The influence of salivary pH on the adhesion of *Candida albicans* to acrylic prosthetic materials obtained using conventional and digital techniques

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Abstract: The influence of saliva pH on the adhesion of *Candida albicans* to PMMA-based prosthetic materials manufactured using conventional and digital techniques (3D printing, milling) was examined. The obtained data were subjected to statistical analysis using one-way ANOVA and the Tukey HSD post hoc test ($\alpha = 0.05$). Materials obtained by 3D printing had the highest surface roughness, while those obtained conventionally had the highest number of CFU (colony-forming unit). The mean CFU value was highest at pH 4.5 and statistically significant compared to other pH values. No significant correlation was found between surface roughness and the average CFU value. The surface of materials obtained using the conventional and milling methods showed lower adhesion of *Candida albicans*.

Keywords: PMMA, *Candida albicans*, CAD/CAM, 3D printing.

Wpływ pH śliny na adhezję *Candida albicans* do akrylowych materiałów protetycznych uzyskanych technikami konwencjonalnymi i cyfrowymi

Streszczenie: Zbadano wpływ pH śliny na adhezję *Candida albicans* do materiałów protetycznych na bazie PMMA, wytwarzanych technikami konwencjonalnymi i cyfrowymi (druk 3D, frezowanie). Otrzymane dane poddano analizie statystycznej za pomocą jednoczynnikowej analizy ANOVA oraz testu post hoc Tukeya HSD (α= 0,05). Materiały uzyskane metodą druku 3D wykazywały największą chropowatość powierzchni, a otrzymane konwencjonalnie charakteryzowały się największą liczbą CFU (*ang. colony-forming unit*). Średnia wartość CFU była największa przy pH 4,5 i statystycznie istotna w porównaniu z innymi wartościami pH. Nie stwierdzono znaczącej korelacji pomiędzy chropowatością powierzchni a średnią wartością CFU. Powierzchnia materiałów otrzymanych metodą konwencjonalną i metodą frezowania wykazywała mniejszą adhezję *Candida albicans*.

Słowa kluczowe: PMMA, *Candida albicans*, CAD/CAM, druk 3D.

Patients with partial or full edentulism often require a removable appliance to replace missing teeth and surrounding structures [1]. For decades, denture base material, specifically polymethyl methacrylate (PMMA), has been commonly used for removable dental prosthesis fabrication [2]. PMMA is classified into chemical, heat, or light-activated types based on polymer activation, which can be fabricated using traditional techniques [3]. In recent years, CAD/CAM technology is being largely implemented in fabrication of dentures and these technologies can be categorized into additive (3D-printing) or subtractive (milling) manufacturing [4, 5].

Polymethyl methacrylate has several benefits, including low density, durability, attractiveness, cost-effectiveness, ease of manipulation, and diverse physical and mechanical properties [6, 7]. However, PMMA has some drawbacks, such as limited biocompatibility, porosity, and surface roughness [8]. Furthermore, removing microorganisms from the tissue surface of a denture is challenging, leading to the proliferation of microorganisms, especially *Candida albicans* [9, 10].

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Candida albicans is the most common microorganism found in the oral cavity [11]. Individuals with weakened immunity, inadequate nutrition, poor dental cleanliness, misuse of medications, trauma, and incorrect removal of removable prostheses are more susceptible to oral infections [12]. Denture stomatitis, characterized by inflammation due to inadequate denture hygiene, is often linked to *Candida albicans* biofilm formation [13]. PMMA-based CAD/CAM polymers have been shown to have lower candida adhesion compared to heat-polymerized PMMA [14].

Saliva has an innate defense mechanism and a physical cleaning effect, but it is unclear if saliva plays a role in *Candida albicans* infection. As dentures impede the flow of oxygen and saliva to underlying tissues, an acidic and anaerobic microenvironment is created, which promotes yeast growth [15]. Salivary pH is lower in complete denture wearers than in non-denture wearers, and the pH of saliva changes after the insertion of a complete denture or a denture supported by implants [13]. Additionally, elderly individuals have a lower oral mucosa pH, contributing to denture stomatitis [15].

Hajisadeghi *et al*. [16] reported that diabetic patients had lower pH and higher *Candida albicans* adhesion compared to the control group, suggesting a role for pH in *Candida albicans* adhesion. However, the literature lacks information regarding the effect of saliva pH on *Candida albicans* adhesion of various denture acrylic materials, especially fabricated using CAD/CAM technologies. Therefore, this study aims to evaluate the impact of saliva pH on *Candida albicans* adhesion of conventional heat-cured, CAD/CAM milled, and 3D-printed denture-based materials.

EXPERIMENTAL PART

Materials

The details of the materials used to fabricate the denture acrylic specimens are presented in Table 1.

Preparation of acrylic discs

Three different types of acrylic materials were used in this study, including conventional heat-cured acrylic materials, CAD/CAM milled acrylic materials, and 3D printed acrylic dentures. To produce forty discs of each type of material measuring 10 mm in diameter and 3 mm in thickness [17], a specific protocol was followed for each type of material as described below. Initially, a virtual disk with fixed dimensions (10 × 3 mm) was designed using CAD software (Zenotec, Wieland Dental Systems Inc., Pforzheim, Germany), and then an STL file was generated and saved.

For conventional heat-cured acrylic discs, a CAD/CAM (Blue, NHT high technology, Dubai, UAE) plastic disc was milled using a saved STL file to create plaster molds to ensure accuracy with sample dimensions. The plastic discs were embedded using plaster (Uni-base 300, type 4, Dentona, Germany) in a dental flask, and then the flask was immersed in boiling water (MultiCure, Vertex Dental, Soesterberg, The Netherlands) to melt the plastic and create a plaster mold. The flask was opened and a separation medium (Technosil, Girona, Spain) was applied to the plaster mold. The mixture of PMMA powder and liquid monomer was mixed to a dough consistency according to the manufacturer's recommendations and packed into the mold. The flask was then placed in a flask press (Hydraulic Press 660, Silfradent, Italy) to remove excess acrylic, and then immersed in boiling water (100°C) for an hour to complete the curing process of the acrylic material [17]. The processed discs were then removed from the flask and any plaster residue adhering to the surface of the discs was removed. The saved STL file was used to produce the 3D printed acrylic discs (Figure 1a).

A Nextdent 3D+ photopolymer printing resin was used to print the discs on a printing machine (ST1600, Satori Ltd., London, UK). The printed discs were immersed in isopropyl alcohol (99.9%) for 5 minutes and then light-cured in a light-curing device (Zyrlux, Ivoclar Vivadent, Schaan, Liechtenstein) for 5 minutes [18].

For CAD/CAM milled discs, PMMA blanks were obtained on a milling machine (Wieland Dental Systems Inc., Pforzheim, Germany) (Fig. 1b) using a saved STL file. For milled and 3D printed discs, excess acrylic attached to the discs was removed using an acrylic dental drill, and then all discs were immersed in water in an ultrasonic bath [19]. The discs were then finished and polished according to the manufacturer's instructions.

T a b l e 1. Materials used in the study

Type	Trade name	Manufacturer	Composition
Conventional PMMA resin	Meliodent	Heraeus Kulzer GmbH, Hanau, Germany	Liquid: methyl methacrylate, glycol dimethacrylate, dimethyl p-toluidine Powder: PMMA, ethyl hexyl acrylate, N-octyl methacrylate
CAD/CAM PMMA blanks	IvoBase CAD	Ivoclar Vivadent, Schaan, Liechtenstein	Industrially polymerized CAD/CAM blocks containing > 90% PMMA
Photopolymer 3D print resin	NextDent Denture 3D+	Vertex-Dental B.V., Soesterberg, The Netherlands	>75% ethoxylated bisphenol A dimethacrylate, 7,7,9(or 7,9,9)-trimethyl-4,13-dioxo-3,14-dioxa-5,12-diazahexadecane-1,16-diyl bismethacrylate, 2-hydroxyethyl methacrylate, diphenyl(2,4,6- trimethylbenzoyl) phosphine oxide, SiO_2, TiO_2 and pigments

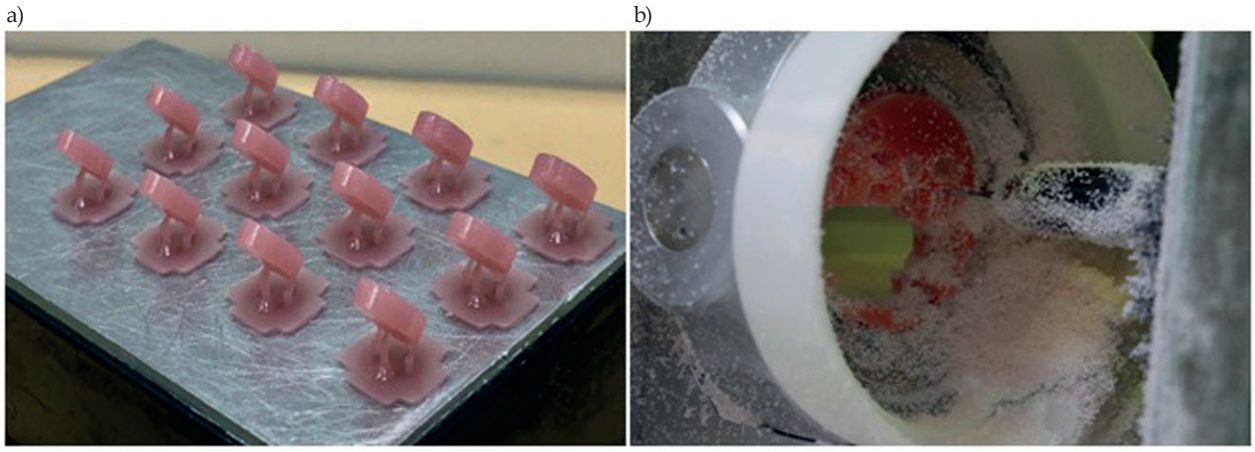

Fig. 1. a) 3D-printed acrylic resin specimens; b) CAD/CAM milling of the PMMA blanks

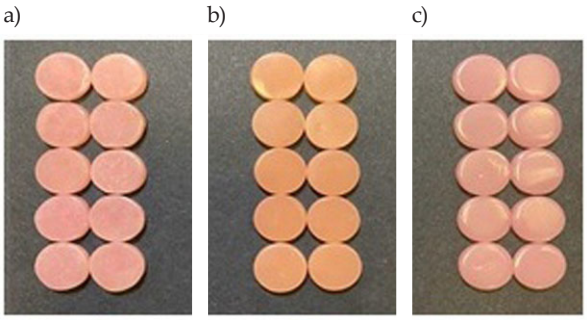

Fig. 2. Acrylic resin specimens fabricated using conventional (a), milling (b) and 3D-printing (c)

They were then polished according to the manufacturer's instructions using dental polishing machines (Ray Foster Dental Equipment, Huntington Beach, CA, USA) (Derotor double speed), using a pumice stone (Whip Mix, Louisville, KY, USA) and a polishing cloth (5 by 50 Rag Muslin Wheel, Kerr Corporation, Brea, California, USA) (Figure 2).

Artificial saliva formulation

To develop an artificial human saliva solution, chemicals as listed in Table 2 were measured by a weighing scale and dissolved in 800mL distilled water in accordance with a previous study [20].

T a b l e 2. Chemical composition of the formulated artificial human saliva

Chemical constituents	Chemical formula	Concentration (g/900mL)
Potassium dihydrogen phosphate	KH_2PO_4	3.062
Disodium hydrogen phosphate	Na_2HPO_4	4.005
Sodium bicarbonate	$NaHCO_3$	13.515
Potassium chloride	KCl	5.260
Magnesium chloride hexahydrate	$MgCl_2$	0.275
Citric acid	$C_6H_8O_7$	4.702
Calcium chloride	$CaCl_2$	1.985

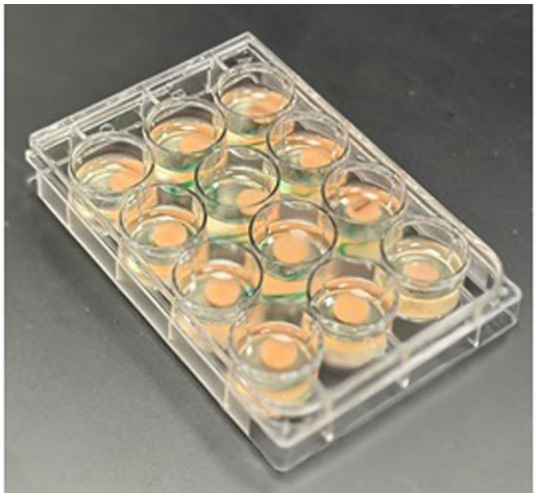

Fig. 3. Specimens immersed in plastic container containing AS

pH level of artificial saliva was adjusted to values of 3.5, 4.5, 5.5, and 6.5 using HCl and NaOH pellets. The pH range of each solution was measured during the adjustments to make sure it reached the desired pH. Once the desired pH values were attained, distilled water was added to each chemical solution to make 900 mL of the solution [17]. The artificial salivary pH was measured using a pH meter (# 2700, OAKTON Instruments, IL, USA).

Incubation

The sample discs were divided into four groups based on their pH values and stored in a plastic container containing 30 mL of artificial saliva. For each of the 3 materials, 10 discs were placed in each pH group (n=10) (Figure 3). The plastic containers were then placed in an incubator at 37°C for 30 days according to the experimental protocol.

Surface profilometry

The characterization and imaging of the samples were conducted using a Contour GT-K 3D optical profilometer

(Contour GT-K 3D, Bruker GmbH, Mannheim, Germany), which uses a non-contact surface metrology system that uses interferometry [21]. The samples were measured by vertical scanning interferometry, with a Michelson lens with 5× magnification, a Gaussian regression filter with parameters of scanning speed 1×, threshold 4 and a measurement area of 2 mm^2. The profilometer software, Vision 64 (Contour GT-K 3D, Bruker GmbH, Mannheim, Germany), controlled the instrument settings, data, and graphical output. The measurement was performed by scanning each sample at 3 equidistant positions. The roughness (Ra) value was then determined by averaging the three readings.

Preparation for inoculum and growth conditions

To prepare *Candida albicans* suspension, each candida cell measuring 0.5 micrometers was incubated in 5 mL of Brain Heart Infusion broth (BHI) (HiMedia Laboratories Private Limited, Mumbai, India) for 48 hours at 37°C. The BHI broth was made by dissolving 37 grams of BHI in 1000 mL of distilled water. A total of 120 acrylic discs were placed in ten wells of a 12-well tissue culture plate (SPL Life Sciences Co., Ltd., Gyeonggi-do, Korea). Each well was injected with 1 mL of BHI broth and 12.5 microliters of *Candida albicans* were placed using a pipette pump (Joanlab Equipment Co., Ltd., Zhejiang, China). The plate was then placed in an incubator (Panasonic, Osaka, Japan) for 72 hours at 37°C. After incubation, a 10× buffered phosphate saline (PBS) solution was prepared by mixing 16 grams NaCl, 0.40 grams KCL, 2.8 grams Na_2HPO_4, and 0.49 grams KH_2PO_4 (Sigma-Aldrich, St. Louis, MO, USA) in 200 mL of deionized water, yielding a buffer solution of pH 7.4. The prepared PBS was further diluted to 1×, autoclaved, and used to wash discs for 2 seconds.

The *Candida albicans* cells adhering to the denture surfaces were dislodged using 2 mL of distilled water. The cell suspension was diluted to four dilutions by transferring 100 mL of the cell suspension to 900 μL of distilled water each time. The third dilution (50 mL) was divided and placed in two separate Sabouraud dextrose agars (SDA) (Neogen, Lansing, MI, USA) with chloramphenicol, and the same was done with the fourth dilution. After 48 hours at 37°C, the colony-forming unit (CFU) counts were calculated (Fig. 4) and used to express the adherent cell count CFU/mL.

Statistical analysis

The measured data were analyzed using SPSS v.22 (IBM® SPSS® Inc., Chicago, IL, USA). The descriptive data of surface roughness and CFU triple and quadruple dilutions was expressed as mean and standard deviation. A one-way ANOVA and Tukey *post hoc* multiple comparison tests were used to analyze and compare the mean CFU about study materials and salivary pH (α=0.05).

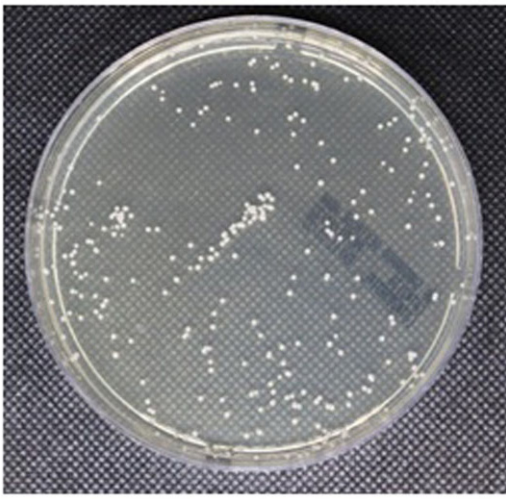

Fig. 4. Image of the candida colony formed in a glass plate

RESULTS

Surface roughness

Table 3 displays the mean and standard deviation (SD) of the surface roughness of three different acrylic materials (conventional, milled, and 3D printed) under four different pH values. The results demonstrate that, regardless of pH level, the conventional material had the lowest surface roughness, followed by the milled and 3D-printed material.

Colony forming units (CFU)

Table 4 presents the mean CFU of *Candida albicans* according to different pH values and material types after third dilution. About conventional acrylic material, the mean CFU was the highest at pH 4.5 and the CFU values were significantly higher than pH 3.5 (p=0.029*), pH 5.5 (p=0.000 significant at 0.01 level) and pH 6.5 (p=0.001 significant at 0.01 level). In the milled materials, the CFU

T a b l e 3. The mean and SD of surface roughness of the three tested materials under four pH value

pH	Material	Mean ± SD
3.5	Conventional	0.215 ± 0.171
	Milled	0.272 ± 0.163
	3D Printed	0.754 ± 0.204
4.5	Conventional	0.230 ± 0.269
	Milled	0.261 ± 0.132
	3D Printed	0.873 ± 0.254
5.5	Conventional	0.223 ± 0.200
	Milled	0.388 ± 0.224
	3D Printed	0.887 ± 0.346
6.5	Conventional	0.194 ± 0.156
	Milled	0.366 ± 0.385
	3D Printed	0.801 ± 0.223

T a b l e 4. Mean CFU of the tested materials at different pH after third dilution

Material	pH				p value
	3.5	4.5	5.5	6.5	
Conventional	2756000 ± 2167277	3577000 ± 2164065	202000 ± 182805	153000 ± 248061	0.000**
Milled	1780000 ± 1387403	2418000 ± 1197903	1172000 ± 809812	3488000 ± 1757800	0.003**
3D-printed	1668000 ± 750019	2300000 ± 1003571	2692000 ± 1188143	1757000 ± 795180	0.070
p value	0.242	0.142	0.000**	0.000**	

** significant at 0.01 level, * significant at 0.05 level

T a b l e 5. Mean CFU of the tested materials against different pH after fourth dilution

Material	pH				p value
	3.5	4.5	5.5	6.5	
Conventional	3680000 ± 2721029	8590000 ± 6243120	320000 ±385284	1250000 ± 3106355	0.000**
Milled	3030000 ± 2365516	5610000 ± 3301666	2480000 ± 2788787	6810000 ± 4232007	0.014*
3D-Printed	3490000 ± 2402059	6610000 ± 7876752	2157000 ± 3425178	330000 ± 222460	0.025*
p value	0.837	0.547	0.146	0.000**	

** significant at 0.01 level, * significant at 0.05 level

were higher at pH 6.5 compared to other pH values, and these values were significantly different from CFU values at pH 3.5 (p=0.033) and 5.5 (p=0.002). In 3D printed material, no statistically significant difference was found between the pH values (p>0.05).

The comparison of materials about different pH showed no statistically significant differences between the materials (milled, conventional and printed, p>0.05) at pH 3.5 and 4.5. However, at pH 5.5, the mean CFU of printed material was significantly higher than milled and conventional materials. The mean CFU of the milled material at pH 6.5 was significantly higher than conventional and 3D printed materials (p>0.05).

Table 5 presents the mean CFU of *Candida albicans* according to different pH values and material types after fourth dilution. About conventional material, the mean CFU at pH 4.5 was significantly higher than CFU at pH 3.5 (p=0.029), pH 5.5 (p<0.000) and pH 6.5 (p<0.001). In milled acrylic material, the CFU at pH 6.5 was significantly higher than CFU at pH 3.5 (p=0.025). The 3D-printed material showed that CFU at pH 4.5 was significantly higher than CFU at pH 6.5 (p=0.025).

The comparison of materials about different pH showed no statistically significant differences between the materials (milled, conventional and printed, p>0.05) at pH 3.5, 4.5 and 5.5. The mean CFU of the 3D printed materials at pH 6.5 was significantly higher than conventional materials. However, it was significantly lower than milled materials (p<0.05).

Table 6 presents the correlation between surface roughness and the mean CFU of the third and fourth dilutions. There was no significant correlation between surface roughness and the CFU of both dilutions.

T a b l e 6. Pearson correlation of the CFU for the third and fourth dilution

Analysis	CFU (third dilution)	CFU (fourth dilution)
Pearson Correlation	0.080	-0.078
Sig. (2-tailed)	0.383	0.395

Correlation is significant at the 0.01 level (2-tailed)

DISCUSSION

Salivary pH plays a crucial role in maintaining oral health and can impact the growth and adhesion of microorganisms like *Candida albicans* [22]. *Candida albicans* exhibits different behaviors and adhesion capabilities at various salivary pH levels. Research studies have demonstrated that acidic pH conditions promote the adhesion of candida albicans to denture acrylic [11, 23, 24]. Acidic environments can alter the surface properties of the denture acrylic, making it more susceptible to fungal colonization and adhesion [9, 13, 25]. The results suggest that the adherence of *Candida albicans* to denture base materials varies depending on the pH and the acrylic material used for manufacturing and therefore the null hypothesis was rejected.

An acidic salivary pH creates an environment that is more favorable for the growth of candida on denture surfaces. *Candida* species, especially *Candida albicans*, thrive in slightly acidic conditions [26]. Acidic pH can disrupt the balance of microorganisms in the oral cavity, favoring the overgrowth of candida [24]. Additionally, acidic conditions can weaken the natural defense mechanisms

of the oral mucosa, making it more susceptible to candida colonization [27].

Several studies have indicated a significant association between lower salivary pH values and higher levels of *Candida albicans* species in certain patient groups. A research conducted by Hajisadeghi *et al*. [16] found that diabetic patients, who are known to have lower salivary pH values, exhibited a greater presence of candida species compared to non-diabetic patients. Additionally, Narendra *et al*. [15] observed that patients with denture stomatitis, a condition often characterized by candida colonization, exhibited low salivary pH levels. It is important to consider that denture stomatitis is typically detected in elderly patients who frequently suffer from systemic disorders and experience dietary changes [15]. *Candida albicans* tends to become more virulent and cause candidiasis more frequently when the host's immune system is compromised by disease [13].

The interaction between candida and denture materials is complex. Dentures provide an ideal surface for candida to adhere to and from biofilms. These biofilms are structured communities of microorganisms encased in a protective matrix. *Candida* biofilms on denture surfaces can be more resistant to antifungal treatments and host immune responses compared to planktonic (free-floating) *Candida albicans* cells [28, 29]. Acidic pH can modify the surface characteristics of denture materials, making them more susceptible to *Candida albicans* adhesion [30-33]. It can also promote the expression of adhesion-related genes in *Candida albicans*, enhancing its ability to bind to the denture surface [26].

Poly(methyl methacrylate) (PMMAs) is a well-researched and utilized class of polymers in dentistry due to its exceptional biomechanical and self-hardening characteristics. PMMAs are now primarily employed in dentures, provisional crowns, customized impression trays, and orthodontic devices [34]. Nevertheless, no denture based polymers possesses all the optimal mechanical, physical, aesthetic, and biocompatibility characteristics [35]. It has been demonstrated that the methyl methacrylate (MMA) monomer from denture material vaporizes, which may have negative consequences through inhalation, irritating lung tissues and impacting the central nervous system [36]. In one study, rats were exposed to MMA vapors, and the results demonstrated histological symptoms, including lung collapse, edema, and emphysema [37]. In a recent study, fish with direct exposure in translucent E3 medium did not exhibit accelerated toxicity endpoints compared to juvenile fish exposed to untreated methacrylate (photopolymers for 3D printing) in ultrapure water [38].

Polymeric biomaterials are typically classified as significant, stable structures with exceptional biodegradability. Numerous studies have indicated polymers could be vulnerable to various biodegradation processes within the oral cavity. Many factors, such as changes in pH and temperature, salivary enzymes, chemical and dietary changes, and chewing, can trigger polymer breakdown and promote biodegradation [35]. For conventional acrylic denture materials, acidic salivary pH can lead to increased surface roughness [30]. These alterations in surface topography provide more sites for *Candida albicans* to adhere to, facilitating its colonization on the denture surface [39]. Acidic conditions can also affect the composition of the acquired pellicle, a layer of salivary proteins that forms on the denture surface. The changes in the pellicle composition can promote *Candida albicans* adhesion [40]. When it comes to milled denture materials, the effect of salivary pH on *Candida albicans* adhesion follows a similar pattern. Acidic salivary pH can induce surface roughness and increase the porosity of the milled materials. These changes create more favorable conditions for *Candida albicans* adhesion and colonization on the denture surface [31].

Regarding 3D printed denture materials, the impact of salivary pH on candida adhesion may vary depending on factors such as the specific printing technique used and the properties of the material itself [41]. Some studies have suggested that certain 3D printed denture materials, particularly those incorporating nanoparticles or antimicrobial agents, can exhibit enhanced resistance against *Candida albicans* adhesion compared to conventional materials [42]. However, the specific effect of saliva pH on *Candida albicans* adhesion to printed acrylic materials is still an area that requires further investigation. Contrary to the mentioned studies, Darwish *et al*. [43] and Darwish and Nassani [44] reported no significant association between the type of denture base materials and *Candida albicans* adhesion. In contrast, Koujan [45] and Meirowitz *et al*. [41] found that milled and conventional acrylic materials exhibited significantly lower *Candida albicans* adhesion compared to printed acrylic material. Additionally, Larijani *et al*. [46] demonstrated that *Candida albicans* presence was significantly lower on CAD/CAM acrylic resins compared to conventional material.

Furthermore, the findings of this study indicate that at a pH of 6.5, the CFU were higher in milled materials compared to conventional and 3D printed acrylic materials. Several studies demonstrated that milled material exhibits lower *Candida albicans* adhesion than 3D printed and conventional materials [45, 47]. The results of this study were obtained through CFU of the third dilution, which were further confirmed by the fourth dilution, except in for pH 5.5 where no significant difference was observed among the tested materials.

It is worth noting that while acidic salivary pH can contribute to *Candida albicans* growth on denture materials, it is not the sole factor responsible for this phenomenon. Other factors such as poor oral hygiene, inadequate denture cleaning, prolonged denture wearing, and systemic conditions like diabetes can also play a role in the development of candida-related denture problems [13].

It is well-established that rough surfaces on denture base materials can increase *Candida albicans* adhesion

[10]. This study revealed that 3D printed material exhibited the highest roughness, followed by milled and conventional materials. These findings are in contrary with a previous study by Al-Fouzan *et al*. [25] who found that the surface roughness of conventional material was significantly higher than CAD/CAM materials. Similarly, another study by Aldwairi [48] reported that conventional material had the highest surface roughness.

This study represents the initial endeavor to elucidate the influence of saliva pH on *Candida albicans* adhesion to various acrylic materials. However, several limitations should be acknowledged when interpreting the current results. Firstly, the multifaceted nature of *Candida albicans* adhesion, encompassing material type, surface roughness, and pH value, introduces complexity, making it challenging to establish clear correlations between these factors. Secondly, the incubation conditions employed in this study were semi static, which do not fully replicate the dynamic environment of the oral cavity. Lastly, the oral cavity is a complex milieu with diverse microbial biofilms that may contribute to *Candida albicans* adhesion. Future studies should investigate the multifaceted factors influencing candida adhesion in greater detail. Additionally, the present in-vitro outcome should be verified with the *in-vivo* settings.

CONCLUSIONS

Different acrylic materials exhibited varying levels of *Candida albicans* adhesion owing to the salivary pH. Acidic pH promotes *Candida albicans* adhesion and modifies the surface characteristics of acrylic materials.

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