: ↓ expression of TLR4 ($p<0.05$) and CD16 ($p<0.01$) in elder group than the young group ↑ expression of CD32, CD11B in young controls compared to elder DS ($p>0.05$)
Gasparoto, 2012[b] (Brazil)	Cross-sectional	B	3	Controls: Group 1: 15 Group 2: 15 (13♂,17♀) Cases: Group 1: 15 Group 2: 15 (14♂,16♀)	 67.9± 1.2 38.9 ± 3.1 69.5 ± 1.6 46.8 ± 1.5	Complete denture wearers	Unstimulated	Salivary levels of IL-4, IL-10, IL-12 & IFN-Ɣ: ↑ level of IL-4 in elder DS group than age-matched controls ($p>0.05$) ↑ level of IL-4 in elders regardless of DS status ($p>0.05$)	↓ levels of IL-12 in elder DS group than matched controls ($p<0.001$) ↓ levels of IFN-Ɣ in elders regardless of DS status ($p<0.001$)	Expression of CD64+, CD66b, CD69, on salivary neutrophils: ↓ expression of CD66b in elderly DS than matched controls($p<0.01$) ↓ CD64+ levels in young DS than

								↑ levels of IL-10 in young DS than age-matched controls (p>0.05)	matched controls. (p<0.001) ↑CD69 expression in DS groups regardless of age (p<0.01)
Bencharit, 2012 (USA)	Cross-sectional	B	3b	Controls: 9 (1♂, 8♀) Cases: Group1: 6 Group 2: 4 (5♂ 5♀)	71.9 ± 9.7 70.3 ± 8.3 64.3 ± 9.8	Complete denture wearers	Unstimulated	Salivary proteins specific to DS: As compared to controls, DS group had threefold ↑ in levels of STAT (p=0.001), two-fold ↑ in KNG-1 (p=0.04), four to sixfold ↑ in DSC-2 (p=0.008), two-fold ↑ in PPIAs (p<0.01), ↑ CYTN & CYTC (p<0.05).		
Byrd, 2014 (USA)	Cross-sectional	C	3	Controls: 15 Cases: 15 (N/A)	N/A	Complete denture wearers	Unstimulated	Differentially expressed proteins associated with DS: ↑ CYTN, CYTC, CAH6 and several IG fragment levels in DS patients (p<0.01)	
Martori, 2014 (Spain)	Cross-sectional	A	2	84 (N/A)	83.7	Complete denture wearers	Unstimulated	Risk factors of DS associated with saliva: low salivary pH associated with DS (p=0.018)	
Pesee, 2015 (Thailand)	Cross-sectional	B	3	128 (42♂, 86♀)	57.1	Partial and complete denture wearers	Unstimulated	Salivary cytokine levels in DS: No difference in levels of IL-6, IL-8, IL-10, IL-17, TNF-α & ICAM-1 in elders and adults regardless of DS status and presence of *Candida* spp. (p>0.05)	

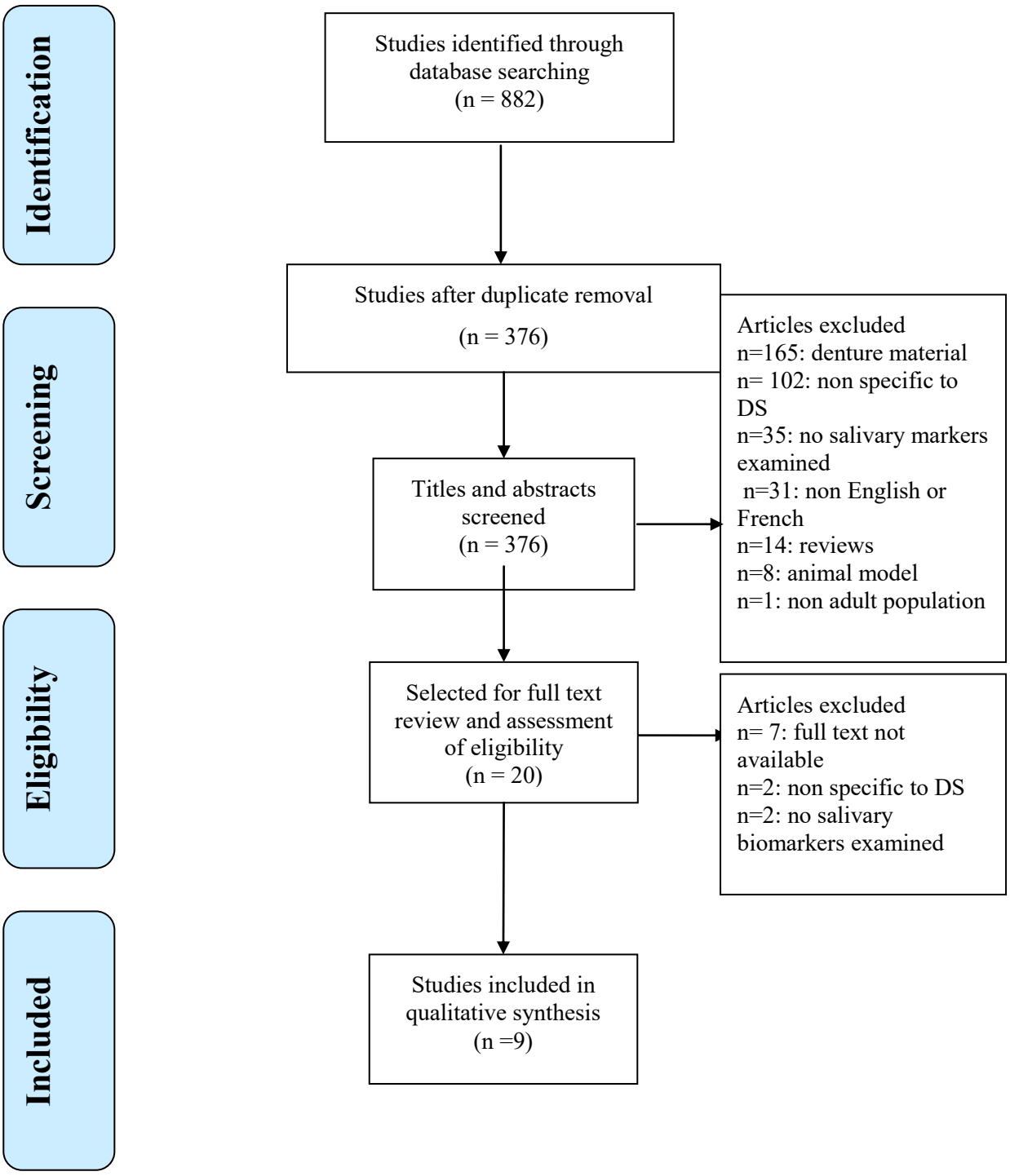

Figure 3: PRISMA flowchart of the systematic review

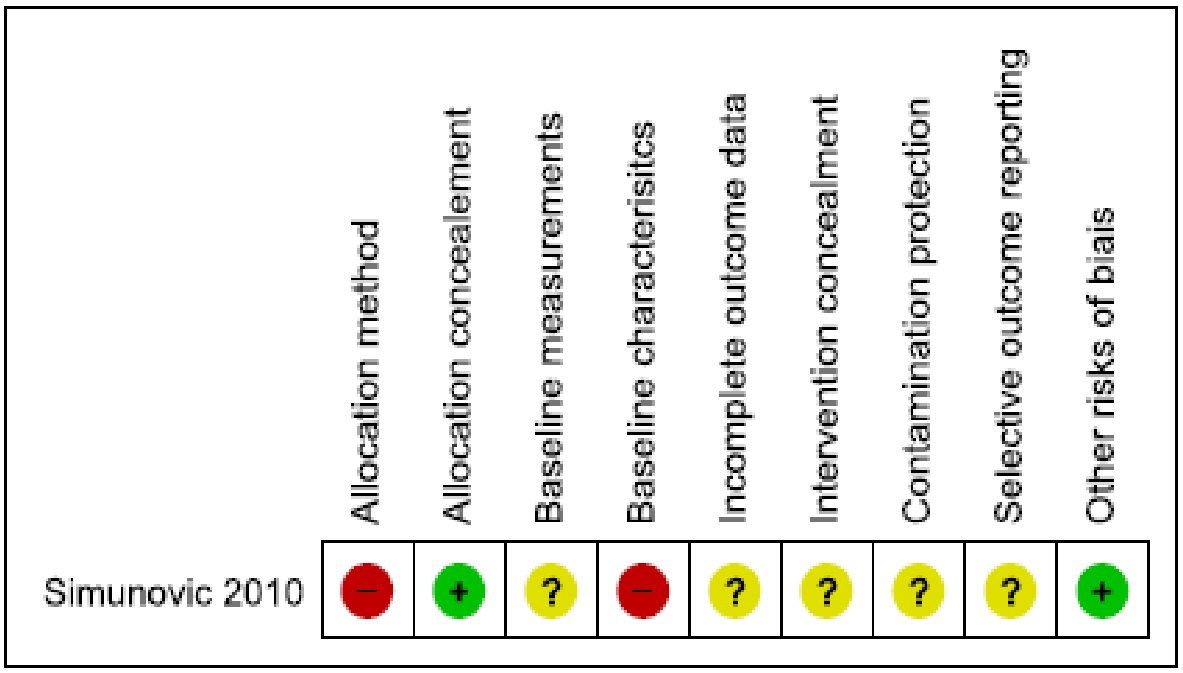

Figure 4: Risk of bias assessment

CHAPTER 4

DISCUSSION

The goal of this master's research project was two-tiered:

1) To compare the diagnostic accuracy of conventional laboratory cultures and DNA-DNA checkerboard hybridization for the detection of *Candida* spp. in patients with DS;
2) To systematically review current literature to examine the differences in the levels of various salivary biomarkers observed in healthy individuals and patients with DS.

The results from this master's project suggest that when considering one technique as the reference method for the other, both conventional culture and DNA-DNA checkerboard hybridization technique show suboptimal sensitivity. However, checkerboard hybridization technique had better specificity for the detection of *Candida* spp.

Furthermore, the results of the systematic review from this thesis suggest that the levels of salivary biomarkers may represent an association with the intensity of inflammation observed in DS.

Finally, further studies are needed to improve the quality of evidence available on the two topics studied in this thesis.

4.1 COMPARISON BETWEEN DNA-DNA CHECKERBOARD HYBRIDIZATION AND CULTURE FOR THE DETECTION OF *CANDIDA* SPECIES

4.1.1 Application for *Candida* detection in denture stomatitis

DNA-DNA checkerboard hybridization is a high-throughput molecular technique used for the successful detection of microorganisms not detected by conventional culture techniques [27, 188, 272, 273, 280]. It allows the simultaneous analysis of samples for a large number of microorganisms including *Candida* spp. and has been used to examine the microbiota in a variety of oral conditions including candidiasis [27, 272-279].

While checkerboard can detect *Candida* spp. on the basis of their genetic variability, microbial culture is limited to the detection of viable *Candida* alone [293, 294]. The ability of the former to detect non-viable *Candida* may substantiate previous reports suggesting that non-viable *Candida* contribute to cellular invasion through induced endocytosis [122] and may play an important role in its pathogenicity in DS. Additionally, non-selective culture media does not permit *Candida* spp. to be differentiated, which appear as convex, smooth, creamy colored colonies [207, 210, 211], requiring selective media for further identification [188, 218, 219]. Consequently, the final identification of *Candida* spp. isolated from DS samples may take up to 24-72 hours [25, 209-211]. In contrast, molecular methods like DNA-DNA checkerboard hybridization are time efficient, and their application complementary to phenotypic techniques may be more cost-effective, and improve the clinical and patient outcomes associated with DS [237, 295-297].

To our knowledge, there are no previous reports comparing the diagnostic accuracy of conventional laboratory cultures and DNA-DNA checkerboard hybridization for the detection of *Candida* spp. in patients with DS. While using each technique as a reference method for the other, we observed that both culture and checkerboard were highly specific in detecting *Candida* spp. However, both techniques lacked the sensitivity to rule out the presence of *Candida* spp. within the samples, with certainty. A significant lack of agreement was observed and the difference between the two techniques was statistically significant. Furthermore, a positive correlation between *Candida* counts and clinical inflammation levels was observed using DNA-DNA checkerboard hybridization, which was statistically significant.

Our results have some similarities with recent studies, although most of these were not focused on *Candida,* nor were they specific to DS. Leonhardt et al. [298] compared the microbial detection frequency of culture and checkerboard hybridization for 18 microbial species, sampled from 15 participants having dental implants. They showed that in comparison to checkerboard, the overall effectiveness of culture was lower for microbial detection. The difference between the two techniques increased as the checkerboard detection threshold increased from $\geq 10^5$ to $\geq 10^6$. This was due to the detection of a much higher number of microorganisms with checkerboard at a threshold of $\geq 10^5$ as compared to $\geq 10^6$. While *Candida* was detected using culture in three of the samples, it was not validated using checkerboard. It was concluded that culture and checkerboard should be used to complement each other.

Nascimento et al. [278] conducted a feasibility study examining the sensitivity of checkerboard for the detection of *Candida* spp. in oral candidiasis. Samples from pseudo-membranous plaques from the oral mucosa of three patients were collected and tested for the

presence of *C. albicans*, *C. dubliniensis, C. glabrata, C. krusei*, and *C. tropicalis*. While no signals were generated for the presence of *C. albicans*, all other species were present in quantities $<10^5$. Due to the small study size, no statistical tests were performed. Similar observations were made in our present study in which all *Candida* spp., if detected using checkerboard, were mostly present in quantities $<10^5$. Additionally, using checkerboard as the reference, culture showed a specificity of 100% for all *Candida* species. This meant that if there where were certain *Candida* spp. not detected by checkerboard, they was also absent using culture, thus having a high number of true negatives. This observation was expected, since as previously mentioned, checkerboard may also detect non-viable microorganisms.

Our results were also similar to a study by Moraes et al. [257] comparing the effectiveness of culture, PCR, and checkerboard for the detection of *Fusobacterium nucleatum* in 13 participants with infected endodontic lesions. They concluded that the three techniques varied to a large extent, and if a microorganism was detected in a sample using one technique, the other technique did not detect it ($p>0.05$). It was further suggested that checkerboard may be prone to decreased sensitivity if the DNA probes were not appropriately sensitized, resulting in incorrect cross-hybridization.

Papapanou et al. [287] examined the effectiveness of culture and checkerboard for the detection of microorganisms in the subgingival microbiota of 70 patients with periodontal disease. Similar to our study, the percentage of each microorganism present in the samples was observed to be higher using checkerboard as compared to culture. The authors suggested that a lower microbial count may have been observed with culture since it was not specifically optimized in comparison to checkerboard. For checkerboard, the authors emphasized the importance of optimized probe sensitivity for the correct detection of microorganisms.

The low presence of *Candida* spp. in the samples examined in our study can be related to the sample site. It has been reported that samples taken from whole saliva and fitting surfaces of dentures yield a higher *Candida* count as compared to swabs from denture bearing palatal mucosa, in patients with DS [25, 57]. Similar observations were also made in our main clinical trial, where 18.8% and 77% of denture sonicate and mucosal swabs respectively, were negative for *Candida* [2]. However, we were unable to find any study that compared *Candida* counts in samples obtained from palatal swabs and denture sonicate of DS patients, using checkerboard technique.

Sachdeo et al. [299] examined microbial counts in samples obtained from saliva and eight soft tissue sites in the oral cavity from 61 patients wearing dentures. The sensitivity of the assay was adjusted to detect counts of $\geq 10^4$. While all microbial species tested were detected on all surfaces, the concentrations varied significantly among sites. The highest concentration was detected on the dorsum of the tongue, and the lowest on the hard palate, buccal and vestibular areas ($p<0.001$). Additionally, microbial quantity was higher in saliva, compared to the swabs samples from the palatal mucosa ($p<0.001$).

Contrary to our observations, few previous studies have shown culture techniques to have optimal sensitivity and specificity. Odds et al. [218] showed that the overall sensitivity of culture using selective media ranged between 95% and 99% for the detection of *C. albicans, C. tropicalis,* and *C. krusei.* The study utilized yeast isolates from samples of the oral cavity, skin and genital area. Similarly, Pfaller et al. [220] showed the sensitivity and specificity of CHROMagar to range between 95% and 99% for the selective identification of *C. albicans and C. tropicalis* respectively, in yeast isolates from clinical samples obtained through stool and rectal swabs. Comparing Albicans ID and CHROMagar, two selective media for *Candida*

identification, Baumgartner et al. [221] utilized yeast isolates from rectal swabs, sputum, eyes, nose, throat, and broncho-alveolar fluid. The sensitivity and specificity were reported to be 93.6% and 99.8% respectively for Albicans ID, and 92.2% and 100% respectively for CHROMagar.

However, it is important to note that these studies were not specific to DS, and a gap in this area of research is evident. Similar contrasting results with some previous checkerboard studies were also noticeable, that successfully identified periodontal and endodontic microbiota, including *Candida* spp. [27, 272-279, 285].

4.1.2 Methodological issues

Comparing the culture and the DNA-DNA checkerboard hybridization techniques it is not possible to consider either one as a standard reference alone, since both these techniques are based on entirely different technologies. This was overcome by using each technique as the standard reference for the other, when examining their diagnostic accuracy [287].

We observed that despite adhering to standard guidelines, most of the samples showed negative results for the presence of *Candida* spp. with the culture technique. Taking into consideration the limitations of culture in comparison to checkerboard and other molecular techniques [293, 294, 300], this may be plausible. Numerous procedural errors may occur during the collection and storage of samples, which may affect the diagnostic accuracy of laboratory techniques [286, 301, 302]. Our observation of a low *Candida* count yielded by both techniques in this study and our previous study [2] is in concordance with previous reports [25, 57]. To minimize these errors, biofilm samples were collected from the inflamed

parts of the palatal mucosa, transported on ice and processed within recommended time frames [2, 25, 58, 242, 303].

Similarly, the choice of apparatus for the collection of samples may affect the accuracy of the detection methods significantly [302, 304, 305]. Therefore, even though standardized sampling methods were used for each technique in this study, the possibility that cotton swabs and brushes used for culture and checkerboard respectively, may have played a role in the differences in *Candida* counts observed between the two techniques should be considered.

Wall-Manning et al. [272] analyzed samples from 13 participants for the detection of gram-positive bacteria and *Candida* spp. in carious lesions using DNA-DNA checkerboard hybridization technique. They recommended that adjusting the probe sensitivity was a crucial step for the correct detection of microbial species. An error at this step may increase the cross-reactions and significantly reduce the sensitivity of the test. In this study, the DNA probes used were set to detect a minimum of 10^5 microbial cells. *Candida* count lesser than 10^5 were therefore not detectable and were graded as zero or absent. As highlighted by other studies, the sensitivity of the DNA probes in checkerboard technique may present a challenge for the correct detection and quantification of microorganisms [257, 294]. A more sensitive DNA probe, detecting smaller microbial quantities in the oral samples could have possibly reflected in our results as an overall increase in the prevalence of *Candida* spp. However, we argue that a lower DNA probe sensitivity is unlikely to hold any clinical significance, since commensal levels of *Candida* spp. are commonly observed among healthy individuals, and does not necessarily indicate a diseased state. Additionally, increased probe sensitivity in the checkerboard technique has been reported to cause an unfavorable increase in the cross-reactions between species leading to incorrect microbial identification [280]. Therefore, the

trade-off between increased sensitivity and specificity must be considered according to the study objective at hand.

4.2 SALIVARY BIOMARKERS IN DENTURE STOMATITIS

Overall, the studies included in our systematic review propose that the predisposition to DS may be linked to an impaired salivary defense mechanism. More specifically, it was suggested that the quantitative and functional imbalances of various salivary components such as the impaired production and function of salivary cytokines and neutrophils, may increase the susceptibility to DS [50, 58, 165, 169, 306]. Since the studies included showed contrasting results in relation to the concentration of various salivary biomarkers, DS type and the age of the study participants, it is unclear whether age plays a significant role in the individual susceptibility to DS [169]. In fact, most studies included in this review suggest that a statistically significant difference in the levels of various cytokines can be observed in both young and elder patients with DS as compared to their age-matched control groups [50, 58, 163, 165, 307].

Interestingly, these observations point towards the role innate immunity may have over acquired immunity, in response to the presence of *Candida* spp. in DS. For instance, neutrophils were observed to be reduced in quantity, exhibiting a higher apoptosis rate and reduced phagocytic activity. Additionally, lower levels of inflammatory modulators like peroxidase, along with impaired expression of various proteins like CD16, CD32 and TLR4, also affect neutrophil function. Similarly, differences in the levels of various cytokines in DS patients compared to those without DS, can also be attributed to neutrophil function regulated by the innate immune system. Likewise, changes in the differential expression of various

proteins including IgG antibodies in DS, also point towards the importance of the role of the innate immunity in determining how the host responds to the inflammation associated with DS.

Results from most of the included studies in this systematic review are also in agreement with other studies that have investigated the role of salivary biomarkers in DS in the presence of systemic diseases. For instance, vascular endothelial growth factor (VEGF) considered to be the underlying biomarker of chronic inflammation and autoimmune conditions in the oral cavity, is decreased in DS type I and increased in DS type II, among individuals with type 2 diabetes mellitus [71, 177, 178]. Collectively, these findings highlight the importance of saliva and its components towards developing and maintaining local immunity to DS.

Studies included in this review did not vary significantly in their inclusion criteria, diagnostic indices, and study design. They recruited adult complete denture wearers, used valid indices for disease classification and collected unstimulated whole saliva for analyzing biomarkers. However, almost all studies had a cross-sectional design, which is not ideally suited for inferring causality and association, as it is not possible to determine the sequence of events [308]. Additionally, due to variations in standardized systems used for the processing of samples and weak methodological quality of most studies, caution is needed in the interpretation and generalizability of the results. For instance, including non-edentate patients wearing partial dentures may lead to different results, given that the presence of teeth may change the microbial environment and hence the immune response and expression of biomarkers [10]. Further investigation of the host immune reaction can lead to a better understanding of the role saliva and salivary biomarkers play in the initiation of denture stomatitis, and the underlying mechanisms governing its pathogenesis.

4.3 STUDY LIMITATIONS AND RECOMMENDATIONS FOR FUTURE STUDIES

Our overall results using the DNA-DNA checkerboard hybridization technique show a positive correlation between *Candida* count and the amount of inflammation seen in DS. Nevertheless, careful interpretation is necessitated due to certain limitations.

As mentioned in Chapter 1, the geographic, socio-demographic and lifestyle associated factors may vary across populations, affecting the regional and global prevalence of DS. As our study was limited to the data collected from a relatively small sample from the Brazil center alone, it is therefore plausible that our results may be localized due to such global variations. However, primary data analysis from our previous clinical trial with the same participants did not show any statistically significant differences between the participants from Brazil and Canada, when examined for socio-demographic and other DS associated risk factors [2]. Differences in denture hygiene practices and age of the dentures were on the other hand evident, which may impact the presence of *Candida* and the associated mucosal inflammation. We therefore suggest that future studies employ culture and DNA-DNA checkerboard hybridization techniques to conduct a comparative analysis on a larger sample size, representative of multiple geographical locations, thus providing an improved global perspective.

The suboptimal sensitivity and specificity observed in this study may either be due to a substantially lower number of samples testing positive for *Candida* spp., reflective of a significantly low microbial count, or due to a bias associated with the technical aspects of sample collection and the choice of sampling site. To address this, we recommend future studies to obtain samples from whole saliva and the fitting surface of dentures. Additionally,

the dorsum of the tongue may also serve as an important site since it has shown to have the highest *Candida* count as compared to other sites in the oral cavity [299]. We theorize that following denture removal at night, the dorsum of the tongue stays in direct and nearly uninterrupted contact with the palate, and may therefore play a role in the palatal inflammation observed. A comparison of the association between *Candida* spp. count obtained from the dorsum of the tongue and the inflammation observed using DNA-DNA checkerboard hybridization and culture techniques could provide new insights into this domain of research.

Using DNA-DNA checkerboard hybridization technique, we observed an absence of signals generated in a majority of samples in this study, and others [272, 278, 298]. The need to develop modified methods specific for the detection of *Candida* spp. in the oral cavity, particularly in DS is therefore evident. However, increasing the probe sensitivity has been reported to cause unfavorable increase in the cross-reactions between species, leading to incorrect microbial identification [280]. Therefore, the trade-off between increased sensitivity and specificity must be considered according to the study objective at hand. We suggest that future studies may consider developing experiments for the optimization of probe sensitivity and standardization of cut-off thresholds based on clinically meaningful quantities of *Candida* in DS lesions.

Salivary diagnostics is an expanding field, and the utility of saliva as a diagnostic fluid and a potential future replacement of blood for the diagnosis of local and systemic conditions is a topic of great interest to clinicians and researchers alike. Therefore, we recommend that future studies explore the role of specific biomarkers pertinent to the inflammation observed in oral diseases, and investigate their potential role in systemic diseases in healthy and particularly immuno-compromised individuals.

CHAPTER 5

CONCLUSION

The results of this master's research project suggest that:

1. DNA-DNA checkerboard hybridization shows greater diagnostic accuracy compared to culture technique for the detection of *Candida* spp. in biological samples obtained from the palatal mucosa of patients with DS.
2. There is a statistically significant positive correlation between *Candida* count as quantified by checkerboard and the extent and severity of inflammation observed in DS.
3. The levels of salivary biomarkers may have an association with the inflammation observed in DS.
4. Individual susceptibility to DS may be affected by an impaired salivary function exhibiting an imbalance in the concentration and function of salivary cytokines and neutrophils.
5. Future studies with a larger sample size and rigorous research design, should be conducted to confirm the diagnostic accuracy of DNA-DNA checkerboard hybridization in patients with DS, and further clarify the diagnostic capacities of salivary biomarkers.

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APPENDICES

APPENDIX I: COMPARISON BETWEEN CULTURE AND DNA-DNA CHECKERBOARD HYBRIDIZATION TECHNIQUES FOR THE DETECTION OF *CANDIDA* SPECIES

C. albicans		DNA-DNA checkerboard hybridization		
	Culture	absent	present	
	absent	23	3	26 (100.0%)
	present	0	0	0 (0.0%)
		23 (88.5%)	3 (11.5%)	26 (100.0%)
Kappa κ	0			
C. dubliniensis		DNA-DNA checkerboard hybridization		
	Culture	Absent	Present	
	Absent	17	9	26 (100.0%)
	Present	0	0	0 (0.0%)
		17 (65.4%)	9 (34.6%)	26 (100.0%)
Kappa κ	0			
C. glabrata		DNA-DNA checkerboard hybridization		
	Culture	Absent	Present	
	Absent	16	10	26 (100.0%)
	Present	0	0	0 (0.0%)
		16 (61.5%)	10 (38.5%)	26 (100.0%)
Kappa κ	0			

C. krusei		DNA-DNA checkerboard hybridization		
	Culture	Absent	Present	
	Absent	18	8	26 (100.0%)
	present	0	0	0 (0.0%)
		18 (69.2%)	8 (30.8%)	26 (100.0%)
Kappa κ	0			
C. tropicalis		DNA-DNA checkerboard hybridization		
	Culture	Absent	Present	
	Absent	13	12	25 (96.2%)
	Present	1	0	1 (3.8%)
		14 (53.8%)	12 (46.2%)	26 (100.0%)
Kappa κ	-0.076			

APPENDIX II: DIAGNOSTIC ACCURACY OF CULTURE FOR THE DETECTION OF *CANDIDA* SPECIES USING DNA-DNA CHECKERBOARD HYBRIDIZATION TECHNIQUE AS REFERENCE

C. albicans		DNA-DNA checkerboard hybridization			
	Culture	absent		present	
	absent	TN (specificity)	23 (100)	FN	3 (100)
	present	FP	0 (0)	TP (sensitivity)	0 (0)
C. dubliniensis		DNA-DNA checkerboard hybridization			
	Culture	Absent		Present	
	Absent	TN (specificity)	17 (100)	FN	9 (100)
	Present	FP	0 (0)	TP (sensitivity)	0 (0)
C. glabrata		DNA-DNA checkerboard hybridization			
	Culture	Absent		Present	
	Absent	TN (specificity)	16 (100)	FN	10 (100)
	Present	FP	0 (0)	TP (sensitivity)	0 (0)
C. krusei		DNA-DNA checkerboard hybridization			
	Culture	Absent		Present	
	Absent	TN (specificity)	18	FN	8
	present	FP	0 (0)	TP (sensitivity)	0 (0)
C. tropicalis		DNA-DNA checkerboard hybridization			
	Culture	Absent		Present	
	Absent	TN (specificity)	13 (92.9)	FN	12 (100)
	Present	FP	1 (7.1)	TP (sensitivity)	0 (0)

APPENDIX III: DIAGNOSTIC ACCURACY OF DNA-DNA CHECKERBOARD HYBRIDIZATION TECHNIQUE FOR THE DETECTION OF *CANDIDA* SPECIES USING CULTURE AS REFERENCE

C. albicans		Culture			
	DNA-DNA checkerboard hybridization	absent		present	
	absent	TN (specificity)	23 (88.5)	FN	0 (0)
	present	FP	3 (11.5)	TP (sensitivity)	0 (0)
C. dubliniensis		Culture			
	DNA-DNA checkerboard hybridization	Absent		Present	
	Absent	TN (specificity)	17 (65.4)	FN	0 (0)
	Present	FP	9 (34.6)	TP (sensitivity)	0 (0)
C. glabrata		Culture			
	DNA-DNA checkerboard hybridization	Absent		Present	
	Absent	TN (specificity)	16 (61.5)	FN	0 (0)
	Present	FP	10 (38.5)	TP (sensitivity)	0 (0)
C. krusei		Culture			
	DNA-DNA checkerboard hybridization	Absent		Present	
	Absent	TN (specificity)	18 (69.2)	FN	0 (0)
	present	FP	8 (30.8)	TP (sensitivity)	0 (0)

C. tropicalis		Culture			
	DNA-DNA checkerboard hybridization	Absent		Present	
	Absent	TN (specificity)	13 (52)	FN	1 (100)
	Present	FP	12 (48)	TP (sensitivity)	0 (0)