Salivary cytokines and levels in denture stomatitis: An exploratory case-control study[*]

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ABSTRACT

We aimed to evaluate the microbial and inflammatory characteristics associated with Denture Stomatitis (DS) analyzing: l) Levels of salivary cytokines and cultivable *C. albicans*; 2) DNA-DNA checkerboard on biofilm associated with mucosal tissue-bearing denture surfaces; 3) Serum C-reactive protein (CRP) levels. Thirty-two subjects were enrolled in the study with control (n = 17) and DS types II and III (n = 15) subjects. Samples were collected from unstimulated whole saliva, serum and swabs from denture surfaces. Salivary levels of inflammatory mediators and CRP were measured by multiplex. Samples from denture and mucosal surfaces were analyzed by DNA-DNA checkerboard. Saliva from DS subjects showed increase in IL-8 (p = 0.04) and IL-1β (p = 0.04) with trend for increase in IL-1β, TNFα and IL-6 levels. *C. albicans* higher counts in DS saliva (p = 0.03) showed association with elevated levels of IL-8 (p = 0.03) and IL-1α (p = 0.01). CRP levels were not different among groups (p = 0.74). DNA-DNA checkerboard analyses indicated typical periodontal pathogens below the detection threshold of 10^4 organisms on both denture and inflamed mucosal surfaces. The data suggest that DS is associated with elevation of salivary IL1 and IL-8 together with increased *C. albicans*. There was no evidence of systemic inflammation as measured by serum C-reactive protein levels.

Keywords: Cytokines; Serum C-Reactive Protein; Denture; *C. albicans*; Stomatitis; Salivary

1. INTRODUCTION

Candida-associated denture stomatitis (CADS) is characterized by inflammation of the palatal mucosa in contact with the maxillary denture and is the most common form of oral candidal infection with *Candida albicans* being the principal etiological agent [1-3]. CADS has been reported in 11% - 67% of the otherwise healthy denture wearers [4], and has been classified according to Newton's work in 1962 [5] into 3 clinical types: Type I—a localized simple inflammation or a pinpoint hyperemia; Type II (mild): an erythematous or generalized simple type presenting as more diffuse erythema, and Type III (severe): a granular or papillary type. This condition has been considered to have a multifactorial etiology, which can include factors related to 1) Trauma such as denture wearing; 2) Local trauma resulting from salivary hypofunction [6,7]; 3) Infection with candida species; or 4) Factors related to impaired host defense as in systemic diseases such as HIV infection, leukemia, lymphoma, radiation therapy for head and neck malignancies, chemotherapy, diabetes, hormonal imbalance, anemia, malnutrition, or long-term use of corticosteroid or antibiotics. The multi-factorial etiology for denture stomatitis is reflected in the current treatment regimens which vary from antifungals [8], to denture hygiene measures, antiseptics, and use of denture reline materials containing antifungals [9].

Although some progress has been made in revealing the mechanisms of epithelial and immunoregulatory interactions with Candida, there are major gaps in our knowledge regarding processes that lead to the establishment of candidiasis as an infection and the relationship between mucosal and systemic inflammatory responses. Primary innate defense mechanisms are known

to play key roles in preventing yeast colonization of the oral cavity by providing epithelium as a physical barrier as well as lingual antimicrobial peptide, defensins, secretory IgA and salivary factors [10]. The main sources of cytokines generated in the oral mucosa during infection with Candida have been reported to be of oral epithelial cell origin, although infiltrating neutrophils are likely important contributors. Cytokines in saliva are also derived from salivary gland cells (secretory, epithelial cells, fibroblasts, endothelial cells), and leukocyte aggregates within the salivary gland tissues, tonsils, and the oral mucosa, including the periodontium. Therefore, cytokine profiles in whole saliva can depict the collective state of cytokine responses to infectious agents by all tissues in the oral cavity, including accessory salivary glands which are major contributors of secretory IgA [11]. However, the influence of whole saliva composition on Candida adherence and invasion is yet to be fully understood.

Biofilms are structured microbial communities in which the cells bind tightly to a surface and become embedded in a matrix of extracellular polymeric substances produced by these cells. Indeed components of saliva or serum proteins, such as mucin, fibrinogen and complementary factors specifically bind to Candida blastospores and germ tubes, possibly modifying the biofilm formation [12]. Biofilms of differing compositions colonize oral surfaces including the teeth, the mucosa, the tongue and acrylic denture surfaces. These self-replenishing biofilms gain notoriety from their ability to resist antimicrobials and immune cell challenge [13,14].

The purpose of this study was to identify the candidate inflammatory mediators present within the saliva of subjects with denture stomatitis. We sought to confirm previous reports establishing the relationship between DS and increased salivary levels *C. albicans* and to determine if the level of *C. albicans* was associated with the levels of salivary cytokine biomarkers. Finally, since DS is such a common infection among edentate people we accessed whether there was any evidence of systemic inflammatory response as indexed by serum levels of C-reactive protein. Although this was a relatively small exploratory pilot investigation, the data suggest that increased levels of certain salivary cytokines are associated with the presence of DS and related to salivary levels of *C. albicans*.

2. MATERIAL AND METHODS

An IRB approved case-control study was performed. Thirty-two subjects were enrolled and grouped into two groups: a control group (n = 17) with no signs or symptoms of denture stomatitis (DS), and a diseased group (n = 15) with type II or III DS (Newton's classification). Inclusion and exclusion criteria, and demographics of this cohort of patients are described in **Table 1**.

Table 1. Inclusion and exclusion criteria.

Inclusion criteria	Exclusion criteria
1. At least 45 years of age.	1. Less than 45 years of age.
2. Male or females without menses for 12 consecutive months or who have had a complete hysterectomy.	2. Have chronic disease with oral manifestations other than denture/mucosal stomatitis.
3. Wear complete maxillary denture (overdentures, implant or tooth retained dentures acceptable) without daily use of denture adhesive.	3. Have overt denture abrasion associated with symptoms.
4. Must have read, understood and signed an informed consent form.	4. Participants with clinically significant organic diseases, including impaired renal function, bleeding disorder, or any condition requiring antibiotic pre-medication for dental visits.
5. Must understand and be willing to comply with all study procedures and restrictions.	5. Participants with active infectious diseases such as hepatitis, HIV or tuberculosis.
6. Must be in good general health; diabetics included.	6. Participants who are immunosuppressed because of medications or condition.
7. Must have Type II or Type III denture stomatitis for denture stomatitis group.	7. Participants who have used antibiotics or antifungals for any medical or dental condition within 1 month prior to screening.
8. Must have no signs of denture stomatitis for control group.	8. Participants using ongoing medications initiated less than 3 months prior to enrollment.
	9. Participants who have used tobacco products within 6 months of screening.
	10. Post-menopausalwomen on hormone replacement therapy.

The selection of candidate cytokines was based upon the findings of enhanced mRNA expression of specific cytokines within mucosal biopsy samplescollected from these subjects using whole transcriptome gene expression arrays.

2.1. Unstimulated Saliva Collection and Cytokines Analysis

Subjects were instructed to remove their dentures and refrain from eating, drinking, smoking, brushing their teeth or chewing gum for 15 minutes prior to salivary collections. All collections were performed between 9:00 and 11:00 AM. Subjects were instructed to swallow to clear the mouth of any accumulated saliva, and whole unstimulated saliva was allowed to pool in a sterile polypropylene graduated collection vial for 5 minutes. A fraction of the sample was sent immediately to the microbiology lab to prevent overgrowth of the Candida species. Samples were aliquoted into Eppendorf tubes, centrifuged for 10 minutes at 3000 g, and the supernatant stored at −80˚C until analysis. Before cytokines analysis, the samples were thawed and centrifuged for 10 minutes at 3000 g to remove any solids. Specific proteins (IL-1α, IL-1β, IL-6, IL-8, and TNFα) levels in Saliva samples were quantified by ELISA and/or using Fluorokine® MAP cytokine multiplex kits (R&D Systems, Minneapolis, MN) and a Bio-Plex 200® analyzer system (BioRad, Inc., Hercules, CA). Protein levels were log-transformed prior to application of multivariate tests comparing subjects with denture stomatitis to those without denture stomatitis, and comparing diseased and non-diseased sites within subjects with clinical stomatitis.

A fraction of the unstimulated saliva was sampled in Sabouraud Dextrose agar containing quemicetine succinate. Candida colonies were counted after 48 h and the patients were classified according to the number of CFU (Colony-forming units) as follows: negative (CFU/ml = 0), carrier (CFU/ml < 400), and positive (CFU/ml > 400).

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2.2. Microbiology

Four bacterial biofilm samples were collected from the tissue and the corresponding surface area of the denture using a plastic curette. The palate was divided into four zones (according to the method described by Barbeau *et al.* [16] as shown in **Figure 1**, and one biofilm sample was collected from each zone and recorded on a diagnostic diagram. The samples were pooled (4 mucosal samples and 4 denture samples) and placed into a separate Eppendorf tube containing 150 μL of TE buffer. 150 μL of 0.5 M NaOH were added to each sample and frozen at −80˚C prior to analyses. The pooled biofilm samples were analyzed quantitatively and qualitatively to measure the presence, absence and levels of 18 dental biofilm organisms by DNA-DNA checkerboard tissue, where median cell count levels of 16 organisms was produced for comparison of subjects with clinical stomatitis to those without clinical stomatitis using a nonparametric multivariate test. Mean Candida CFU counts were produced for comparison of the diseased and non-diseased groups. Data has been published elsewhere [15].

2.3. DNA-DNA Checkerboard

2.3.1. Sample Deposition on Nylon Membranes

The samples were boiled in a water bath for 5 minutes and neutralized using 0.8 ml 5 M ammonium acetate. The released DNA was placed into the slots of a Minislot device (Immunetics, Cambridge MA) and thereby deposited as "lanes" onto a (formerly Boehringer Mannheim, now Roche Diagnostics nylon membrane). The DNA was then permanently fixed to the membrane by exposure to ultraviolet light, followed by baking at 120˚C, for 20 min. The Minislot device permits the deposition of up to 30 different bacterial samples in individual "lanes" on a single 15 × 15 cm nylon membrane. In order to create reference signals on each membrane, two of the 'lanes' were used to deposit pooled DNA of known bacterial numbers (10^5 and 10^6) for each one of the microorganisms included in the analysis.
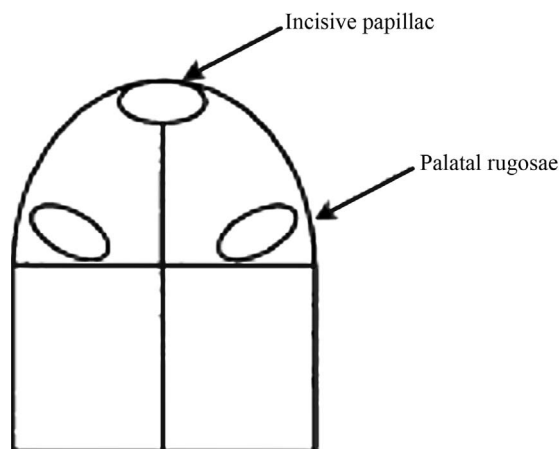

Figure 1. A diagram showing the division of the palate area into quadrants for bacterial biofilm collection from each of the 4 zones.

2.3.2. DNA-DNA Hybridization

The membranes were prehybridized in "prehybridization" buffer [50% formamide, $5 \times$ SSC ($1 \times$ SSC = 150 mM NaCl, 15 mM Na citrate, pH 7.0), 1% casein (Sigma), $5 \times$ Denhardt's reagent, 25 mM sodium phosphate (pH 6.5) and 0.5 mg/ml yeast RNA (Boehringer-Mannheim)], at 42˚C, for 1 hour and then placed back into a "Miniblotter 45", turned 90˚ to the original orientation. A 30×45 "checkerboard" pattern was produced. Digoxigenin-labeled DNA probes dissolved in "hybridization" buffer [45% formamide, $5 \times$ SSC, $1 \times$ Denhardt's reagent, 20 mM Na phosphate (pH 6.5), 0.2 mg/ml yeast RNA, 20 ng/ml of labeled probe, 10% dextran sulfate and 1% casein], were laid in individual lanes of the Miniblotter and the whole apparatus was placed in a sealed plastic bag. Hybridization was allowed to occur overnight, in a shaking water bath, at 42˚C. Membranes were then washed once at low stringency to remove loosely bound probe and then twice at high stringency (68˚C, $0.1 \times$ SSC, 0.1% SDS, 20 min), in a Disk Wisk apparatus (Schleicher and Schuell).

2.4. Blood Collection (Serum CRP)

Venous blood (5 - 7 ml) was collected under a sterile technique in a purple top vaccutainer. Blood was processed into serum within 2 hours after collection: the whole blood was kept at room temperature for 30 - 45 minutes to allow a clot to form, and then centrifuged for 12 minutes to separate the serum from the clot. Serum was then aliquoted into barcode labeled microfuge tubes and quickly frozen at −80˚C and stored until analysis using ELISA method. Mean serum CRP levels (mg/ml) were compared in diseased and non-diseased groups.

3. STATISCAL ANALYSIS

Salivary cytokine levels were not normally distributed and between group comparisons were made using the non-parametric, non-paired Wilcoxon test. Associations between salivary *C. albicans* CFU/ml and cytokine levels were compared by one-way ANOVA and regression analyses. The association between the presence or absence of *C. albicans* and the presence (pooling Type II and Type III) or absence of DS was determined by Chi Square test. A p value < 0.05 was considered significant, however no adjustments were made for multiple comparisons and the results should be interpreted with caution.

4. RESULTS

4.1. Salivary Markers Comparing Control and Denture Stomatitis

We measured salivary levels of IL-1α, IL-1β, IL-6, IL-8

and TNFα. The data from the DS and control subjects appear in **Table 2**. There was a statistically significant increase in salivary levels of IL-8 (p = 0.04) and IL-1β (p = 0.04) in DS as compared to health. In DS the apparent increase in salivary levels of IL-6, TNFα and IL-1α were not statistically significant. These increases in protein expression were consistent with the increased tissue mRNA expression (data not shown).

A statistically significant association between salivary counts of *C. albicans* and salivary levels of IL-8 ($r^2 = 0.16$, P = 0.03), and IL-1α ($r^2 = 0.20$, p = 0.01) was seen as illustrated in **Figure 2**.

C. albicans was detected in saliva of 80% (12/15) of the DS subjects, and in 35.3% (6/17) of the Healthy subjects, (p = 0.03), using the positive cut-off value of (CFU/ml > 400).

4.2. Bacterial Composition of Mucosal and Denture Surface Biofilm Determined by DNA-DNA Checkerboard

Tissue and denture surfaces were sampled using a plastic curette to measure the presence, absence and levels of 16 dental biofilm organisms by DNA-DNA checkerboard. Biofilm samples from the palatal tissue surfaces did not show any detectible organisms, suggesting a level below 10^4 counts. Organisms were detected in the biofilm samples collected from dentures with (3/17) control subjects, (1/ 8) DS Type II, and (1/7) DS Type III. The data for the 3 healthy are compared to the Type II and Type III DS in **Table 3**. There were no significant differences in the counts comparing Health to Diseased samples.

Table 2. Salivary Cytokines levels in control and DS groups.

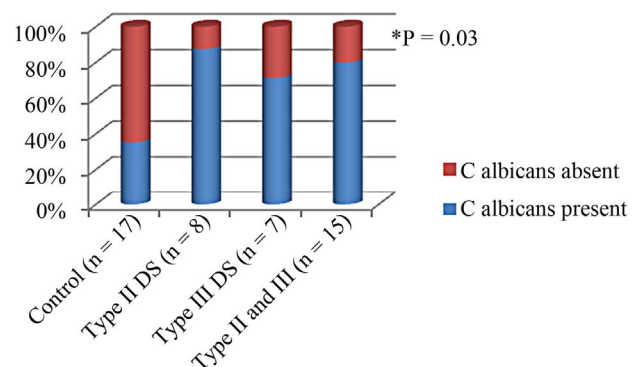| Cytokine	Control group Median (IQR) [ng/mL]	DS group Median (IQR) [ng/mL]	Wilcoxon p value
IL-1α	342 (202 - 752)	508 (140 - 1170)	0.47
IL-1β	**146 (77 - 328)**	**293 (119 - 1100)**	**0.04**
IL-6	4.2 (0.0 - 12.6)	5.2 (0.0 - 8.4)	0.50
IL-8	**446 (198 - 847)**	**792 (447 - 1970)**	**0.04**
TNFα	0.0 (0.0 - 6.2)	5.8 (0.0 - 14.2)	0.12

Figure 2. Salivary *C. albicans* prevalence according to DS severity.

Table 3. Organisms detected with DNA-DNA checkerboard of biofilm from denture surfaces. Numbers reflect counts $\times 10^6$ CFU.

Microorganism	Control (Mean ± SD)	DS (Mean ± SD)	P value
P. gingivalis	0.8 ± 1.6	0.9 ± 2.2	0.93
P. intermedia	0.8 ± 1.6	2.2 ± 4.9	0.42
P. nigrescens	1.8 ± 3.9	2.4 ± 6.0	0.80
B. forsythia	3.4 ± 6.5	3.3 ± 7.4	0.97
T. denticola	2.0 ± 4.0	2.4 ± 5.1	0.84
A. a	2.5 ± 5.0	3.7 ± 7.9	0.64
C. rectus	5.3 ± 10.2	9.1 ± 21.9	0.62
E. corrodens	1.8 ± 3.5	2.2 ± 5.00	0.82
F. nucleatum	15.1 ± 29.1	17.9 ± 38.3	0.85
S. noxa	2.5 ± 5.0	4.0 ± 9.4	0.65
C. ochracea	0.8 ± 1.6	1.4 ± 3.2	0.61
V. parvula	1.3 ± 3.1	1.9 ± 4.9	0.76
S. sanguis	2.2 ± 4.3	2.5 ± 5.4	0.86
S. intermedius	3.1 ± 6.6	3.1 ± 6.5	0.98
S. oralis	1.6 ± 3.1	3.0 ± 6.4	0.53
A. viscosus	6.3 ± 12.6	8.3 ± 17.8	0.75

4.3. Serum CRP

There was no significant difference in the serum CRP level comparing health to DS individuals (p = 0.74). We also examined levels of clinically elevated CRP values using CRP < 3 mg/ml as a threshold, and no statistical differences between the groups could be detected (**Table 4**).

5. DICUSSION

We investigated levels of the selected markers: IL-1α, IL-1b, IL-6, IL-8, and TNFα in saliva and found a statistically significant two fold increase in median salivary levels of IL-8 (p = 0.04) and a 1.8-fold increase in IL-1β. These increases are in accordance with other studies that looked at these pro-inflammatory cytokines in oral epithelial cells infected with *C. albicans* [17-19]. Increased levels of GM-CSF, IFN-γ, TNF-α IL-8, IL-18 and IL-1α, IL-1β, IL-6, have been identified in the oral mucosa epithelial cell infection models and saliva of subjects with oral candidiasis and in experimental models of oral candidiasis [11,18,19]. Recognition of *C. albicans* by oral epithelial cells through specific pattern-recognition receptors (PRRs) leads to activation of intracellular signaling pathways that modulate inflammatory responses including release of cytokines such as IL-1α, IL-6, IL-8, IL-15, IL-18, TNFα as well as upregulation of synthesis of specific antimicrobials such as defensins and nitrous oxide (NO) [19]. These mediators could also exhibit a regulatory effect on the secretion of each other, for example TNF-α, which showed a, increase, albeit no significant, in the DS group is known to activate secretion of other cytokines, such as IFN-γ, IL-1, IL-6, and IL-12 which further amplifies the protective immune response to *C. albicans* infection [19,20]. Furthermore, IL-1β which was significantly increased in the DS group,

Table 4. CRP levels in control and DS groups.

Serum CRP (µg/ml)		
Groups	Mean ± SD	p value
Control (n = 17)	4980.1 ± 2601.1	
DS (n = 15)	4687.7 ± 2271.8	0.74

is known to play a crucial role in activating production of IL-1inducible cytokines, which attract and activate cells of the immune and inflammatory systems, e.g., IL-6, IL-8, granulocyte-macrophage (GM)-CSF, and G-CSF [21]. We found a significant association between the salivary counts of *C. albicans* and the levels of IL-8 and IL-1α (**Figure 2**). We have recently found regarding *C. albicans* colonization in denture stomatitis patients, that the denture appears to be the primary source of salivary *C. albicans* rather than the inflamed mucosa. *C. albicans* was detected in saliva of 80% (12/15) of the DS subjects, but only 41.2% (7/17) of the Healthy subjects, (p = 0.03). Thus, *C. albicans* was twice as likely to be present in saliva if mild or severe DS was present. Therefore, the high levels in saliva likely represent the organisms released from denture surfaces with minor contribution from the mucosal surfaces. IL8 levels in saliva ranged from 447 - 1970 ng/ml in the DS group, this cytokine acts as a potent chemokine for neutrophils at nanomolar concentrations, thus this increase in IL-8 levels would lead to neutrophil recruitment within the inflamed mucosa and passage into the saliva via transepithelial migration. Such an increase in neutrophils within the saliva has previously been reported in mucositis and in DS subjects [11,18,22].

It is well understood that adequate attachment of candidal organisms to denture surface is a crucial step in the pathogenesis of denture stomatitis. The formation of biofilms on the surface of the denture protects these organisms from detachment and removal by salivary

flow and physical forces [4,14]. It's also worth noticing that quorum sensing (QS) occurs both in single species bacterial communities and in complex mixed bacterial-yeast communities [23,24]. However, the role played by saliva or serum pellicles during the colonization process and subsequent multilayer biofilm formation is poorly understood [12,24-26].

Measuring the levels of 16 dental biofilm organisms by DNA-DNA checkerboard in the mucosa and dentures, only samples collected from dentures were within the detectable range of 1×10^4 counts, however, organisms could be detected in the biofilm samples collected from dentures in three control cases, 1-DS Type 2, and 1-DS Type 3 patients and there were no significant differences in the counts comparing Health to Diseased samples. Those findings suggest that a more sensitive method, like 16 s RNA, should be utilized to better assess changes in the bacterial mucosal bacterial flora when comparing DS and control samples [27-33].

Sachdeo *et al.* [34] have used a similar methodology to the one performed in our study to characterize the microbiota of edentulous oral cavities using checkerboard DNA-DNA hybridization, analyzing samples from mucosal and denture surfaces, and saliva from edentulous patients for 41 bacterial species where they found that *A. actinomycetemcomitans* and *P. gingivalis*, which were thought to be eliminated with the extraction of all natural teeth, were seen in significant numbers in the edentulous subjects. These pathogens were detected in our study as well. Also, when those authors compared the mean total DNA probe counts among the dorsal surfaces of the tongue, denture palates, and the subjects' palates, the highest mean counts were found on the tongue dorsum, followed by the polished (exterior) surface of the denture palate, and were lowest on the hard palate. This finding is also consistent with our study where no organisms were detected from the mucosal samples. However, no samples were taken from the polished surfaces of the denture in our samples, as we collected the tissue-bearing surface.

Other studies have shown that the predominant cultivable flora of denture plaque was Gram-positive cocci (*Streptococci*) and rod-shaped bacteria [35-37]. Results from such studies suggest similarity between denture plaque bacterial composition and the periodontal niche in dentate patients. Therefore, consistent with the association of periodontal disease and specific periodontal pathogens on conditions such as cardiovascular disease and poor glycemic control among diabetics it has been suggested that denture cleaning is important to oral and systemic health and that the denture-wearing population requires care and follow-up similar to their non-denture-wearing counterparts [34].

Analyzing levels of CRP in those patients, we found no significant difference in the levels of CRP between the two groups of patients. The findings are consistent with those of Kostial el. al who reported that CRP values in patients with acute fungal stomatitis in non-immuno-compromised patients were within normal ranges [38]. In contrast, another study, Ajwani *et al.* reported elevated levels of CRP and microbial counts in edentulous individuals with signs of oral candidiasis or denture stomatitis [39]. It has been reported that CRP levels are higher in patients with periodontitis [40-44], and our data showed there is similarity between the microbial content of denture plaque and that associated with periodontitis. Thus, it is plausible that fungal infections, which activate a different microbial pathogen recognition system than bacteria, may specifically trigger the release of hepatic acute phase reactants, other than CRP, such as alpha antitrypsin, transferrin or orosomucoid synthesis.

6. CONCLUSION

Elevated levels of salivary IL-8 and IL-1β are associated with DS, and IL-8 is positively associated with increased levels of *C. albicans* in saliva. Our findings suggest that the overgrowth and colonization of *C. albicans* on acrylic denture surfaces may act as an early colonizer in the succession of biofilm formation (replacing many streptococci) and may serve to facilitate the emergence of anaerobic microorganisms. These data suggest that an unclean denture can serve as the primary source of *C. albicans* that enhances mucosal inflammation, pointing to the importance of denture hygiene in reducing denture stomatitis.

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