Original Article

In situ Multispecies Colonization of An Acrylic Resin: Comparison to Oral Microbiome and Potential for Inflammatory Response

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Abstract

Background: Conventional acrylic resin is prone to microbial colonization and may cause inflammatory and allergic response. **Aims:** This study aims to research the initial microbial adhesion *in situ* and tissue response to an acrylic resin used in prosthodontics. **Materials and Methods:** Disks of a commercial acrylic resin were prepared and included on the surface of individual intraoral splints fabricated for 50 participants. The splints were used for 4 h, under clinical conditions. Beforehand, each participant was swabbed to provide a control for microbiological comparison. A cytological control sample was also taken from the palate. After the time elapsed, each splint was removed and growth of anaerobes, aerobes, *Pseudomonas*, oral streptococci, staphylococci, yeasts, and *Streptococcus mutans* was determined by plate counts and compared to the oral microbiome. A cytological sample was taken from the contact zone, stained using the Papanicolaou technique, analyzed in light microscopy, and classified accordingly. Means and standard deviations were calculated, and a nonparametric Wilcoxon test was employed to compare experimental groups. The significance level was set at 0.05 (95% confidence interval, and statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) version 20.0. **Results:** Nuclear-cytoplasm ratio increase was found in 84% of the smears retrieved from the contact zone. Over 60% showed nuclear alterations. With exception to yeasts and *Pseudomonas*, all microbial groups colonized the resin. No statistically significant differences were found between the oral microbiome and the acrylic resin's colonization except regarding yeasts ($P > 0.05$). **Conclusions:** Cellular alterations were found but a diagnosis of inflammation is inconclusive. Microbial adhesion to the acrylic resin was substantial, with multiple species adhering.

Keywords: *Bacterial load, dental materials, inflammation, polymethyl methacrylate, prosthodontics*

Introduction

Polymethyl methacrylate (PMMA) is the most frequently used acrylic resin in the fabrication of removable dentures mainly due to its optimal characteristics such as overall esthetics, biocompatibility, color stability, and ease in repair.[1] Despite recent alternative options for denture base materials, PMMA did not lose its popularity.[2] Inherent to its polymeric nature, PMMA has important surface characteristics such as porosity, surface roughness, or water sorption.[3] These properties influence biofilm formation and render this material prone to microbial colonization, similarly to tooth structures.[4]

Recent rehabilitation with a removable denture is responsible for modifying the oral environment, leading to an accumulation of denture plaque on the surface of the denture.[4] The composition of denture plaque is analogous to dental plaque, even though scarce studies seem to report the microbiology of denture colonization.[5] This denture plaque is responsible for the onset of dental diseases such as caries, periodontal diseases, or even respiratory tract infections such as aspiration pneumonia, in elderly patients.[5,6]

The predominant bacterial species colonizing acrylic resin are mainly the Gram-positive bacteria such as *Streptococci* spp., *Actinomyces* spp., and *Lactobacilli*.[6] Gram-negative rods are infrequent and generally fewer in number.[7] Other species including Staphylococci can also be found, and studies reporting colonization of *Pseudomonas* in dentures of institutionalized patients are present in the literature.[6,8]

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Recent attention has also been drawn to yeasts, namely Candida spp., being one of the responsible factors for the development of denture stomatitis, a condition characterized by mucosal inflammation beneath the fitting surface of the denture.[9] Several recent researches have focused on addressing the colonization of *Candida* spp. in removable partial dentures. This yeast has the capacity to adhere tenaciously to the acrylic resin's surface[10] although it depends specifically on coaggregation mechanisms to the existing biofilm, such as complex lectin-like interactions with oral streptococci.[9,11]

It is urgent to determine the pathogenic potential of the biofilm that colonizes acrylic resins, not only in the elderly or diseased population alike in past studies,[11] but also in healthy controls.

Regarding inflammatory response due to rehabilitation with acrylic resins, it is generally known that acrylic materials, irrespective of its polymerization method, all have the potential to elicit mucosal irritation, cellular damage, and allergic response due to the content of residual monomer.[12,13] Theories have been proposed to justify the cytotoxic potential of acrylic resins since the mechanism is thought to be associated with direct toxicity from the residual methyl methacrylate or oxidative stress generated by free radicals.[14,15]

In situ studies are required to extrapolate results that are more approximate to what we can find in a real-life setting since *in vitro* studies do not accurately reproduce the complex environment of the oral cavity.[16] Up to date, there is no study simulating *in situ* biofilm formation and assessing the differences in initial colonization on acrylic surfaces. This study aims to highlight the differences between the resident microflora in healthy controls and the initial microorganisms that colonize surface of the acrylic resin together with the inflammatory response assessment of the same material, also *in situ*, through exfoliative cytology.

Materials and Methods

This study was approved by the Ethics Comittee of Instituto Superior de Ciências da Saúde Egas Moniz, in January 2017, and was conducted in accordance with the Helsinki Declaration. The study sample were 50 participants in total, that attended Instituto Superior de Ciências da Saúde Egas Moniz as students, randomly selected based on the inclusion and exclusion criteria. The inclusion criteria were the presence of both upper premolars and molars, with no active carious lesions, no apparent mucogingival lesions, nonrehabilitated with removable partial dentures and with no systemic pathologies such as immunosuppressive diseases or taking any anti-fungal, antibiotic or immunosuppressive therapies.

An informed consent was presented and signed by each participant, and after this, data were collected individually: gender, age, oral hygiene habits, and a random number were assigned to a datasheet so that the study remained blind. Following this, a thorough clinical examination was conducted on the oral cavity of each participant so that the inclusion criteria could be applied, and other possible inflammatory conditions could be screened and eventually excluded, based on the observations. In the experimental phase of the study, maxillary alginate impressions (IQ Chrome, Lascod) of the upper arch were taken for each participant with a standard universal tray previously selected. These were subsequently poured to obtain a working cast used to fabricate of an individual intraoral splint for each participant. The working cast was identified with the random number previously assigned.

Individual intraoral splints were custom made ranging from the first premolar to the second molar from thermoplastic acrylic clear foils (Keystone) of 125 mm of diameter and 1.5 mm of width, vacuum formed, as previously stated in the studies of Claro-Pereira *et al*.[17] and Gomes *et al*.[18] Two disks of ProBase® Hot (Ivoclar), 4 mm in diameter and 2 mm in height, were included on the external lingual surface of the splints for each of the participants. These were prepared from a cylindrical standardized mold, in equal calibrated dimensions each, and were polished according to their clinical use instructions using a polishing machine equipped with 600 grit SiC paper (LaboPol-4, Struers A/S, Denmark). The samples were included 24 h after their preparation so that real clinical time between laboratory and clinical conditions was simulated. Before intraoral exposure of the splint with the specimens, an ultrasonication procedure (Branson 2200, Sotel) in a bath of 70° ethanol for 15 min followed by a distilled water wash was conducted on the splints and specimens. This was performed on each specimen to guarantee they were disinfected and free of microorganisms before the intraoral exposure.

Before the exposure, a microbiological swab was taken on the buccal sulcus distal to the first molar on the upper arch so that each participant served as a control comparison of himself. A cytological smear was also taken from the palatal area using a microbrush. The microbiological swab was deposited on a sterile Eppendorf with 500 µl of NaCl at 0.9%, and the cytological sample was deposited inside a sterile tube containing a fixative solution (CytoLyt®).

The exposure period of the intraoral splint was between 9 A. M. and 13 P. M. to standardize the study. The participants were instructed not to drink or eat during this time frame.

After the 4 h elapsed, the splints were removed, and a new cytological smear was taken from the zone in contact with the acrylic resin. The cytologic microbrush used for the sample collections was deposited inside a sterile tube containing a fixative solution (CytoLyt®) and was then poured onto funnels which were mounted and aggregated

together with the other samples inside a cytocentrifuge machine (Cytospin®). A cytocentrifugation technique was employed for all the cytological samples for 4 min at 1200 RPM. Each cycle comprised 12 samples. The smears obtained from this technique were analyzed under a light microscope in search for inflammatory parameters and classified accordingly using a semiquantitative score scale by an anatomopathologist. For the microbiological analysis, the acrylic resin disks were removed and placed in sterile Eppendorfs containing 500 µl of NaCl at 0.9%. Each sample was coded with the number previously attributed followed by the algorithm "C" for the control sample and "B" for the acrylic resin. The Eppendorfs were vortexed for 5 s and sonicated in an ice bath to promote desorption of microorganisms, and this procedure was repeated three times. The suspensions obtained were then diluted, with 0.9% NaCl until 10^{-1}. (100 uL of suspension with 900 uL of NaCl). From the resulting solutions, 20 µl was streak plated onto both rich and selective growth mediums. Initial microbial colonization of total aerobes, total anaerobes, *Enterobacteriaceae*, *Staphylococci* spp., *Pseudomonas* spp., yeasts oral streptococci, and streptococci of the mutans group was assessed by plating the following growth mediums: brain heart infusion (BHI), Columbia agar +5% sheep blood, drigalski, chapman, cetrimide, Candida Select, Mitis Salivarius agar, and selective Mitis Salivarius agar with 0.2 mL/unit of bacitricine – selective for streptococci belonging to the mutans group. In order to research the presence of aerobe species, the following growth mediums were incubated in aerobiosis (35°C–37°C) – BHI to determine total aerobes, drigalski to assess the presence of *Enterobacteriaceae*, cetrimide for *Pseudomonas*, and Chapman for staphylococci. The anaerobe species were determined with the remaining growth mediums and were incubated inside sealed jars containing a CO_2 generator to replicate an anaerobe atmosphere at 37°C for 4 days. All the samples were processed immediately after their initial retrieval.

After the incubation period, plate counts were determined, and the results were expressed in colony-forming units (CFUs) per square millimeter.

Means and standard deviations were calculated. Normality was tested by means of the Kolmogorov–Smirnov test. Since data did not follow a normal distribution, a nonparametric Wilcoxon test was employed to compare both experimental groups. Percentages were calculated for the cytological parameter frequency. A significance value of 0.05% was set, to indicate statistical significance, at a confidence interval of 95%. Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) version 20.0 (IBM, Armonk, NY, USA) and exported from this same program.

Results

The mean age of the participants included in this study was 23 years, with 32% being males (n = 16) and 68% of the participants (n = 34) females. Considering the oral hygiene habits, the participants reported a mean of 2 daily brushings.

When analyzing the plate counts, overall, the anaerobe species prevailed (PB: 23.8 CFU/mm^2; C: 34.6 CFU/mm^2) over the aerobes (PB: 12.7 CFU/mm^2; C: 10.5 CFU/mm^2) in both experimental groups.

The most predominant bacteria found both in the oral microbiome of the participants and on the acrylic resin's surface were the oral streptococci (PB: 13.7 CFU/mm^2; C: 23.5 CFU/mm^2), followed by bacteria belonging to the *Enterobacteriaceae* (PB: 8,5 CFU/mm^2; C: 7,7 CFU/mm^2) as illustrated in Table 1. The bacteria with the lowest counts were the Staphylococci since only one participant colonized these bacteria on the acrylic resin sample. *Pseudomonas* spp. was not isolated in any of the samples retrieved from the participants of the study. In respect to yeasts, *Candida* spp. colonies were only found on the control samples of some of the participants. No *Candida* spp. colonized the acrylic resin for the studied population sample.

Table 1: Colony-forming units per square millimeter of both experimental groups

Microorganism	CFU/mm^2
Total counts	
PB	Mean - 36.6±33.3
C	Mean - 45.1±38.8
Total aerobes	
PB	Mean - 12.7±35.0
C	Mean - 10.5±13.2
Total anaerobes	
PB	Mean - 23.8±22.4
C	Mean - 34.6±16.1
Oral *Streptococci*	
PB	Mean - 13.7±12.4
C	Mean - 23.5±28.3
Streptococci (*mutans* group)	
PB	Mean - 9.2±11.2
C	Mean - 13.8±22.4
Enterobacteriaceae	
PB	Mean - 8.5±11.8
C	Mean - 7.7±12.2
Staphylococcus	
PB	Mean - 0.003
C	-
Pseudomonas spp.	
PB	-
C	-
Candida spp.	
PB	-
C	Mean - 1.265±6.08

PB: Acrylic resin Probase Hot; C: Control group - oral microbiome of the participants; CFU: Colony-forming units

No significant differences were observed between the resident bacterial flora counts and the bacteria which colonized the acrylic resin disks (Wilcoxon test, $P > 0.05$). The only significant differences found belonged to the yeasts since no yeasts colonized the acrylic resin (Wilcoxon test, $P < 0.05$) [Table 2].

Concerning the cytological analysis, the contact zone with the acrylic resin revealed a high percentage (84%) of cells exhibiting a high nuclear-cytoplasm ratio, with >50% of these showing moderate severity [Table 3]. Considering the nuclear morphology alteration, three parameters were assessed: karyolysis, karyorrhexis, and karyopyknosis. All of these mentioned parameters had a higher percentage of positives when compared to the control zone. Looking into detail at the percentage of cells which presented karyolysis, 60% of the smears retrieved from the contact zone were positive whereas only 28% of the smears from the control zone showed the same result [Table 3].

Discussion

In this clinical pilot study, we aimed to disclose the acrylic resin ProBase® Hot (Ivoclar) potential to suffer colonization and cause local inflammatory response in the host. It is important to highlight that the present material was colonized by all of the identified microorganisms that inhabited the oral microbiome of the participants except for the yeast *Candida*. This observation highlights the great susceptibility of this material to biofilm formation.[18] In addition, considering no differences were found when comparing the CFU counts obtained in the oral microbiome of the participants to the acrylic resin's surface, it is important to stress that the biofilm which formed over the acrylic resin's surface resembles the normal oral biofilm.

In this research experiment, the oral *Streptococci* showed the highest bacterial counts in both the oral microbiome and the acrylic resin samples. These results are expectable since these bacteria are known to be the predominant species in the oral cavity and are the initial colonizers of the biofilm.[19] Relating to the high *Enterobacteriaceae* CFU counts, this is considered a curious finding since these bacteria are exogenous to the oral cavity and are not typically found in the oral microbiome. Some authors suggest they are able to colonize the bristles of the toothbrush, which in turn means that their presence might be due to extraoral factors such as poor hygiene of toothbrushes or even their incorrect storage with lack of aseptic conditions.[20,21] Pesci-Bardon *et al*.[22] have also reported adhesion of bacteria from the *Enterobacteriaceae* family onto the surface of acrylic resins.

Candida spp. was not able to colonize the acrylic resin in this time frame, probably since this yeast depends on coaggregation mechanisms and binding to preexistent bacteria, which explains why 4 h maybe insufficient for this to happen.[10,23] Furthermore, the CFU counts of *Candida* spp. were very low, owing to the young age, health status, and good oral hygiene habits of the participants included in this research.[17]

Although there is potential for colonization of pathogenic species, *Staphylococcus* spp. and *Pseudomonas* spp. did not show any CFU counts in the studied population. This may once again be due to the optimal characteristics of the study sample or may indicate that the time frame is too premature to permit colonization of these species. This goes in accordance with previous studies which state that these bacteria are present mainly in the elderly population or in patients with respiratory diseases.[6,24]

Furthermore, anaerobes colonized the acrylic resin more readily than aerobe species in the population sample. Anaerobe species are traditionally preceded by aerobe species, and given the time frame (4 h), it would be

Table 2: Wilcoxon nonparametric test for the comparison of the acrylic resin samples versus oral microbiome (control) of the participants (*Z* - test and asymptotic significance (bilateral))

Wilcoxon test	*Z*	significance
Total counts (control vs. resin)	−1.183	0.108
Aerobes (control vs. resin)	−0.497	0.619
Anaerobes (control vs. resin)	−1.607	0.108
Oral *Streptococci* (control vs. resin)	−1.870	−0.061
Streptococci mutans (control vs. resin)	−1.382	0.167
Enterobacteriacae (*control vs. resin*)	−0.356	0.722
Pseudomonas (control vs. resin)	0.000	1.000
Staphylococci (control vs. resin)	−1.000	0.317
Candida (control vs. resin)	−2.207	0.027

Table 3: Semiquantitative scale used for the cytological parameter score under light microscopy

	Cytological parameter	(-)	(+)	(++)	(+++)
Contact zone	Nuclear-cytoplasm ratio (%)	16	20	52	12
	Cytoplasmic amphiphilia (%)	26	64	10	0
	Multinucleation (%)	56	24	20	0
	Perinuclear halos (%)	48	44	6	2
	Karyorrhexis (%)	28	22	48	2
	Karyolysis (%)	40	52	8	0
	Karyopyknosis (%)	6	64	28	2
Control zone	Nuclear-cytoplasm ratio (%)	56	32	12	0
	Cytoplasmic amphiphilia (%)	14	10	64	12
	Multinucleation (%)	84	10	6	0
	Perinuclear halos (%)	28	44	26	2
	Karyorrhexis (%)	40	46	14	0
	Karyolysis (%)	72	28	0	0
	Karyopyknosis (%)	10	18	60	12

-: Negative; +: Mild; ++: Moderate; +++: Severe

expectable to encounter more aerobes than anaerobes, but the contrary happened. This suggests that the biofilm maturation on the acrylic resin's surface happens more rapidly than biofilm formation on normal oral surfaces. This feature is alarming when it comes to the high potential of pathogenic environment that could, theoretically, be established over the acrylic resin's surface.[25,26]

The cytologic findings for the experimental group highlighted a certain degree of nuclear alterations when the cells were in contact with the acrylic resin's surface. High nuclear cytoplasm ratios are a manifestation of cellular atypia, indicating that cellular pathologic changes might be taking place.[27] Furthermore, on analyzing the nuclear morphology alterations, the contact zone revealed a high number of positives. These nuclear alterations are frequently evidence of processes of cellular death,[28] which may prove the acrylic resin was cytotoxic, *in situ*, to the contacting cells. Other parameters such as multinucleation or perinuclear halos were considered less reliable in the interpretation of an inflammatory diagnosis since there was a certain degree of cellular overlapping which made the microscope analysis difficult.[29] Another limitation to point out was that the majority of the cells collected were superficial squamous cells which, in turn, might mask an underlying inflammatory condition. An acute inflammatory process tends to start in the deeper layers of the subjacent oral tissues.[30] Future studies should address this issue, by comparing various brushes and enhancing their capacity in collecting deeper mature cells.

Upcoming research should focus on surface electron scanning microscopy analysis of the biofilm formation over the acrylic resin's surface and future comparison between different commercial products of acrylic resins. This will allow the determination of each material's susceptibility to colonization. Immunohistochemical assays could also provide insight on the characterization of the inflammatory process and cellular damage of these materials.

Conclusion

From this *in situ* study, taking into consideration all of the limitations in the study and experimental design, we can infer that the colonization of acrylic surfaces exposed intraorally, to the microorganisms tested, is similar quantitatively and qualitatively to the oral biofilms. It was also possible to verify *in situ* cellular alterations in the contact zone with the acrylic resin samples showing that this material might be cytotoxic to the oral mucosa in the initial hours.

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Conflicts of interest

There are no conflicts of interest.

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