PREVELANCE OF ORAL AND RESPIRATORY MICROORGANISMS ON THE CAD-CAM VERSUS PRESS FORM OF POLY-ETHER-ETHER KETONE MATERIAL FOR ORAL AND MAXILLOFACIAL PROSTHESIS

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ABSTRACT

INTRODUCTION: Biofilm formation differs in accordance to material type and processing technique, which has a crucial impact on maxillofacial prosthetic materials' practicability.

OBJECTIVES: to compare the prevalence of the upper respiratory tract and oral microbial flora colonization on the two commercially available forms of polyetheretherketone (modified PEEK); the conventional press form and the CAD-CAM form.

MATERIALS AND METHODS: : forty eight circular discs, were processed forming four groups: Group I: twelve BioHPP discs were prepared with CAD-CAM form, Group II: twelve BioHPP discs with conventional press form, Group III: twelve heat polymerized poly methyl meth acrylate (PMMA) to mimic the polished surface Group IV: twelve heat cured PMMA to mimic the fitting surface. Microbiological procedures were performed including microbiological sampling, isolation, purification and identification, biofilm formation and assessment of the normal oral and respiratory flora on modified PEEK and PMMA discs.

RESULTS: Acrylic resin fitting surface group had the highest biofilm formation when compared with the three other groups. No statistical significant difference was found between each pair of the three remaining groups, CAD-CAM BioHPP group showed the lowest biofilm formation of all groups according to mathematical data, but it didn't approach the level of being statistically significant.

CONCLUSIONS: Both BioHPP processing techniques were positive for biofilm formation, though BioHPP CAD-CAM showed a rougher surface than pressed BioHPP, it showed the least biofilm formation of all groups. Fitting surface of acrylic resin group showed the worst results for biofilm formation.

KEYWORDS: Biofilm, PEEK, Acrylic resin, Respiratory flora.

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INTRODUCTION

Management of the patient with congenital or acquired maxillofacial defect resulting in communication between the oral cavity, nasal cavity, and/or maxillary sinus, presents challenge to the clinician. The maxillofacial defect involving maxilla can be rehabilitated either by surgical correction with plastic surgery or by an obturator prosthesis.

The maxillofacial prosthesis can rehabilitate the defect and a successful rehabilitation would go a long way in improving the quality of life of the patient, and in certain circumstances, it may be an alternative for plastic surgery repair (1).

Silicone and poly methyl meth acrylate (PMMA), have been used as maxillofacial prosthetic materials for ages, with several advantages of superior esthetics, seamless transitions between the natural and artificial and a cosmetic restoration as close to natural as possible. The cardinal disadvantage of the well-established materials, silicone, and (PMMA), is the fact that microorganisms find the most suitable conditions for growth on the surface. This microbial contamination could result in local or systemic infections via biofilm formation (2).

Moreover, presence of acrylic denture biofilms in contact with oral tissue surfaces is a significant cofactor in the pathogenesis of denture stomatitis. This pathology involves an erythematous condition of the oral mucosa seen under the acrylic dentures (3).

Adherence of microorganisms is an essential first compulsory step for colonization, and the currently used materials may present suitable ground by virtue of some physical properties, such as uncontrolled surface roughness. In fact, fitting surfaces of maxillofacial prostheses, are processed against dental stone and the resultant surface is not smooth in general; several studies have shown that rougher surfaces may result in greater microorganisms adhesion, while other studies have proved that surface roughness and mature biofilm formation are quiet irrelevant, as it only affects initial adhesion force and early attachment instead of whole stages of biofilm formation, moreover surface roughness appeared to have no effect on the number of adherent bacteria (2).

Unfortunately, it is not easy to avoid adhesion of microorganisms to the surface of dental materials. Because of the disadvantages associated with the commonly used materials, the search for the ideal material continues, so searching for an alternative material with an enhanced properties became a necessity.

A potential candidate is modified polyetheretherketone (PEEK) (BioHPP)®. BioHPP is a semi crystalline polyaromatic linear polymer that exhibits an excellent combination of strength, stiffness, durability and environmental resistance (4). The biocompatibility of peek has been established and subsequent prosthetic application

of the material have followed. It has also been used intraorally as a maxillofacial prosthesis (5).

Since modified PEEK is a promising alternative, we hypothesized that biofilm formation on the two commercially available forms of BioHPP; CAD-CAM form and press form in comparison to the gold standard PMMA might be promising.

According to our current knowledge, no research has been published yet studying BioHPP's microbiological properties to determine its biocompatibility with the upper respiratory tract flora (oral, nasal and nasopharyngeal) owing to its complex nature. Hence, further researches are needed to investigate the practicability of using BioHPP as a removable maxillofacial prosthetic material. The null hypothesis tested for this study was that the prevalence of upper respiratory tract flora is equal for both modified PEEK and PMMA materials.

MATERIALS AND METHODS

This study was a comparative laboratory study, in which the biofilm formation on different available commercial forms of BioHPP (Bredent GmbH & Co. KG, Senden, Germany) was assessed in comparison to PMMA.

For this study, 48 circular specimens were prepared forming 4 groups (Group I, II, III and IV), group I (study) includes 12 specimens fabricated using CAD-CAM milling technique, group II (study) includes 12 BioHPP specimens fabricated using the Press technique, group III (control) includes 12 PMMA specimens fabricated by conventional processing technique (mimicking the polished surface) and group IV (control) includes 12 PMMA specimens fabricated by conventional processing technique (mimicking the fitting surface). Each specimen was a circular disc of 8mm diameter and 3 mm thickness. Each specimen was microbiologically evaluated for biofilm formation, bacterial colonization (Aerobic and Anaerobic) and fungal growth.

Specimen Preparation

A- CAD-CAM technique (Group I).

Twelve BioHPP specimens were prepared from breCAM circular blank for processing in a CAD/CAM Workflow. On a computer software (Dental Wings DWOS. CAD-CAM designing and milling software) and milling machine (SHERA eco-mill 5x.Germany), circular study disc of 8mm diameter and 3 mm thickness was virtually designed and used to standardize the dimensions of all specimens for all four groups. Finally, with the help of the CAD-CAM workflow twelve circular 8 mm diameter and 3 mm thick BioHPP discs were milled from the breCAM blank (figure 1). A rubber cup bur was used to smoothen the milled specimens after cutting the connector, in order to mimic the clinical conditions (5).

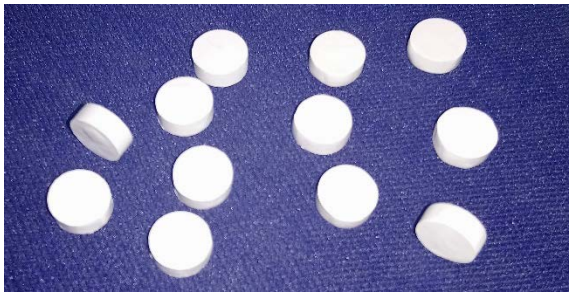

Figure (1): Showing twelve milled BioHPP circular discs.

B- Conventional press technique (Group II)

Twelve circular BioHPP discs of the same dimensions were created using conventional press method utilizing BioHPP pellets using *for2press* device (figure 2).

Figure (2): BioHPP press procedure.

Processing technique

A circular wax blank14 mm thickness that burns out without leaving any residue (Katana wax disc, Kuraray Noritake Dental Inc., 300 Higashiyama, Mioshi-cho, Japan) was used to produce twelve circular wax discs of 8 mm diameter and 3 mm thickness.

Spruing was done then the muffle formers were filled with the investment material (special for *for2press*) while it was being placed on a vibrator. The muffle was placed into the preheating furnace (Apex Burn-out, Apex Dental Technologies, 850 N Dorothy Dr. Richardson, TX 75081, USA) after an expansion time of 20 minutes.

Once the material has melted, a disposable press plunger which was preheated up to 400°C was attached to the muffle, which was placed into the for 2 press unit (BioHPP®, Bredent GmbH & Co. KG, Senden, Germany) and the pressing table was closed manually.

Devesting process was accomplished. Investment material residue was removed with a fine sandblasting unit (6) µ aluminum oxide, at a pressure of 3 bars). Finishing and polishing was done following Bredent protocol finally, twelve circular BioHPP discs of 8 mm diameter and 3 mm thickness, produced via pressing method were created.

C- Heat cured PMMA (Group III and IV)

Group III

Twelve heat cured PMMA circular discs (8mm diameter, 3mm thickness) were processed by conventional processing technique to be a control group for the study (7).

Procedures:

1. From a circular wax blank (Katana wax disc, Kuraray Noritake Dental Inc., 300 Higashiyama, Mioshi-cho, Japan) twenty four circular discs were milled by CAD-CAM subtractive milling procedure by the help of the previously used software module to standardize this control group with the previously explained two study groups I and II.

2. After subtractive milling using the exact same previously used software 24 circular wax discs were produced.
3. The produced wax discs were flasked using conventional processing methods, each 6 discs were flasked separately.
4. The wax was eliminated by placing the flask in the wax boil out and elimination unit.
5. Curing was done in a curing unit done via a short curing cycle then the produced discs were finished with sand paper after removing the excess flashes of acrylic resin that was forced out between the two halves of the flask.
6. They were finished and polished following traditional acrylic resin finishing and polishing protocol (8), in order to mimic the polished surface clinical condition.

Group IV

The other twelve samples were created to mimic the fitting surface. They were created with the exact previous steps; no further polishing of the samples was applied in order to mimic the oral conditions of the fitting surface.

Microbiological Preparations and Methodology

I- Microbiological sampling (9)

Oral, nasal, and nasopharyngeal samples were collected with a sterile cotton swab. Samples were taken from a healthy volunteer they were collected carefully to avoid touching non-involved surfaces or mucosa.

II-Isolation of microorganisms

The isolated microbial flora were cultured on (blood agar, chocolate blood agar, MacConkey's agar, Sabouraud dextrose and mitis salivarius agar) and anaerobic conditions as well (blood agar in restrict anaerobic conditions).

After the plates were incubated aerobically and anaerobically for 72 hours at 37°C.

III- Specimen purification and identification

Prior to identification by Matrix assisted laser desorption time of flight mass spectrometry (MALDI-TOF MS) (Ultra Flex Extreme, Bruker Corporation, Massachusetts, USA) (figure 3). The isolated bacterial strains were purified by sub-cultures on blood agar to obtain a young and fresh culture. The sub-cultures were identified to the species level by the use of MALDI-TOF MS identification device.

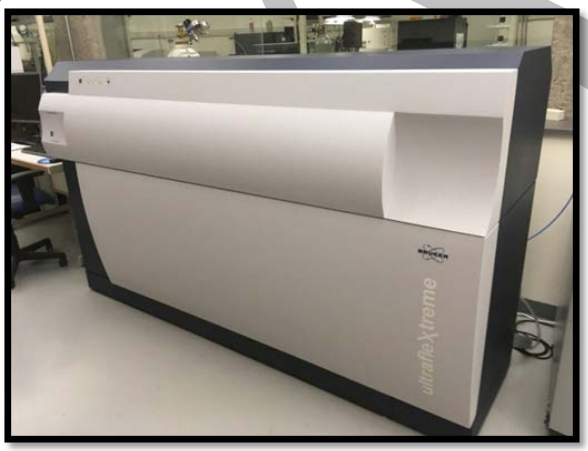

Figure (3): Bacterial strains identification device MALDI-TOF MS.

As MALDI utilizes a soft ionization mechanism, a saturated low mass organic solution called matrix (a UV-absorbing MALDI solution) (Bruker Matrix; α-Cyano-4-hydroxycinnamic acid. Billerica, Massachusetts, US) was used.

Once ionized, proteins within the specimen were analyzed by a component of the mass spectrometer called the mass analyzer to reveal characteristic information about the composition of the sample in the context of mass-to-charge ratios, which are electrodynamic measurements of how quickly charged ions from a sample move through the time of flight (TOF) tube and reach a detector. Once spectra were generated, comparison to a data base of defined reference spectra lead to microbial identification (10).

After MALDI-TOF MS identification, 13 oral, nasal and naso-pharyngeal species were identified to the species level, based on which the following steps proceeded.

All the study discs (BioHPP and PMMA) were sterilized by gas plasma sterilizer; low-temperature, hydrogen peroxide plasma sterilizer (HUMANMEDITEK Plasma Sterilizer medical devices & equipment companies –Gas plasma sterilizer. Toronto, Ontario M3B 3P9 Canada).

IV- Biofilm formation on BioHPP and PMMA Discs

After identification, another fresh subculture was done. From the 13 isolates, bacteria was harvested from each plate and inoculated onto a sterile flask containing 500 ml nutrient broth (Thermo Fisher Scientific, Oxoid Products and Remel, 100-1926 Merivale Rd, NEPEAN, Ontario K2G 1E8), with 1% glucose, turbidity was adjusted to 108 (0.5 McFarland turbidity standard).

The flask was incubated for 24 hours, at 37°C, then the culture was diluted 1/100. A rack with 48 sterile test tubes was prepared and divided to four groups of sterile test tubes (twelve for the press form BioHPP **group I**, twelve for the CAD-CAM form BioHPP **group II**, twelve for polished surface PMMA **groups III** twelve for the fitting surface PMMA **group IV**).

From the diluted broth 10 ml were added to each corresponding tube. 48 sterile BioHPP and PMMA discs were aseptically added.

All tubes with discs were incubated at 37°C for 72 hours aerobically to help biofilm establishment (figure 4). After incubation, the discs were removed from the test tubes and transferred into another dry sterile test tubes prior to washing.

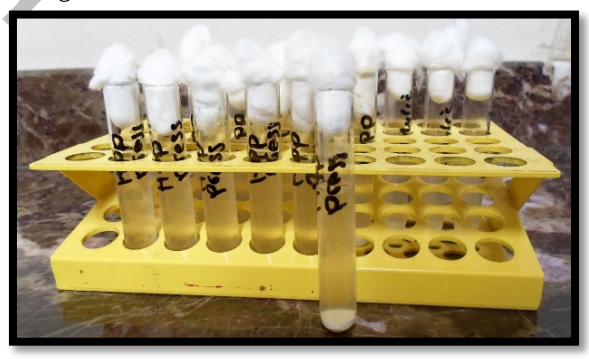

Figure (4): Showing discs in turbid mixture after incubation (biofilm formation).

The discs were washed with sterile saline 4 times with gentle shaking every time, to remove the free floating excess bacteria. Biofilm formed on discs was fixed with 1% methanol for 15 minutes, then it was stained with 0.1% crystal violet (5ml) on each disc for another 15 minutes in sterile cups (11, 12).

Excess crystal violet stain was removed by washing the discs in sterile distilled water four times (avoiding direct application on the specimen), then it was allowed to dry

overnight room temperature. Two ml at 30% acetic acid was added to each disc to elute the crystal violet stain from the biofilm formed on the surfaces (12) in sterile labelled test tubes (figure 5).

Figure (5): Showing different acetic acid elusion color owing to different stain uptake, corresponding to different amounts of biofilm formation.

V- Quantitative evaluation of biofilm formation (colorimetric staining assays)

The stain eluted from each disc was subjected to an enzyme-linked immunosorbent assay (ELISA) reader at an optical density (OD) of 570 (13). to read the stain absorbance (figure 6). The amount of dye solubilized by the solvent (acetic acid), was directly proportional to the amount of biofilm formation (12).

Statistical analysis

Data were collected and entered to the computer using SPSS (Statistical Package for Social Science) program for statistical analysis (ver 21). Data were entered as numerical or categorical, as appropriate.

Kolmogorov-Smirnov test of normality revealed no significance in the distribution of the variables, so the parametric statistics was adopted.

Data were described using minimum, maximum, mean, standard deviation.

Comparisons were carried out between more than two independent normally distributed subgroups using one-way ANalysis Of VAriance (ANOVA) test. When F ratio of ANOVA was significant Levene test of homogeneity of variances was done, and if significant Brown-Forsythe Robust test was adopted. Post-hoc multiple comparisons was done using Games-Howell method. An alpha level was set to 5% with a significance level of 95%, and a beta error accepted up to 20% with a power of study of 80% (14).

Figure (6): ELISA micro well auto reader.

RESULTS

Optical density (OD) results representing biofilm formation in CAD-CAM BioHPP (Group I) ranged from 0.0100 to 0.0210 with a mean value of 0.0141 ± 0.0034, while in Press form BioHPP (Group II), it ranged from 0.0090 to 0.0260 with a mean value of 0.0196 ± 0.0063. While, in Acrylic resin polished surface (Group III), OD values ranged from 0.0100 to 0.0260 with a mean value of 0.0172 ± 0.0053. Finally in Acrylic resin fitting surface group, it ranged from 0.0290 to 0.0860 with a mean value of 0.0464 ± 0.0169.

Pair-wise comparison using Games-Howell multiple comparison method proved that there was a statistically significant difference among the four study group (F=29.070, p=0.000). Acrylic resin fitting surface group had the highest biofilm formation with the highest OD when compared with the three other groups, (Having different superscript letters). No statistical significant difference was found between each pair of the three remaining groups (having same superscript letters) (Table 1and 2).

Table (1): Showing optical density results for all groups indicating biofilm formation.

Group	*Specimen number*											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
CAD-CAM PEEK(group I)	0.01	0.017	0.013	0.019	0.014	0.021	0.015	0.012	0.014	0.013	0.01	0.011
Press form PEEK (group II)	0.023	0.024	0.011	0.025	0.010	0.020	0.018	0.024	0.009	0.020	0.026	0.025
Polished surface PMMA (group III)	0.015	0.026	0.017	0.014	0.018	0.011	0.010	0.023	0.016	0.020	0.012	0.025
Fitting surface PMMA (group IV)	0.061	0.048	0.0186	0.029	0.043	0.067	0.036	0.031	0.038	0.033	0.041	0.044

Table (2): Comparison of biofilm formation among all four groups.

	Group			
	CAD CAM PEEK(Group I)	**Press form PEEK (Group II)**	**Acrylic resin polished surface (Group III)**	**Acrylics resin fitting surface (Group IV)**
Biofilm formation				
n	12	12	12	12
Minimum	0.0100	0.0090	0.0100	0.0290
Maximum	0.0210	0.0260	0.0260	0.0860
Mean	0.0141[a,b,c]	0.0196[a,b,c]	0.0172[a,b,c]	0.0464[d]
±S.D.	0.0034	0.0063	0.0053	0.0169
Test of significance p value	$F_{(BF)(df=3)}$=29.070 *p*=0.000*			

n: Number of specimens
S.D.: Standard deviation
BF: Brown-Forsythe test.
Different superscript letters (a, b, c, d) indicate significant difference using Games-Howell multiple comparison method.
*: Statistically significant ($p<0.05$)
NS: Statistically not significant ($p\geq0.05$).

DISCUSSION

The successful function of maxillofacial prosthodontic appliance requires the fabrication of a biocompatible prosthodontic appliance that meets patients' demands of comfort, light weight and function as well. Regarding maxillofacial prosthodontics PMMA has been declared as the gold standard as it has proven success through years of clinical service, but, it has been reported that PMMA has a great potential for harboring microorganisms owing to its porosity (23 pores at 0.01 µm) hence the need for a new candidate that will overcome this drawback (15).

In the current study, prevalence of oral and respiratory flora was tested on the two commercially available forms of (BioHPP)® (CAD-CAM form and press form) that was successfully used in maxillofacial prosthodontics, in comparison to heat cured (PMMA) (Acrostone) the gold standard for maxillofacial prosthodontics.

The aim of this work was to assess whether both types of modified PEEK has lower susceptibility for biofilm formation or not.

Poly ether ether ketone (PEEK) was chosen as it's the new era in prosthodontics, it has proven a favorable clinical outcome by many investigations like: Santiago Costa-Palau et al. (5) who has published a clinical report of the fabrication of an obturator prosthesis for a patient with large oral-nasal defect using PEEK-Optima and reported that it was a good alternative to conventional materials.

On dental wings computer software a circular study disc of 8 mm diameter and 3 mm thickness was designed and created virtually which was used for all groups to standardize dimensions.

The reason why it was designed circular with those specific dimensions was related to the use of 10 mm diameter test tubes, so circular discs of narrower diameter were chosen to facilitate the discs' entry and exit throughout the test tubes. Circular dimensions were recommended by Pei Yu et al (16), who designed round zirconia discs with dimensions of 10mm × 2 mm, in order to study the influence of surface properties on adhesion forces and attachment of Streptococcus mutans to zirconia.

In the current study, finishing and polishing protocols were not the same for all the four groups, as the study was designed to mimic the clinical conditions for materials' use, because different materials with different processing procedures necessitate different finishing and polishing protocols.

For heat cured acrylic resin, the study design was to test biofilm formation on both fitting and polished surfaces, in order to simulate the exact clinical conditions for using PMMA. Specimens replicating polished surfaces (Group III) were finished and polished following conventional protocol (8). But, those replicating fitting surfaces were not touched after processing and finishing.

Regarding BioHPP specimens, using CAD-CAM (Group I), specimens were fabricated and were already shiny after milling, only a rubber cup was used to smoothen the specimens as only minimal polishing was needed (5).

As for press form BioHPP specimens (Group II), finishing and polishing was a must as well, because pressing conditions entail sand blasting after devesting, which leaves the surface quite rough, hence to mimic the clinical conditions at which pressed BioHPP was used, following the four steps Bredent finishing and polishing protocol was inevitable as well.

Gas plasma sterilization method was chosen for all specimens to avoid inducing any chemical or mechanical changes to the material as suggested by previous studies that used other sterilization techniques.

Amit Kumar (17) studied The effects of the sterilization process on medical grade thermoplastic BioHPP, Test results on the medical device concluded that there is a decrease of ~20% in the compression force after 30 autoclave cycles and a decrease of ~6% in the lateral dimension after 50 autoclave cycles.

Thus, this thermal reliability study on BioHPP suggests that if a reusable medical device is fabricated using BioHPP, which will be subjected to repeated sterilization processes, the change in mechanical properties of BioHPP needs to be accounted for.

At the same time, T.J.A.G. Münker et al. (18) studied the Effects of sterilization on the mechanical properties of poly (methyl methacrylate) based personalized medical devices, and concluded that for the sterilization of PMMA-based materials, only ethylene oxide, hydrogen peroxide gas plasma, and γ-irradiation appear to be suitable techniques to sterilize PMMA-based personalized medical devices. This conclusion supported our choice of gas plasma sterilization method.

From an ecological perspective a bacterial mixture containing oral, nasal and nasopharyngeal microorganisms; aerobes, anaerobes and fungi was performed in one study (test) to get a diversified perspective of all the microorganisms related to the maxillofacial prosthesis.

Choosing quantitative evaluation of biofilm formation (colorimetric staining assays) Using ELISA micro well auto reader, was supported by considering that it is the gold-standard method for biofilm detection as recommended by various microbiological investigations (11,12).

Concerning biofilm formation assessment our study proved that acrylic resin fitting surface group had the highest biofilm formation when compared with the three other group.

Meanwhile, mathematical data of optical density have proved that CAD-CAM specimens had the lowest biofilm

formation of all groups this observation might be related to variable surface roughness of the different specimens, as the acrylic resin fitting surface (Group IV) was not polished, accordingly it had higher surface roughness and showed higher biofilm formation. The other three groups showed less biofilm formation which may be attributed to polishing the specimens that promoted less biofilm formation secondary to less surface roughness.

These results are in agreement with the published study by Kawai et al. (19) which found a positive correlation between surface roughness and the amount of plaque accumulation. Also, Maryam Gharechahi et al. (20) which showed that roughening of the surface increases the area available for bacterial adhesion. Also, Mei et al. (21) evaluated the streptococcal adhesion forces with composite resins with different surface roughness. They confirmed that Streptococcal adhesion forces to composite increase with increasing roughness of its surfaces.

Regarding acrylic resin, Morgan and Wilson (2) investigated the effects of surface roughness and type of denture acrylic on the early development of a Streptococcus biofilm found the same finding that the number of bacteria adhering to acrylic increased linearly with mean surface roughness.

The results of biofilm formation interpreted by optical density in our study revealed that there was no significant difference between (Group I) CAD-CAM BioHPP, (Group II) Press form BioHPP and (Group III) acrylic resin polished surface.

This result might be attributed to another explanation for the relation between bacterial colonization onto materials with different manufacturing method with different topographic view. It was declared that distinct topographies were produced by different manufacturing methods. Previous studies revealed that CAD-CAM BioHPP is considered as machined and Press form BioHPP is considered as injection molded (22).

In our study it was observed that CAD-CAM BioHPP specimens with machined topography showed less biofilm formation compared to Press form BioHPP with injection molded topography.

On the contrary, the findings of T.J Edward et al. (22) declared that machined topography of the CAD-CAM BioHPP leads to increased bacterial adhesion in vitro, particularly around the larger surface features present while, injection-molded BioHPP topography lacks these large features and therefore staphylococcus aureus bacteria adhere at a lower density in a more random manner.

Their results might be due to the great difference between our study and theirs regarding the PEEK type; BioHPP versus PEEK-OPTIMA which was utilized by their study. Also, they focused on one bacterial type (staphylococcus aureus) while our study tested the effect of a bacterial and a fungal mixture rather than a single bacterial strain. Or the difference is owing to the fact that biofilm formation is a complex multi factorial process as it depends on many other factors.

CONCLUSION

Within the limitations of this study it can be concluded that;

1. Biofilm formation on CAD-CAM BioHPP (Group I), Pressed BioHPP (Group II) and polished surface acrylic resin (Group III) was lower than Fitting surface acrylic resin (Group IV) as shown by the OD values and the colorimetric staining assays.
2. Biofilm formation was the highest on the fitting surface acrylic resin (Group IV).
3. There's no significant difference among the other three groups (I, II and III) regarding biofilm formation when compared using Games –Howell pair wise comparison method.
4. CAD-CAM BioHPP showed lowest biofilm values when compared to press form BioHPP but, it didn't approach the level of being statistically significant.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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