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MICROBIOLOGICAL EVALUATION OF BIOFILM FORMATION ON DENTURE BASE MATERIALS MADE USING COMPUTER AIDED DESIGN/COMPUTER AIDED MANUFACTURING (CAD/ CAM) TECHNOLOGY. AN IN VITRO COMPARATIVE STUDY

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ABSTRACT

Background: CAM/CAM technology is widely used in prosthetic and implant dentistry; however, the influence of the material being used for construction of denture base on microbial adherence remains inconclusive.

Aim of the work: The purpose of this study was to compare the amount of biofilm formation of Candida albicans and Staphylococcus aureus on different denture base materials formed by CAD/ CAM technology.

Materials and Methods: The study was performed on 90 controlled type II diabetic patients from Prosthodontics Department for isolation of Staphylococcus aureus and Candida albicans, then detection of biofilm producing isolates and assess biofilm formation on different denture base materials formed by CAD-CAM technology.

Forty disc-shaped (PMMA) samples were divided into two groups as follow:

- Twenty 3D printed (PMMA) disc-shaped samples constructed by CAD/CAM technology.
- Twenty milled (PMMA) disc-shaped samples constructed by CAD/CAM technology.

The adherent cells and formation of Candida albicans and Staphylococcus biofilm were measured by using a microplate reader. At the end of the study all data were collected, tabulated, and statistically analyzed by IBM-SPSS statistics software.

Results: In the current study biofilm formation on 3D printed specimens was more than milled specimens regarding Candida albicans, but there was no significant difference in both groups regarding Staphylococcus aureus.

Conclusions: Additive 3D-printing technology resulted in increased microbial biofilm formation compared to CAD/CAM milling techniques on acrylic denture base resin.

KEYWORDS: PMMA, CAD/CAM milling techniques, Additive 3D-printing Candida albicans, Staphylococcus aureus, biofilm formation.

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INTRODUCTION

Teeth, tongue, and oral mucosa are common habitats for a special microbiota that lives in the mouth. Oral microbes are often present in communities known as biofilms. A biofilm is a structure that firmly attaches to a surface and is composed of cells that are submerged in an extracellular polysaccharide matrix. Dental plaque is one of the most well-known types of biofilms.[1]

Secondary caries and pulp disease can result from microorganisms that cling to dental implants and other types of prosthetic restorations.[2] within 24 hours, a noticeable biofilm forms on oral biomaterials; shortly within three to five days, a biofilm involving many species forms; and within two to three weeks, the biofilm reaches maturity.[3]

Staphylococcal biofilms may quickly colonize dentures since they do not shed saliva. Because it may attach to so many various surfaces of the mouth cavity, Staphylococcus aureus is more commonly detected as normal flora of the mouth in people who wear dentures than in people who do not. It is also known that denture stomatitis can develop when microbes colonize the mouth and denture surfaces, which is an issue for those who wear dentures.[4]

Even though denture wearers are at increased risk for Candida-associated stomatitis, the yeast is really a common component of the oral cavity's natural commensal flora and is responsible for an increase in Candida colonization.[5] Research has shown that the removable denture acrylic surface's porosity, permeability, and roughness can influence Candida colonization and biofilm development.[6] Patients who utilized dental appliances had a higher frequency of Staphylococcus aureus and Candida.[7]

Dentures and prosthetic teeth are most commonly made from polymethyl methacrylate (PMMA) due to its many desirable qualities, such as its biocompatibility with soft tissue, manufacturing simplicity, stability, and aesthetically pleasing appearance.[8] Earlier research has shown that conventional acrylic dentures have a number of drawbacks, including poor mechanical strength, fractures after a few years of use, shrinkage during polymerization, allergic reactions primarily to the unreacted monomer, which can lead to the formation of fissures, and further structural damage to the denture base, which can serve as an entry point for microorganisms. Noticeably, denture surface pore count, microbial colonization susceptibility, and biofilm development might be affected by the kind of curing.[8,9]

The field of prosthodontics is one that is rapidly adopting the use of computer-aided design and computer-aided manufacturing (CAD/CAM) techniques, despite their long history of availability. In dentistry, computer-aided planning (CAP), computer-aided design (CAD), computer-aided analysis (CAA), and computer-aided manufacturing (CAM) are all parts of computer-aided technology (CAT), which is an umbrella word for the use of computers to assist with engineering and analytical activities.[10] In the field of fixed prosthodontics, removable prosthodontics, and implant prostheses, computer-aided design and computer-aided manufacturing (CAD/CAM) have been and will be used.[11&12] Removable dentures may now be made utilizing fast prototyping or milling thanks to recent technological breakthroughs that enable the use of various CAD/CAM systems.[13]

Improving the quality of finished complete dentures and decreasing chair time are the two primary goals of using CAD/CAM method in manufacturing. Modern innovations in both materials and technology have made this a reality, paving the way for a departure from the traditional materials used to make full dentures.[14]

With the use of computer-aided technology, the number of patient visits for complete denture treatment is greatly reduced, which improves and simplifies the process for edentulous patients.

[15,16] Better quality control, more robustness, and digital data storage are a few other possible benefits. [17] The current study aimed to investigate the production of biofilm on various denture base materials created using CAD-CAM technology. Denture foundation materials made of milled and 3D-printed polymethyl-methacrylate (PMMA) were hypothesized to be same.

MATERIALS AND METHODS

Materials and methods can be summarized as following:

1. Selection of the study design and inclusion and exclusion criteria for the participants of the study.
2. Preparation of the patients.
3. Preparation of CAD-CAM acrylic resin samples (3D printed and milled).
4. Processing of oral mucosal swab samples for isolation of Staphylococci and Candida respectively.
5. Detection of biofilm formation by Tissue culture plate method (TCP).
6. Quantification of Biofilm biomass on Denture base materials.
7. Grouping of the CAD-CAM acrylic resin samples.
8. Testing

Study Design and Participants:

This study was carried out over a period of six months in the Medical Microbiology and Immunology Department, Tanta University. The study was performed on 90 controlled type II diabetic patients from Prosthodontics Department, Tanta University, for isolation of *Staphylococcus aureus* and *Candida albicans* then detection of biofilm producing isolates and assessment of biofilm formation on different denture base materials formed by CAD-CAM technology. The study was approved by the Research Ethics Committee faculty of Dentistry, Tanta university (Approval code #R-RP-2-23-2).

Inclusion criteria:

1. Adult patients who were medically diagnosed as controlled type II diabetic patients. Diabetic patient is immunocompromised patient with high risk to colonization of oral cavity with risk of bacterial and fungal infection with biofilm formation due to defect in host clearance mechanism also high rate of implant failure in diabetic patient, so it is mandatory to evaluate different denture types.
2. Age ranged from 45-65 years old were enrolled in this study.
3. The selected patients should be edentulous for at least one year and have an old denture.

Exclusion criteria:

1. Patients suffering from any other metabolic, systemic, and endocrinal diseases rather than diabetes
2. Smoking patients were excluded from this study.

Patients' preparation:

All patients were subjected to preoperative laboratory investigations to confirm that they were controlled diabetics including testing the plasma glucose level every 20 days to indicate the glucose concentration at the time of sampling. For controlled diabetics, the American Diabetes Association recommended fasting plasma glucose level (at least 8h) up to 140 mg/dL and post prandial (2h after meal) up to 180 mg /dL.

Samples preparation:

Forty CAD-CAM acrylic resin discs with dimensions 25x2 mm. were fabricated for this

study and divided into two groups according to material. Two types of digital denture acrylic resin materials were used; twenty 3D printed acrylic resin (HARZ labs 3D printing material-Dental Pink using CHITUBOX Pro V1.3.0 software) and twenty milled acrylic resin discs using Millbox Cam software from Bristol CAD-CAM company according to the predetermined dimensions.

Sample processing: [18]

Oral mucosal swab samples were taken from all patients and were seeded on blood culture agar plates and sabaroud dextrose agar plates for isolation of *Staphylococci* and *Candida* respectively. All plates were incubated at 37°C for up to 24-48 hours. On the following day, the produced colonies were identified by conventional methods including microscopic examination, culture characteristics and biochemical reactions. Identification of *Staphylococci* were done by Mannitol salt agar and Coagulase test for confirmation of *Staphylococcus aureus* while identification of *Candida albicans* isolates were done by germ tube test and carbohydrate fermentation.

The confirmed *Staphylococcus aureus and Candida albicans* isolates were stored at -20°C in brain heart infusion broth containing 20% glycerol and subcultured for prior testing.

Detection of biofilm formation by Tissue culture plate method (TCP): [19]

One of the most common and long-established methods for detecting biofilm development is the tissue culture plate (TCP) experiment. After overnight growth on nutrient agar and sabouraud, a loopful of test organisms was inoculated into 10ml of trypticase soy broth (TSB) containing 1% glucose. For one day, the dextrose agar was kept at a temperature of 37º C. After incubating at 37ϰ C for 24 hours, the culture was further diluted 1:100 with new media. As seen in Figure 1, the sole control group received sterile broth. After the incubation period, the plates were gently tapped. The wells were rinsed four times with 0.2 ml of phosphate buffer saline (pH 7.2) in order to eliminate any bacteria that could be floating in the solutions. Staining with 0.1% crystal violet and fixing with 2% sodium acetate were applied to biofilms that remained attached to the well walls and bottoms. After the plates were adequately dry, any excess discoloration was rinsed away using distilled water. The dyed adherent biofilm's optical densities (OD) were measured at 570 nm using a micro-ELISA reader. We ran the experiment three times to ensure accuracy. Biofilm intensity was defined as a strong biofilm producer for OD values over 0.240, a non-biofilm producer for OD values below 0.120, and a moderate biofilm producer for OD values between 0.120 and 0.240.[20]

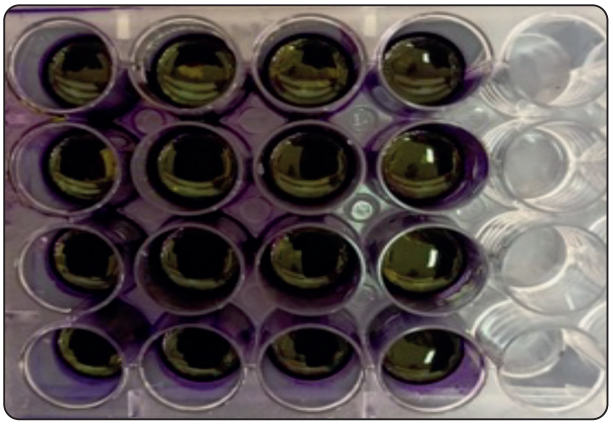

Fig. (1) Tissue culture plate for biofilm detection after staining with crystal violet.

Quantification of Biofilm biomass on Denture base materials:

All isolates (*Staphylococcus aureus* and *Candida albicans*) with strong biofilm forming ability were tested also for biofilm formation in the presence of denture based materials synthesized by CAD/CAM technique.

Sample's grouping:

Forty disc-shaped (PMMA) samples were constructed by CAD/CAM technology divided into two groups as follow:

Group (1): Twenty 3D printed (PMMA) disc-shaped samples subdivided into:

- Ten 3D printed (PMMA) disc-shaped were tested against *Staphylococcus aureus*.
- Ten 3D printed (PMMA) disc-shaped were tested against *Candida albicans*

Group (2): Twenty milled (PMMA) disc-shaped samples were subdivided into:

- Ten milled (PMMA) disc-shaped were tested against Staphylococcus aureus.
- Ten milled (PMMA) disc-shaped were against Candida albicans

To start, single colonies were obtained by cultivating each strain for 24 hours at 37°C on sheep 5% blood agar. Following this, the inoculum was made. To achieve an optical density (OD) 600=0.025±0.005, Staphylococci strains were mixed with C. albicans in the Sabouraud Dextrose Medium with 1% glucose (Oxoid, Termo Scientifc) and Brain Heart Infusion (BHI) medium (Oxoid, Termo Scientifc) with 50 mM glucose.[21] One milliliter of bacterial inoculum was poured over the tested disks after they were inserted in their respective wells of the fat-bottom polystyrene plate (Nest Scientifc Biotechnology). The incubation period for the cultures was 72 hours at 37 °C with gentle shaking at 50 rpm. Every 24 hours, the medium was replaced or renewed. To eliminate the cells that did not stick to the disks, they were gently washed twice with 1mL of phosphate buffered saline after incubation. Crystal violet was used to stain the biofilm on the surface of the sample. Next, the disks' biofilm was fixed in 1 mL of 10% formalin for 5 minutes. After that, 1 mL of Phosphate buffer saline (PBS) was used for rinsing. After 15 minutes of staining with 1 mL of 0.1% crystal violet, the biofilm was washed three times with PBS to remove any excess color. For 15 minutes, the samples were left at room temperature to dry. An ELISA reader was used to measure the absorbance at 590 nm. A positive control was an isolate devoid of discs, whereas a negative control was sterile disks. Triplicate runs of each test were carried out. [22]

RESULTS

Statistical analysis

Statistical analyses were performed using Statistical Package for Social Sciences (IBM SPSS Statistics version 26). Numerical variables are expressed using mean and S.D. P-value <0.05(*) was considered significant difference & P-value <0.001(**) was considered highly significant difference. The tests used in this analysis:

- One Way ANOVA test was used to compare the studied groups at *Candida* isolates and *Staphylococcus* individually.
- The multiple comparison (Tuckey test) was used to compare each of two groups after using ANOVA test.
- The independent t-test was used to compare the Biofilm Formation % between studied groups at *Candida* isolates and *Staphylococcus* individually.

Out of 90 patients with controlled type II diabetes, 76 patients (84.4%) showed positive culture growth while 14 patients (15.6%) had no culture growth. *Staphylococcus aureus* were 50% of the isolated species while *Candida albicans* were represented in 34.2%**.** Regarding biofilm formation among isolated species, 68.4% and 84.6% of isolated *Staphylococcus aureus* and *Candida* respectively were biofilm producers. According to the level of biofilm formation, 10 *Staphylococcus aureus* isolates were strong and moderate biofilm producers also 10 *Candida albicans* isolates were strong and moderate biofilm producers as shown in (**Table 1).**

TABLE (1) Distribution of isolated species from patients with Diabetes as regards Biofilm formation.

Percentage of Staphylococcus aureus and Candida albicans from oral cavity of patients with controlled type II diabetes			
	Negative growth	Positive growth	Total
	N(%)	N(%)	N(%)
Isolate	14 (15.6%)	76 (84.4%)	90(100%)

Distribution of *Staphylococcus aureus* and *Candida albicans* as regard biofilm formation				
	Staphylococcus aureus	*Candida albicans*	Others	Total
	N(%)	N(%)	N(%)	N(%)
Type	38(50%)	26(34.2%)	12(15.8%)	76(100%)
Non biofilm producer	12 (31.6%)	4 (15.4%)	16(25%)	
Biofilm producer	26 (68.4%)	22 (84.6%)	48(75%)	
Total	38 (100%)	26(100%)	64(100%)	
Strong (OD> 0. 24)	4 (15.4%)	3(13.6%)	7(14.6%)	
Moderate (OD >0.12<0.24)	6 (23.1%)	7(31.8%)	13(27.1%)	
Weak (OD<0.12)	16 (61.5%)	12(54.6%)	28(58.3%)	
Total	26(100%)	22(100%)	48(100%)	

The descriptive analysis of the Biofilm optical density **(OD)** for the *Candida albicans* and *Staphylococcus areus* between both studied groups (milled and 3D print) using one way ANOVA-test, revealed a significant difference between the studied groups with p-value 0.004* at *Candida albicans*, while there was no significant difference between the studied group at *Staphylococcus areus* with p-value 0.356 as shown in (**Table 2**).

The percentage of biofilm formation among *Staphylococcus aureus* isolates were detected higher than the percentage among *Candida albicans* isolates in both milled group and 3D printed group as represented in (**Table 3**).

Regarding the comparison between milled group and 3D group at biofilm formation percentage using independent t-test, there was a significant difference between the studied groups with p-value 0.024* at *Candida albicans*, where there is no significant difference between the studied groups at *Staphylococcus areus* with p-value 0.449. p-value was the result of comparing biofilm OD and the Biofilm formation % for the *Candida albicans* and *Staphylococcus areus* using mean and standard deviation, as shown in (**Table 4**).

TABLE (2) Descriptive comparison between the Biofilm OD for the Candida and Staphylococcus using one way ANOVA-test.

Type	**Biofilm OD**			**ANOVA test**	
	OD±S.D	Milled group	3D group	F	P-value
Candida isolates	1.13±1.04*	0.081±0.03*	0.417±0.43	6.773	0.004*
Staphylococcus	0.196±0.07	0.094±0.06	0.193±0.29	1.074	0.356

TABLE (3) The percentage of Biofilm Formation among isolated *Candida albicans* and *Staphylococcus aureus* isolates for each sample.

Sample	Biofilm Formation %			
	Candida isolates		Staphylococcus	
	Milled group	3D group	Milled group	3D group
sample1	2.76%	6.55%	44.97%	36.91%
sample2	2.02%	43.72%	75.00%	63.73%
sample3	4.14%	60.36%	39.63%	60.98%
sample4	4.79%	14.37%	7.14%	85.71%
sample5	6.38%	73.05%	80.75%	49.07%
sample6	4.17%	70.83%	44.60%	3.60%
sample7	50.00%	40.91%	45.08%	74.18%
sample8	52.17%	65.22%	48.51%	74.63%
sample9	45.83%	37.50%	7.14%	85.71%
sample10	42.86%	52.38%	66.12%	16.53%

TABLE (4) Comparison between the Biofilm Optical density and the Biofilm Formation percentage for *Candida albicans* and *Staphylococcus aureus* using independant t-test.

Type	Biofilm OD			Biofilm Formation % ± S.D		
	OD±S.D	Milled group	3D group	Milled group	3D group	p-value
Candida albicans	1.13±1.04	0.081±0.03	0.417±0.43	21.51±22.71	46.49±22.66	0.024*
Staphylococcus aureus	0.196±0.07	0.094±0.06	0.193±0.29	45.89±22.66	55.11±28.36	0.449

p-value is the result of comparing milled group and 3D group at Biofilm Formation.

DISCUSSION

One of the most common materials used for denture bases is acrylic resin polymethyl methacrylate, or PMMA. [23]. It characterized by Easy repair, attractive look, and low pricing are just a few of its many features. It's easy-to-work-with properties stem from the fact that it is a polymer formed by combining monomers of methyl and polymethyl methacrylate. [24]

Shortened fabrication time, data archiving, and automated denture production are just a few of the benefits that may be gained by using either milling or 3D-printed CAD/CAM processes instead of the more traditional approaches. With 3D printing, there is less material waste, production costs are reduced, and the possibility of creating intricate, personalized structures increases, making it a more environmentally friendly method of creation. Moreover, 3D-printing allows for the fabrication of products with complicated structures, in contrast to milling, where tolerance is an issue with milling equipment. [25]

The surface topography of the material is affected by the production procedure through surface flaws, irregularities, fissures, and porosities.[26] Because these flaws offer a surface for germs to cling to, the base material is more likely to get infested. On curved surfaces, the stair-stepping phenomenon becomes most noticeable because to the sequential nature of the 3D-priniting process. [27,28]

Maxillary full dentures' palatal surfaces are very important. The parallel oriented lines produced by milling burs are a defining feature of milled surfaces. [26] In contrast, linear and volumetric shrinkage are intrinsic to the traditional processing method, which results in tiny, microscopic cavities, porosities, and roughness in the final product. [29&30] It is crucial to study the oral micro flora of people who use removable dental prosthesis. [31]

Since the denture base is susceptible to colonization by both intra- and extra-oral species of bacteria, fungi, and other microorganisms, the cultivable flora of the detachable dental prosthesis revealed a diverse bacterial community. It has been proven that the removable dental prosthesis serves as a breeding ground for a variety of bacterial biofilms. [32-34]

Research has shown that the most common bacteria that may cling to the oral mucosa, create biofilms, and cause denture stomatitis are streptococcus mutans and Candida albicans. [35,36] Furthermore, Staphylococcus aureus is more commonly found in the oral natural flora of those who wear dentures compared to those who do not [37]

Many surfaces of the oral cavity can become infested with Staphylococcus aureus, including dental prosthesis.[38] Staphylococcal biofilms colonize dentures more readily than other oral surfaces because dentures do not shed. [39] This study aims to determine which CAD-CAM denture materials could potentially promote biofilm formation of Staphylococcus aureus and Candida albicans in controlled type II diabetic patients, considering the fact that these microorganisms colonize and persist through biofilm formation and their virulence properties.

In the present study, 76 out of 90 patients (84.4%) with controlled type II diabetic patient showed a positive growth of *staphylococcus aureus* and *Candida albicans* in their oral cavity. *Staphylococcus aureus* were the predominant isolates followed by *candida albicans* representing (50%), (34.2%) respectively. These results were matched with results Oral and Enla that showed 39.3% of the individuals exhibited *Staphylococcus* species and 28% of patients had *candida* species mainly *candida albicans*. [40]

This population's high prevalence of Staphylococcus and Candida species indicates that these microbes infiltrate the mouth on a frequent basis, particularly in periodontal pockets. This finding suggests that this colonization might serve as a reservoir for the population, potentially spreading to other parts of the body. [41]

Regarding biofilm formation, most of isolated staphylococcus aureus (68.4%) in the current study were biofilm producers. Moreover, (84,6%) of *Candida albicans* were biofilm producers. Consistent with these findings, Viksne et al. found that 61% of Staphylococcus aureus strains in healthy people's mouths produced biofilms to varying degrees. [42] In a similar vein, Penesyan and colleagues characterized biofilm as the primary lifestyle of microbes. Importantly, it provides a safe space for microorganisms to develop genotypic and phenotypic variety before releasing them into the wild [43].

In addition, laboratory investigations have shown that Candida albicans is the most commonly isolated strain from oral mucosa that has the ability to attach, form biofilms, and ultimately cause denture stomatitis. [44,45]

The percentage of biofilm formation of Staphylococcus aureus and Candida albicans on

various CAD/CAM-formed denture base materials differs significantly between the milled and 3D groups (p-value 0.004* for Candida, p-value 0.356 for Staphylococcus). These findings were in agreement with those of Koujan et al., who had previously shown that the three polymers had different degrees of success in preventing Candida albicans adhesion. Candida albicans adhered far less to PMMA that had been heat-cured and fabricated using CAD-CAM than to PMMA that had been 3D printed [46]. Our investigation found that the adhesive ability of this yeast is unaffected by the 3D printing manufacturing technique; all that changes is the surface roughness after the fact. The development of hyphae and phenotypic switching, which play a role in the pathogenicity of the most dangerous Candida species, are responsible for this [47].

We also looked at how Staphylococcus aureus forms biofilms, as people who have periodontal disease tend to have more of these bacteria in their mouths[48]. In addition to its role in dental implant failure, Staphylococcus aureus can cause angular cheilitis, staphylococcal mucositis, and other oral disorders [49].

Staphylococcus aureus was able to establish biofilms on both of the materials that were examined in this investigation. However, biofilms developed on milled materials at an alarming rate. As expected, these outcomes matched the modified roughness settings. One of the most important elements affecting microbial adherence and colonization on biomaterials is surface roughness. More ideal colonization sites can be found in the depressions of roughened surfaces. [50]

These results were matched with ***Mohammed et al.,*** who also showed most of *Staphylococcus aureus* isolates can form biofilm on the different denture materials Acrylic, Plastic and Metallic denture materials. [51] This can be explained by formation of polymicrobial biofilm which had a complex structure, as *Candida albicans* serving as a scaffold where *Staphylococcus aureus* adheres, preferentially to the hyphal form of the fungus. Future improvements in therapy will need the ability to detect polymicrobial illnesses and characterize biofilms. [52] Furthermore, microbial adhesion patterns in vitro might not be indicative of their in vivo counterparts. Surface free energy as it relates to wettability and hydrophobicity of the examined surfaces, among other physical properties, should be investigated further to shed light on this finding [53]. Consistent with previous research, this study found that biofilm formation was greater on 3Dprinted specimens than on milled ones. This finding lends credence to the idea that the printed specimens exhibited stair-stepping phenomena due to the stepwise linking of layers, which led to increased porosities and deep grooves in the surface structure. The 3D-printed group showed substantially more biofilm growth compared to the other group, and this roughness is thought to be the cause [53&54].

CONCLUSIONS

The microbiological characteristics and surface topography of the manufactured denture base resin material were affected by the production process. When contrasted with CAD/CAM milling methods, additive 3D printing led to a greater rise in biofilm growth.

Limitations of the current study: comprise a single department's sample size that is relatively tiny. In comparison to more traditional approaches, the material is expensive, and laboratory expenses are higher as a result. Also, before the denture is finally fabricated, it is vital to try it out, but some systems don't offer this. Despite the fast development of digitally produced dentures, there is a lack of data on their laboratory, clinical, and patient-centered results. Biofilm production and adhesion patterns in living organisms vary from those in laboratory settings, which is another drawback.

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