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Biofilm formation on denture liners in a randomised controlled *in situ* trial

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ABSTRACT

Objectives: This randomised clinical trial assessed how biofilm development and composition is affected by time and denture material type in denture wearers with and without denture stomatitis.

Methods: Specimens of acrylic resin (control) and denture liners (silicone-based or acrylic resin based, depending on the experimental phase) were inserted into the surface intaglio of 30 denture wearers. Biofilm was formed in two phases of 21 days, and counts of viable micro-organisms in the accumulating biofilm were determined after 7, 14 and 21days of biofilm formation. Data were analysed by three-way ANOVA followed by Tukey test to assess differences among health condition (healthy or with denture stomatitis), materials and time point.

Results: Non-*albicans Candida* species counts were higher in diseased patients with silicone-based denture liners ($p = 0.01$). Denture stomatitis patients showed higher mutans streptococci counts after 7 days ($p = 0.0041$).

Conclusions: Longer biofilm formation time periods did not result in differences on biofilm composition. The denture liners evaluated in this study accumulate greater amount of biofilm, and therefore their use should be carefully planned.

Clinical significance: The silicone-based denture liner tested should be used cautiously in patients with denture stomatitis as it showed increased non-*albicans* species counts, known to be difficult to treat.

1. Introduction

Biofilm formation and the presence of *Candida* species are strongly associated with high prevalence of denture stomatitis in denture wearers.[1,2] Fungi colonisation can interfere with dental treatment and be a barrier to the patient's health,[1,3] since dentures can serve as a reservoir of micro-organisms for new infections.[4–7] Epidemiological studies report denture stomatitis prevalence up to 70% among denture wearers.[1] The adhesion of micro-organisms on the surface of acrylic resin and denture liners depends on the surface topography and the composition of these biomaterials.[8–10] In this context, denture liners have been found to be more prone to microbial adhesion than acrylic resin used as denture base materials.[11–13]

Currently, denture liners are available as silicone-based and acrylic resin-based. The adhesion on these materials depends on the properties of the surface of the microbial

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cells,[8,14,15] which will adhere and form biofilm forming a complex three-dimensional architecture.[16] One of the problems directly related to these materials is still the accumulation of biofilm[17] while there is no consensus on how long these materials last considering longer clinical service. *C. albicans* and non-*albicans* species are often found on the dentures and oral mucosa of individuals without any signs of denture stomatitis,[18] but a quantitative presence of *Candida* has been found to be associated with the onset of the disease. It is possible that the etiological role in denture stomatitis occurs in combination with other factors.[19] However, the interaction among substratum surfaces, oral bacteria, and the differences between healthy and diseased patients is yet poorly understood, especially considering the latter,[19] with few clinical studies evaluating materials directly inserted into the denture base.[20–22] Therefore, this randomised *in situ* clinical trial evaluated the effect of time, substratum and health condition on biofilm composition and surface characteristics of acrylic resin and denture liners. The hypothesis tested was that there would be influence of time, denture liner and health condition on the biofilm formed *in situ*.

2. Materials and methods

2.1. Experimental design

This *in situ*, crossover, double-blinded (patient and biofilm analysis) study had a completely randomised design with substratum type (acrylic resin or denture liner), biofilm aging (7, 14 and 21 days) and health condition (healthy or denture stomatitis) as factors. The study was approved by the Local Research and Ethics Committee (protocol 191/2011). The oral health of the volunteers was assessed, and all participants signed written informed consent before being accepted into the study. Sixty-six patients wearing complete dentures that were looking for treatment in the Dental School were evaluated. After explaining the study, 36 patients accepted to participate while 6 patients were immediately excluded because they had taken antibiotics in the three months prior to the beginning of the experiment. Thus, the 30 other patients had their mouths and dentures swabbed for *Candida* species where 15 were identified as *Candida* carriers, and 15 diagnosed with denture stomatitis. During the experiment, 3 patients from the group diagnosed with denture stomatitis, were excluded because one had a surgery and could not return to the appointments and the other two had taken antibiotics (Fig. 1).

During 2 phases of 21 days each, 30 adult volunteers wearing complete dentures agreed to participate (26 women, 4 men, mean age: 60.9 ± 9.6 year-old), and had inserted in recesses created in their palatal denture's flange 6 acrylic resin specimens and 6 temporary denture liner specimens (silicone or acrylic resin, depending on the randomly assigned experimental phase). In each phase, after 7, 14 and 21 days of clinical service, 2 specimens of each material were randomly chosen and removed. The biofilm formed on the specimens was processed for microbiological composition analysis, and the results were expressed in colony forming units (CFU)/mm^2. Specimens were analysed by scanning electron microscopy (SEM).

2.2. Panellists and ethical aspects

One examiner carried out intra-oral examination of oral soft tissues and dental prostheses of all patients from June to September 2011. These patients were screened for *Candida* species presence. This step allowed the inclusion of volunteers who had *Candida* species in their oral habitat, without however, having the disease, while the other group was classified according to Newton's classification: the clinical appearance of the inflamed mucosa was considered with diffuse hyperemia and micropapules, inflammation and widespread, the mucosa was smooth and swollen, covering the entire region covered by the prosthesis. Swabs from the palate were cultured in CHROMagar™ *Candida* (Difco, Sparks, MD, USA) at 37 °C for 48 h.

Sample size was calculated presuming that ANOVA would be performed with 80% power and $\alpha = 0.05$. Data from previous publications[22] resulted in $n = 12$. However, considering losses during the experiment, we considered $n = 15$. Inclusion criteria included adults of both genders, with complete dentures but who had not had a new or modified prosthesis within the previous 6 months, normal salivary flow rate (0.3–0.5 ml/min), good general and oral health, ability to comply with the experimental protocol, not having used antibiotics during the 3 months prior to the study, and not using any other type of intraoral device. For the denture stomatitis patients, good general and oral health did not apply, as they presented denture stomatitis. The exclusion criteria eliminated those taking antifungal agents or any medication that could predispose to the disease (for the healthy group) or serve as treatment (corticosteroids, for instance), either systemically or locally, using antiseptic mouth-washes and had a medical history that revealed any disease or medical condition predisposing to oral candidosis (*e.g.* diabetes mellitus or iron and vitamin deficiencies) that could insert a bias in the study.

Patients were instructed to wear the dentures at all times and to brush their dentures 3x/day after the main mealtimes with a soft toothbrush and toothpaste (provided by the researchers) except for the area containing the specimens, where only the slurry from the toothpaste was spread on the specimens during the experimental period and 7 days pre-experimental period.

2.3. Preparation of specimens

All materials were prepared by a single operator at room temperature (25 ± 1.0 °C and $50 \pm 5\%$ relative humidity), under aseptic conditions. Specimens (5 mm × 5 mm × 2 mm) were prepared according to manufacturers' recommendations: acrylic resin (Acron MC, GC America, Alsip, IL, USA), Elite® Super Soft Reling (silicone based; Zhermack GmbH, Germany), and Soft Confort (acrylic resin based; Dencril, Pirassununga, Brazil). The acrylic resin was processed as previously described[22] and ground using progressively smoother aluminum oxide papers (320-, 400-, and 600-grit) in a horizontal polisher. For the soft denture liners, surface roughness was standardized by the contact with the glass slides.

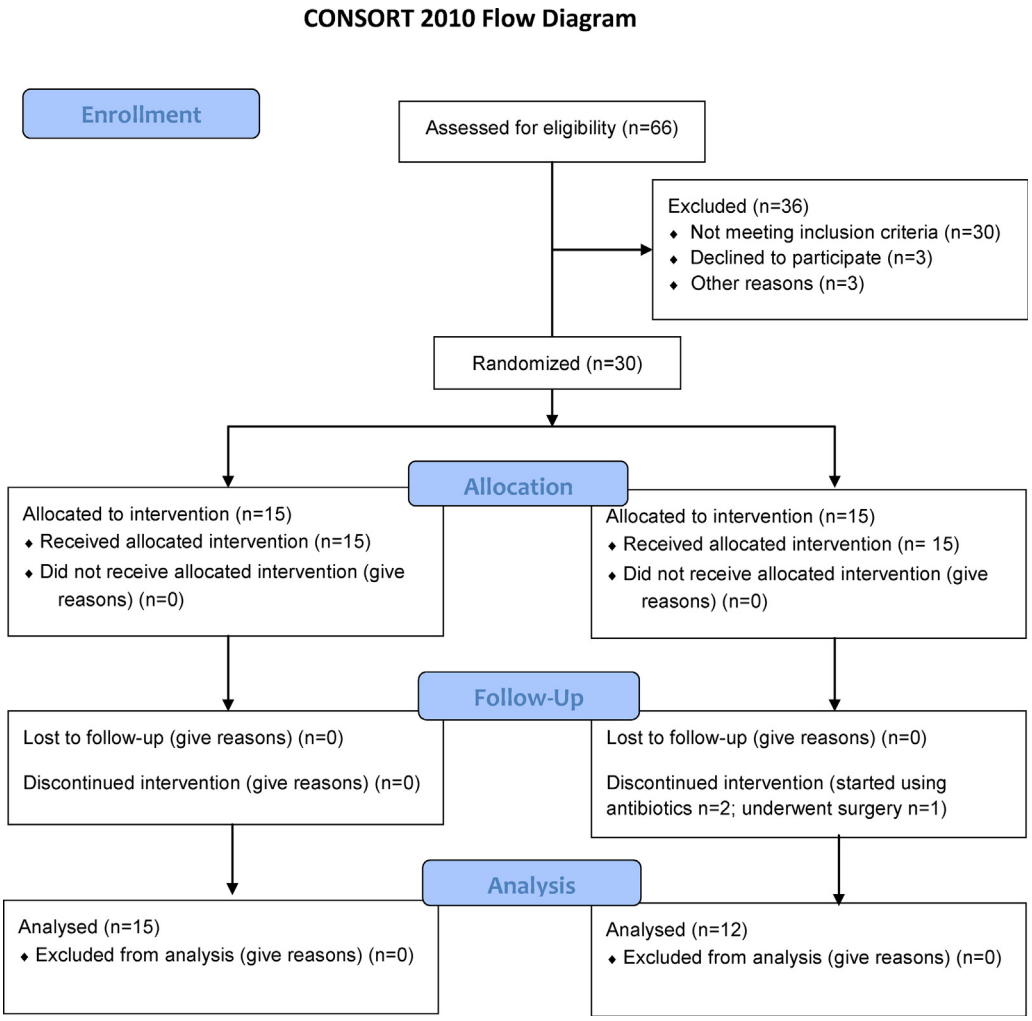

Fig. 1 – Selection criteria according to CONSORT statement.

Surface roughness of the samples were measured using a profilometer (Surfcorder SE 1700 Kozaka Industry, Kozaka, Japan) with a 0.01 mm resolution, calibrated at sample length of 0.8 mm, 2.4 mm percussion of measure, and 0.5 mm/s. Three readings were taken for each sample and a mean value was calculated.[23] The surface roughness of the specimens was measured for standardization purposes of the specimens before the experiment, with acrylic resin, silicone-based and acrylic resin-based denture liner mean values (±10%) of 0.6, 1.2 and 1.0 μm, respectively.

2.4. *Denture preparation and clinical phase*

Initially, the original patients prostheses received a standardised mechanic polishing with a lathe, a brush wheel with pumice slurry and a felt cone with chalk powder were used so that all the surfaces presented the same smooth baseline condition. Six recesses of 6 mm × 6 mm × 3 mm depth were made at each side of the intaglio surface of the maxillary denture in contact with either normal or inflamed mucosa. Each specimen was positioned and fixed with wax in the recess created. The specimens were randomly distributed according to the phase the patient was designated using a computer generated allocation program. Considering that the study followed a crossover design, with the patients participating in both phases, the subjects did not receive any instructions regarding their daily diet. A washout period of 7 days was allowed between the two phases to eliminate possible residual effects from the materials. Specimens were not reinserted and the recess was cleaned and filled with wax.

2.5. *Microbiological analysis of the biofilm*

The biofilm formed and the specimens were collected on the 7th, 14th and 21st day of each experimental phase, in the morning and approximately 2 h after the last meal and hygiene procedures. Two specimens of each substratum type (acrylic resin or denture liner) were randomly selected to be removed. Specimens containing the biofilm were sonicated at 40 W and 5% amplitude with three pulses of 10 s each, serially diluted and inoculated on specific media, and incubated at 37 °C in (anaerobiosis – blood agar, rogosa agar and mitis salivarius bacitracin agar; aerobiosis – CHROMagar *Candida*) for 24–96 h. The CFU were counted using a stereomicroscope,

and the results expressed in CFU/mm^2. Different colony morphologies were identified by Gram staining and morphology and biochemical tests of sugar fermentation were used to confirm mutans streptococci and *C. albicans* and non-*albicans* species. At the end of the second phase, all recesses were completed with acrylic resin, finished and polished until a new pair of dentures was manufactured.

2.6. SEM analysis

In order to observe the surface characteristics of all materials, extra specimens were also added to the dentures of two individuals from each group in the same way as previously described for each time point and type material ($n = 9$). Specimens were fixed with 2.5% glutaraldehyde for up to 12 h at 4 °C and then washed three times in 0.1 M phosphate buffer at 4 °C (pH 7.3) for 10 min each. After fixation, all samples were dehydrated further in an ethanol/water mixture of 50%, 70%, 80%, 90%, 95% and 100% for 20 min each. Finally, the dehydration in 100% ethanol was done and the specimens mounted on a stub, air-dried, sputter-coated with gold (Balzers Union MED 010 evaporator; Walluf, Germany) and examined with a scanning electron microscope (SSX-550; Shimadzu, Japan) at an accelerating voltage of 20 kV for surface characterization after the biofilm formation focusing on surface morphology and biofilm at each time point.

2.7. Statistical analysis

Statistical analyses were done using SAS software (SAS Institute Inc., version 9.0, Cary, NC, USA) employing a significance level fixed at 5%. The hypothesis assumed differences among substrata, time point and health condition assessed. A randomised block design was used for the statistical analysis, considering the patients as statistical blocks, and time points, substratum types and health condition as factors under study. For microbiological analysis, data that violated the assumptions of equality of variances and normal distribution of errors were transformed by rank and after transforming the data, they were then analysed by three-way ANOVA, followed by Tukey test.

3. Results

Three patients withdrew the experiment (one had a surgery and could not return to the appointments and the other two had antibiotics). Qualitative assessment of the three materials

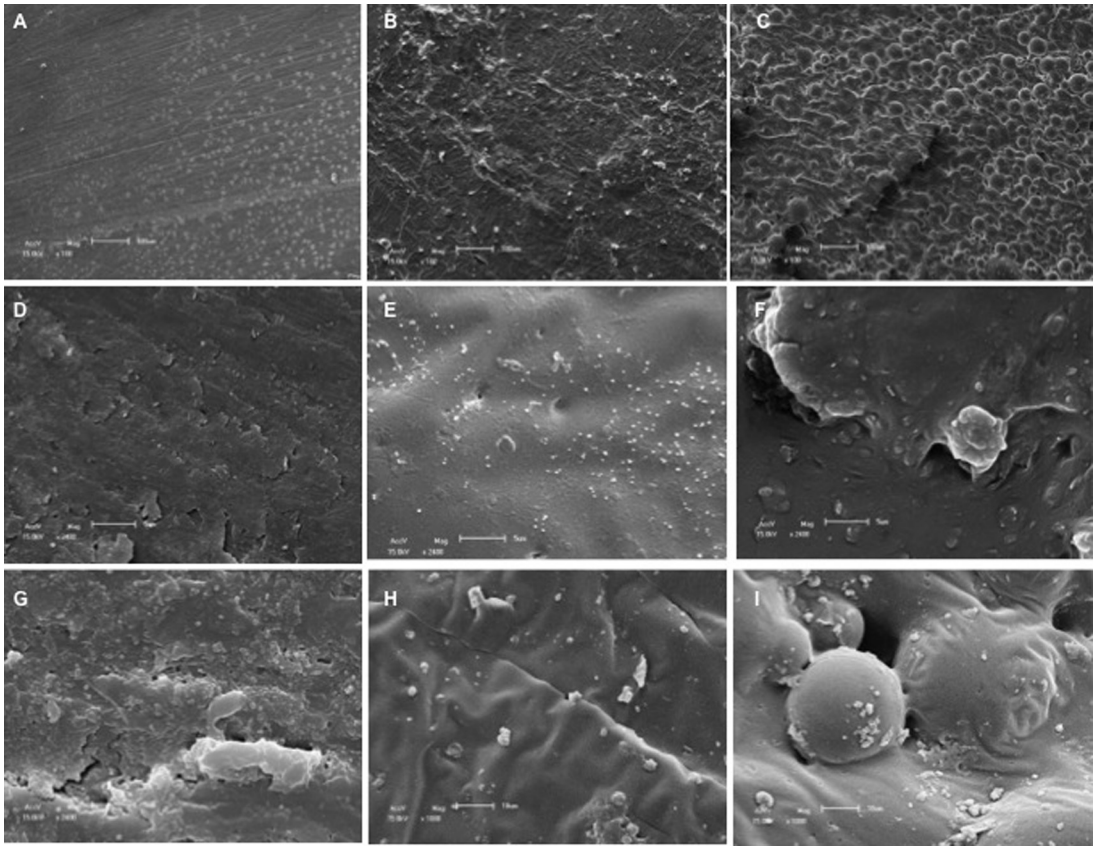

Fig. 2 – Representative SEM images of surface characteristics for each time point and material. 7 days: A, acrylic resin; B, silicone-based denture liner; C, acrylic-based denture liner (original magnification ×100, respectively). 14 days: D, acrylic resin; E, silicone-based denture liner; F, acrylic-based denture liner (original magnification ×2400 respectively). 21 days: G, acrylic resin; H, silicone-based denture liner; I, acrylic-based denture liner (original magnification ×2400, 10,000, 10,000, respectively).

Table 1 – *C. albicans* and non-*albicans* species counts in the biofilm according to the experimental conditions (CFU/mm², average ± SD).

Day	*C. albicans* (×10²)			*C.* non-*albicans* (×10²)		
	Control	Acrylic liner	Silicone liner	Control	Acrylic liner	Silicone liner
Denture stomatitis						
7	2.1 ± 11.0	0.9 ± 2.7	0.4 ± 1.0	4.6 ± 23.7	4.2 ± 13.4	2.2 ± 6.4[a]
14	12.2 ± 78.1	5.2 ± 14.3	4.7 ± 16.9	9.8 ± 50.1	2.1 ± 6.2	11.5 ± 36.0[a]
21	7.7 ± 27.6	1.1 ± 4.9	2.6 ± 8.5	10.3 ± 37.7	0.1 ± 0.2	12.4 ± 26.8[a]
Healthy						
7	1.7 ± 6.4	1.8 ± 6.1	0.4 ± 1.1	1.1 ± 4.9	4.2 ± 17.5	0.6 ± 2.4
14	4.8 ± 18.5	3.4 ± 9.7	3.6 ± 9.8	0.9 ± 4.2	1.3 ± 4.0	1.8 ± 6.4
21	6.9 ± 31.9	6.7 ± 21.0	7.7 ± 22.0	0.6 ± 2.4	3.4 ± 12.0	1.3 ± 4.2

There were no statistically significant differences for *Candida albicans* counts considering all variables tested.
[a] Statistically significant differences among materials and disease for non-*albicans Candida* species (three-way ANOVA followed by Tukey test, $p < 0.05$).

with SEM images showed the materials' different surface topographies. Lesser smoothness was observed in the denture liner samples, with micro-organisms clusters on the surfaces. In general, *C. albicans* adherence was observed in cluster forms and whole attached cells were viewed in blastospore morphology (Fig. 2).

Table 1 shows the microbiological results for *C. albicans* and non-*albicans* species. There was no difference in *C. albicans* counts in all materials and time points studied ($p > 0.05$). Also, healthy or diseased patients did not show differences in *C. albicans* counts ($p > 0.05$). However, non-*albicans Candida* species counts showed statistically significant differences in the silicone-based liner, with higher proportions of these species; diseased patients showed highest counts of non-*albicans* species in the silicone based denture liner ($p = 0.0111$).

For mutans streptococci counts, there were statistically significant differences between healthy and diseased patients only in the beginning of the experiment, *i.e.* 7 days, where mutans streptococci counts were higher in denture stomatitis patients ($p = 0.0041$); however, when the biofilm matured for 14–21 days, this difference was no longer observed, irrespective of the material tested (Table 2).

For lactobacilli counts, the silicone-based liner showed higher counts when compared to the other denture liner, in both healthy and diseased patients and for all time points assessed ($p = 0.032$). When considering total micro-organisms, the resin based denture liner showed higher counts, irrespective of the time point assessed or the health condition of the patient ($p = 0.0404$).

4. Discussion

This clinical study has shown that non-*albicans Candida* species are responsible for higher counts in denture stomatitis patients. In addition, it seems from our study that liners always present higher counts compared to acrylic resin regularly used to fabricate dentures. However, the time

Table 2 – Microbiological results for bacteria in the biofilm according to the experimental conditions (CFU/mm², average ± SD).

Day	Mutans streptococci (×10³)			Lactobacilli (×10⁵)			Total micro-organisms (×10⁶)		
	Control	Acrylic liner	Silicone liner	Control	Acrylic liner	Silicone liner	Control	Acrylic liner	Silicone liner
Denture stomatitis									
7	40.2 ± 278.4b	6.5 ± 15.6b	4.3 ± 7.6b	2.2 ± 5.2a	2.1 ± 2.9a	3.4 ± 5.1b	1.9 ± 3.5a	2.9 ± 3.8b	3.3 ± 5.7a
14	4.1 ± 9.3a	2.0 ± 5.1a	5.9 ± 25.6a	3.5 ± 8.7a	2.9 ± 5.0a	6.2 ± 15.4b	3.7 ± 6.8a	2.0 ± 3.3b	2.8 ± 3.8a
21	3.5 ± 10.4a	24.4 ± 102.5a	4.5 ± 10.4a	2.6 ± 4.6a	5.5 ± 16.1a	2.8 ± 2.7b	1.9 ± 4.3a	1.9 ± 2.2b	1.5 ± 1.6a
Healthy									
7	1.2 ± 3.3a	1.9 ± 4.6a	0.6 ± 1.9a	4.7 ± 11.6a	2.1 ± 3.3a	4.8 ± 7.5b	2.0 ± 3.1a	2.7 ± 2.4b	1.5 ± 2.0a
14	4.7 ± 13.1a	0.3 ± 0.7a	6.6 ± 31.0a	4.0 ± 12.1a	3.5 ± 8.0A	3.0 ± 6.1b	2.5 ± 3.2a	2.2 ± 3.3b	2.6 ± 4.7a
21	5.0 ± 11.4a	2.3 ± 4.4a	4.5 ± 11.0a	2.3 ± 3.9a	7.4 ± 14.2a	3.0 ± 3.6b	1.8 ± 2.6a	3.4 ± 5.8b	1.7 ± 2.7a

Lower case letters represents statistically significant differences among materials for lactobacilli ($p = 0.0302$) and total micro-organisms fixing time and health condition ($p = 0.0404$) and among time periods for Streptococci fixing health condition and material (three-way ANOVA followed by Tukey test, $p < 0.05$). In day 7, mutans streptococci counts were higher in denture stomatitis patients; however, after 14–21 days, this difference was no longer observed, irrespective of the material tested; for lactobacilli, the silicone-based liner showed higher counts when compared to the other denture liner, in both healthy and diseased patients and for all time points assessed; for total micro-organisms, the resin-based liner showed higher counts when compared to the other denture liner, in both healthy and diseased patients and for all time points assessed.

elapsed since the commencement of biofilm formation does not seem to change biofilm composition. The present study evaluated denture wearers with and without denture stomatitis to understand how biofilm composition could be affected by time and denture material type in healthy and diseased subjects. The biofilm was grown up to 21 days to better understand if time would be responsible for changes in biofilm composition especially in diseased subjects, as manufacturers usually indicate the use of these liners for very short periods of time. Therefore, our hypothesis was accepted since there was a difference among time periods for mutans streptococci counts, differences between liners and between health conditions in the biofilm formed *in situ* in this clinical trial.

These new results are important since *in vitro* studies had already shown that denture liners are easily colonised and deeply infected by *Candida* species[15,23] but no attempt to evaluate mature biofilms or to compare the differences between subjects has been made. Furthermore, intraorally a denture is rapidly coated with a salivary pellicle, modifying the properties of the exposed surfaces, which is the reason why *in vitro* studies fail to show this trend, as they rarely account for all the factors which likely play a role during biofilm development.[24]

In this study, the surface smoothness could be viewed by SEM images after the biofilm formation focusing on surface morphology and biofilm at each time point. The morphology of the materials' surface was examined and the images corroborated the microbiological findings that the surface topography could affect microbial adhesion, with higher numbers of cell clusters retained on the rougher surfaces (denture liners). The aging process probably increases the surface irregularities and the likelihood of micro-organisms on the surface. After 21 days, the biofilm will keep maturing and, with an increase of the surface irregularities of the denture liners, the cells will be entrapped in the denture liners' porosities, thus making it more and more difficult to remove biofilm either mechanically or chemically.[25]

Biofilm formation is an important virulence factor for a number of *Candida* species, as it confers significant resistance to antifungal therapy by limiting the penetration of substances through the matrix and protecting cells from host immune responses.[26–28] Moreover, biofilms formed by *C. albicans*, *C. parapsilosis*, *C. tropicalis* and *C. glabrata* isolates have been associated with higher morbidity and mortality rates compared with isolates unable to form biofilms.[28]

Although our study has shown no differences in *C. albicans* counts in any of the conditions tested, *C. albicans* is recognized as a contributing factor in the cause of denture stomatitis since these fungi are capable of proliferating in healthy hosts by surviving immune factors, demonstrating increased resistance to commonly used antifungal drug therapies.[27,29–31] Moreover, in this study, for mutans streptococci counts, there were differences between healthy and diseased patients only in the beginning of the experiment, *i.e.* where mutans streptococci counts were higher in diseased patients. These results are important since mutans streptococci appear in the initial phases of biofilm development are known to have synergism with *Candida* species and are also related to peri-implant diseases.[25,32–34]

For lactobacilli counts, the silicone-based liner showed higher counts when compared with the other denture liner, in both healthy and diseased patients and for all time points assessed. When considering total micro-organisms, the resin based denture liner showed higher counts, irrespective of the time point assessed or the health condition of the patient. Although these findings seem contradictory, the substratum may influence the composition and the formation of the pellicle, together with host characteristics, which may be less important than the surface properties of the dental materials.[35] In addition, most studies showing these differences are *in vitro* and again, may not account for the numerous factors involved *in vivo* in biofilm formation, while antimicrobial properties of saliva may contribute to the tissue/patient factors influence biofilm formation, not the substrate.[20] A change in a key environmental factor (or factors) will trigger a shift in the balance of the resident plaque microflora, and this might predispose a site to disease, resulting in a loss of the balance of the resident microflora, predisposing a site to disease.[36] Microbial specificity in disease would be due to the fact that only certain species are competitive under these shifted environmental conditions as it happened with non-*albicans Candida* species and mutans streptococci. Although local antimicrobials are reported to be useful,[37,38] it is likely that non-*albicans* species where found in higher number in diseased patients due to repeated fungal therapies, as non-*albicans* species are more likely to be resistant to the first line antifungal agents.

In our study, denture hygiene was standardized with the same toothbrush and toothpaste for all individuals, which had the same hygiene instructions. However, poor denture hygiene is clearly accepted as a critical risk factor for denture stomatitis. Thus, it is important to carry out studies comparing different hygiene methods and the effect they will promote in denture liners. While access to dental care is improving and teeth are still present in the elderly patients, there is still a high incidence of individuals with complete dentures. Preventing the disease that is still a cause of high morbidity when there is a widespread of the fungi to the body[39] is ultimately necessary.

Further studies are needed to increase our understanding of the oral ecosystem and the clinically important micro-organisms/materials interactions. Moreover, it is important to emphasize that the results obtained in this study should be interpreted with caution, since we have only tested three materials and more importantly individual factors may influence the findings, according to age, gender, income, general health, oral hygiene, daily period of use of prosthesis, time of use of the prosthesis alcohol consumption, trauma, diet and salivary components.

5. Conclusions

The use of the silicone liner tested should be carefully planned in patients with denture stomatitis due to an increase in non-*albicans Candida* species, known to be difficult to treat. In general, denture liners evaluated in this study accumulate greater amount of biofilm, and therefore their use should be cautious.

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