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Anti-adherence modified denture base resin using polyethylene glycol (PEG) and chlorhexidine diacetate salt (CHX)

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Abstract:

Context: Acrylic resin denture base material is the most commonly used dental material for removable prostheses, although its high affinity to form biofilms on its surface results in serious health problems. **Aims:** Isolation and identification of aerobic bacteria and Candida from biofilms formed on denture base surfaces. Quantifying the biofilm-forming ability and studying the anti-adherence effect of Polyethylene glycol (PEG) and chlorohexidine diacetate salt (CHX) added to heat-cured denture base material. **Methods and Material:** Microbial isolates were collected from denture base surfaces, and further identified by morphological, and biochemical tests, VITEK-2 system, and DNA sequencing. The biofilm-forming potential was examined by Microtiter Plate Assay. The anti-adherence effect of PEG and CHX added to denture base materials was evaluated. **Results:** The investigated microorganisms had different biofilm-forming potential on denture base surfaces ranging through weak, moderate and strong. The PEG and CHX had antibacterial and anti-fungal potential, and the addition of PEG and CHX to denture base resin significantly decreased the adhesion of selected microorganisms compared with control groups. The statistical results were considered significant at $p \leq 0.05$.

Conclusions: This study revealed that modifying denture base resin by the addition of PEG and CHX significantly enhances the anti-adherence property. The combining of PEG and CHX resulted in the enhanced decline of microorganism adherence.

Keywords: Denture base resin, PMMA, PEG, CHX, Biofilm forming, Adherence.

Introduction:

Polymethyl methacrylate (PMMA) is widely utilized to produce dental prostheses [1]. One drawback is poor antifungal and antibacterial properties, which allow microbes to adhere and build biofilms [2]. Denture wearers require antimicrobial base materials and antifouling coatings to manage colonization and biofilm formation [3]. Few bacterial cells that adhere to non-bactericidal antifouling surfaces may multiply forming a biofilm. Conversely, bactericidal surfaces lacking antifouling qualities may accumulate dead bacteria. Thus, combined antifouling and bactericidal strategies are more effective in improving long-term anti-biofilm activity [4-11].

The study aims to evaluate the antibacterial and antifungal effect of Polyethylene glycol (PEG), chlorohexidine diacetate salt (CHX), and the anti-adherence effect of adding them to denture base material.

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Methods:

Ethical consideration:

This research was achieved at the Prosthodontics Department/College of Dentistry/University of Mosul, from the period between April/2023-April/2024, obtaining the agreement of the ethics committee institution (Approval no.: UoM. Dent. 23/12).

Selection of subjects:

Inclusion criteria: Completely edentulous denture wearers for a minimum of 6 months.

Exclusion criteria: Patients under treatment with local or systemic antibiotics or antifungal medications before collection of samples (within 30 days). Patients with a smoking habit. Hence, completely edentulous denture wearers (n = 14 patients) were selected for the study.

Collection of Samples:

The samples were collected using sterile swabs (Microzo transport swabs), from the denture's surface, requiring the patient to wear their denture and not rinsing their mouth for 3 hours before the collection of swabs. The denture surfaces, (occlusal, tissue, and polished) surfaces were swabbed using a single swab. Then the swab was submitted to laboratory microbiological investigations.

Preparation of Culture Media:

All the cultural media (Mannitol Salt Agar, MacConkey's Agar, Sabouraud Dextrose Agar + chloramphenicol, Blood Agar, tryptic soy broth), were fabricated in adherence to the guidelines provided by the manufacturing company. Initially, the components were dissolved in distilled water (D.W), followed by heating on a hot plate with a magnetic stirrer to ensure complete dissolution. Subsequently, the pH of the solution was adjusted to 7.2 using either Hydrochloric acid (HCl) or Sodium Hydroxide (NaOH) with the aid of a pH meter. The sterilization process was carried out by subjecting the media to autoclaving at a temperature of 121°C for 15 minutes under a pressure of 15 bar/inch^2. The prepared media were then dispensed into sterile Petri dishes and left to cool at room temperature. After cooling, the Petri dishes were placed in an incubator set at 37°C for 24 hours to confirm sterility. Finally, the Petri dishes were stored in a refrigerator at a temperature of 4°C until required for use.

Isolation of bacteria and Gram staining:

The colonies' morphology and characteristics were well examined including size, shape, and pigment. Gram stain was done to identify the microscopic morphology of the isolates. Gram stain is the first step in identifying an unknown bacterium [12].

Biofilm Quantification by Microtiter Plate Assay:

The biofilm-forming abilities of the isolated microorganisms were studied by microtiter plate assay. Biofilms were established in pre-sterilized polystyrene Microtiter plates with 96 well-U-shaped bottoms. The isolates were grown in tryptic soy broth and incubated statically overnight at 37°C to secure appropriate growth. The cultures in each tube were diluted to the 0.5 MacFarland standard. For each microorganism, bacterial dilutions (200 µl) were placed in 96-well plates in triplicate and incubated without stirring, at 37°C for 24 h. As a control (C), a non-inoculated culture medium was used. After the incubation period, the culture medium was discarded, a sterile saline was used to wash the wells three times, and the plate allowed for dryness for 1 h at 60°C for cell fixation. One percent (1%) crystal violet (200 µl) was applied to the wells and the plate was left for 45 min at room temperature. The plate was washed with sterilized distilled water till there was no illusion of color and maintained for dryness at room temperature for 45 min. The crystal violet was solubilized by adding 200 µl of 99% ethanol to each well with stirring. Micro ELISA auto reader (Biobase, China) was used to measure the optical density (OD) at the wavelength of 570 nm. The optical density values were considered as an indicator of attachment to the surface. The test was performed in triplicates and an average reading was taken [13,14].

The biofilm-forming microorganisms were categorized into groups:

Negative = OD ≤ OD C Weak = OD C < OD ≤ 2*OD C

Moderate = 2*OD C < OD ≤ 4*OD C Strong = OD > 4xOD C

Vitek-2 system to confirm bacterial identification to species level:

A single isolated colony of pure bacteria is suspended in a sterile test tube, containing 3 ml of saline. The suspension of microorganisms and the conventional turbid standard solutions were compared. The concentration must be between 0.5 and 0.63. The VITEK-2 card or cassette was chosen according to the diagnostic Gram stain. The cassette and test tube racks are delivered to the system (VITEK-2, bioMerieux), the boxes are automatically filled with bacterial suspension after being placed in the first filling area (filler), and the device sends out an end signal. The cassette is left on at 37°C for 24 hours. The results are ready to identify bacteria [15,16].

DNA sequencing:

Steps of genomic DNA extraction were followed as recommended by the manufacturer directly from microbial samples, by using an isolation kit (genomic DNA) provided by the (Gene aid company). The purity and concentration of (genomic DNA) were calculated and then maintained at -20 °C till usage [12].

Identification by PCR technique through using (GoTaq G2 Green Master Mix) provided by (Promega/USA). The PCR was performed in a 20 µL volume reaction. The universal primers 27FAGAGTTTGATCMTGGCTCAG and 1522R AGGAGGTGATCCARCCGCA were utilized to amplify the whole region of the 16S rRNA gene for bacteria [17] and universal primers ITS1, ITS3, and ITS4[18] for fungi.

The PCR program was set at (initial denaturation) for 3 min at 95 °C, then (denaturation step) for 30 seconds at 95 °C, (annealing) for 1 minute at 55 °C and (extension) for 1 minute at 72 °C. A (final extension step) for 5 minutes was set at 72 °C. The concentration of primers (1 µM each) and the total amount of template DNA (100 ng) were added according to the instructions of the manufacturer. On 1% agarose gel, the PCR products were separated and stained by the application of Midori Green Advance DNA stain. Then PCR products were purified and sent for DNA sequencing at Psomagene sequencing company (USA).

Minimum Inhibition Concentration:

The tube dilution method was used to determine the minimum inhibitory concentration [19]. The PEG and CHX were tested as anti-microbial and antifungal agents against isolated microorganisms. Stock solutions for the examined materials were prepared by the addition of PEG and CHX to the nutrient broth. The amount needed of PEG and CHX and the nutrient broth in which it can be dissolved can be calculated by using the following equation: Powder Weight (mg) = Solvent Volume (ml) * Concentration (µg/ml) / Powder Potency (µg /mg)

The PEG potency is 1120 mg/ml starting from this concentration we take 2ml of PEG and 2ml of nutrient broth in the first tube, then 2ml from the first tube is taken and addition of 2ml of nutrient broth in the second tube and so on to achieve PEG concentrations (562.5, 281.25, 140.6, 70.31,35.15, 17.5, 8.78, 4.39, 2.19, 1.09 mg/ml). For CHX stock solution preparation 100 mg of CHX dissolved in 1 ml ethanol obtaining 100 mg/ml stock solution. To obtain a stock solution of 1000 µg/ml concentration by using the following formula: C1*V1=C2*V2. A 20 µl is added to 2ml of nutrient broth in the first tube then 2ml from the first tube is taken and the addition of 2ml of nutrient broth is in a second tube and so on to achieve CHX concentrations (500, 250, 125,

62.5,31.25,15.6,7.8,3.9, 1.95, 0.97 µg/ml), the ethanol (concentration less than 1% after dilution).

The microorganisms were cultured and incubated in nutrient broth at 37°C for 24 hours for growth, then the suspension to produce turbidity equal to 0.5 McFarland standard which is about 1.5×10^8 CFU/ml. A comparison was made between the inoculum and 0.5 Macfarland standard against a card with white and black lines [20,21].

Transferring 0.1ml of the inoculum suspension into all serially diluted tubes that contain broth and PEG or CHX suspension, then mixing well to get a uniform mixture. A positive control tube is included in the test that contains inoculum and nutrient media with no additives. The negative control consists of nutrient broth with PEG or CHX and without microorganism suspension. The inoculated tubes were incubated at a temperature of 37°C for 24 hours and thereafter detected for growth or turbidity [22].

Adherence test:

The PEG and CHX were incorporated into denture base resin material and tested for their anti-adherence effect against selected microorganisms. The denture base resin samples, four hundred fifty (450) samples were prepared with 10*10*2.5 mm dimensions, and separated into groups: A control group (denture base resin without additives) and an experimental group (denture base resin with additives). All the samples of Triplex heat polymerized denture base resin were prepared by mixing of monomer and polymer of heat-cured resin following manufacturer instructions for the control group the powder-to-liquid mixing ratio was (23.4 g powder to 10 ml liquid), and the addition of PEG and or CHX for experimental groups. The PEG was incorporated at four different concentrations by weight depending on minimum inhibitory concentration results, where the MIC represents 20% of total monomer weight, and since it is the large percentage to be incorporated, the concentrations of PEG tested were (5%, 10%, 15% and 20%). The CHX was incorporated at four different concentrations by weight depending on minimum inhibitory concentration results, where the MIC represented 78 µl/10 ml of monomer, so the concentrations of CHX tested were (78, 156, 312, and 625 µl/10 ml of monomer), as CHX is added to polymer complex so its action may be affected. The materials were added in separate groups to identify the best anti-adherence effect gained. Then PEG and CHX were mixed and incorporated into denture base resin to identify the best anti-adherence effect gained against the chosen microorganisms. The mixing ratios are illustrated in Table (1).

Table (1): The concentration (µl) of PEG and CHX addition to experimental groups.

PEG concentration	CHX 1 µl	CHX 2 µl	CHX 3 µl	CHX 4 µl	Monomer ml
PEG 1 (5%) = 0.5 ml	78	156	312	625	9.5
PEG 2 (10%) = 1 ml	78	156	312	625	9
PEG 3 (15%) = 1.5ml	78	156	312	625	8.5
PEG 4 (20%) = 2 ml	78	156	312	625	8

Initially, a specific amount of methyl methacrylate (MMA) monomer was poured into a glass container (MMA alone for the control group and MMA with CHX for experimental groups) and the particular percent of PEG was added to it and mixed by glass rod until a homogenous mixture was obtained. The powder was added slowly and mixed, and then the acrylic resin dough mass was packed into the metal mold and closed under slow, constant pressure (200 MPa) for 10 minutes using a hydraulic bench press. After that, the molds were screwed tightly ready for processing. A short curing cycle was used (heat up gradually to 100°C for 90 min and let boil for 45 min, according to the manufacturer's instruction) using a thermostatically controlled water bath curing unit. Then the molds were removed and left to cool down slowly on the bench before opening (ADA No.12, 2002).

For anti-adherence evaluation, the microorganisms tested were *Staphylococcus warneri* (as gram-positive weak biofilm-forming bacteria), *Klebsiella oxytoca, Klebsiella pneumoniae, Citrobacter farmeri, Pseudomonas aeruginosa* (as gram-negative moderate and strong biofilm forming bacteria), and *Candida albicans* (as representative of fungi species). All denture base specimens were sterilized in sterile Petri dishes. The specimens of each group were placed in sterile plates containing (20ml) of the suspension specified for each microorganism tested and incubated at room temperature for 24 hours. Following removal from the suspension, the specimens were rinsed by phosphate buffered saline solution (PBS) twice for 1 minute to eliminate the non-adhered cells, with sonication for 15 min, the specimens were allowed to dry and fixed with 1 ml of methanol 99.9% for 5 min., and allowed to dry and stained using Gram's staining technique. The staining was performed using crystal violet for 30 seconds, washed with (PBS) solution again for 30 seconds, and dried to be inspected under a light microscope 100X (Optika, Italy). The adhered cells were counted for each specimen in five fields of view (0.25 mm^2 per field), and the results were expressed as microorganism cells/mm^2 of each of the denture base samples [23].

Statistical analysis: After data collection, the following statistical methods were performed for analysis and assessment of the results of the study by utilizing SPSS statistic software version 25 (IBM, USA).

1. Test the Normality Distribution to determine Whether the data are Parametric or Non-Parametric.
2. Descriptive statistics of the measurements including means, standard deviations, maximum and minimum values.
3. The analysis of variance (Kruskal-Wallis) and Post Hoc test for non-normal distribution data to assess if there was a significant difference between groups under test. The statistical results were considered significant at $p \leq 0.05$.

Results:

The collected microorganisms from patients' swabs after primary isolation and identification by Gram stain, oxidase, catalase tests, and the biofilm-forming abilities for the isolated microorganisms that were studied by microtiter plate assay showed that from 43 isolates, 19 isolates were of weak biofilm-forming ability, 17 isolates were of moderate biofilm-forming ability and 7 isolates had strong biofilm forming ability. The results of the biofilm-forming ability of microorganisms revealed that the optical density (OD) considered:

Negative if OD $\leq$ 0.058
Weak if 0.058 $<$ OD $\leq$ 0.116
Moderate if 0.116 $<$ OD $\leq$ 0.232
Strong if OD $>$ 0.232

Thirty isolated microorganisms with strong, moderate, and weak biofilm-forming ability and suspected gram-positive bacteria and candida isolates were selected for further identification, twenty-one by DNA sequencing and nine isolates by VITEK-2 method. The results of identification are displayed in Table (2).

Table (2): Identification of microorganisms using DNA sequence analysis and VITEK-2, with biofilm-forming ability.

M.O.	Identification	(O.D.) Biofilm	Result
1	*Klebseilla pneumoniae 81.82%* **	0.266	strong
2	*Klebsiella pneumoniae 91%* †	0.144	moderate
3	*Klebsiella pneumoniae 91%* †	0.203	moderate
4	*Klebsiella pneumoniae 91%* †	0.167	moderate
5	*Klebseilla pneumoniae 91%* †	0.210	moderate
6	*Klebsiella pneumoniae 91%* †	0.211	moderate
7	*Klebseilla oxytoca 96.76%* **	0.240	strong
8	*Klebseilla oxytoca 97.36%* **	0.228	moderate
9	*Klebseilla oxytoca 96.98%* **	0.270	strong
10	*Klebseilla oxytoca* †	0.205	moderate
11	*Klebseilla oxytoca* †	0.121	moderate
12	*Citrobacter farmeri 85.52%* **	0.237	strong
13	*Citrobacter farmeri 87.6%* **	0.131	moderate
14	*Candida albicans strain 99.27%* **	0.085	Weak
15	*Candida albicans strain 99.57%* **	0.083	Weak
16	*Candida albicans strain 98.73%* **	0.086	Weak
17	*Pseudomonas aeroginosa 98.95%* **	0.234	strong
18	*Pseudomonas aeruginosa 93%* †	0.216	moderate
19	*Stenotrophomonas maltophilia 89.76%* **	0.218	moderate
20	*Stenotrophomonas maltophilia 93.71%* **	0.207	moderate
21	*Acinetobacter pitti 97.81%* **	0.268	strong
22	*Acinetobacter lwoffii* †	0.182	moderate
23	*Enterobacter homaechi 95.71%* **	0.286	strong
24	*Enterobacter cloaecae 99.18%* **	0.223	moderate
25	*Staphylococcus Warneri 96.5%* **	0.112	Weak
26	*Bacterium enrichment 93.08%* **	0.382	strong
27	*Bacillus subtilis 88.39%* **	0.083	Weak
28	*Bacillus cereus 80%* **	0.105	Weak
29	*Lysinibacillus mangiferihumi 87.14%* **	0.147	moderate
30	*Nakeseomyces glabratus 99.86%* **	0.077	Weak
Control		0.058	

** DNA sequencing, † VITEK, M.O. = microorganism, O.D. = optical density

The results of isolated microorganisms showed that *Klebsiella pneumoniae* represents 20% of the identified microorganisms with moderate to strong biofilm-forming ability, *Klebsiella oxytoca* represents 15% of the identified microorganisms with moderate to strong biofilm-forming ability, *Citrobacter farmeri*, and *Pseudomonas aeruginosa* represent 6.6% of the identified microorganisms with moderate to strong biofilm forming ability and *Stenotrophomonas maltophilia* represents 6.6% of the identified microorganisms with moderate biofilm-forming ability. *Candida albicans* represents 10% of the identified microorganisms with weak biofilm-forming ability. Other types of microorganisms represent 3.3% of the identified species, with different biofilm-forming abilities, figure (1).

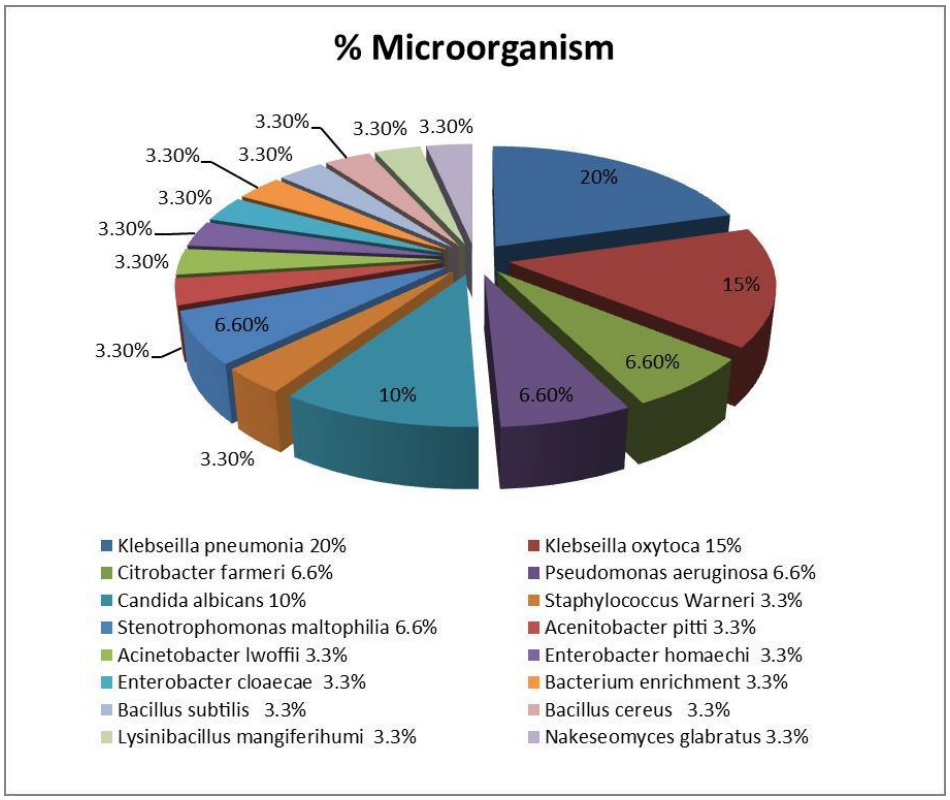

Figure (1): Percentage of isolated Strains under Study.

Minimum Inhibition Concentration:

The antibacterial and antifungal activity of PEG and CHX were tested. The results revealed that the MIC of PEG was 281.25 mg/ml and the MIC of CHX was 7.8 µg/ml as shown in Table (3).

Table (3): MIC of PEG (mg /ml) and CHX (µg/ml) for isolated Strains under Study.

M.O.	MIC PEG mg/ml	MIC CHX µg/ml
Citrobacter farmeri	**281.25**	**7.8**
Staphylococcus warneri		
Klebsiella oxytoca		
Klebsiella pneumoniae		
Pseudomonas aeroginosa		
Candida albicans		

Adherence test:

For evaluation of the anti-adherence ability of PEG and CHX incorporated into acrylic resin denture base materials the following microorganisms were selected, *Staphylococcus warneri* (as gram-positive biofilm-forming bacteria), *Klebsiella oxytoca, Klebsiella pneumoniae, Citrobacter farmeri, Pseudomonas aeruginosa* (as gram-negative biofilm-forming bacteria), and *Candida albicans* (as representative of fungi species). The mean values of adhesion of *Candida albicans, Staphylococcus warneri, Klebsiella oxytoca, Klebsiella pneumoniae, Citrobacter farmeri, and Pseudomonas aeruginosa* on control and experimental groups surface were demonstrated in Table (4). It revealed that the lower adhesion value in experimental groups of *Candida albicans* was (0.0 cell/mm²) for groups (PEG4, CHX3, CHX4, and PEG4:CHX4), while the highest value was (73.33 cell/mm²) for the control group.

The mean values of adhesion of *Staphylococcus warneri* showed that the lower adhesion value of experimental groups was (0.0 cell/mm²) for the group (PEG4:CHX4), while the highest value was (353.33 cell/mm²) for the control group. While for Klebsiella *oxytoca* it revealed that the lower adhesion value in experimental groups was (2.66 cell/mm²) for the group (PEG4:CHX4), while the highest value was (93.33 cell/mm²) for the control group.

The mean values of adhesion of *Klebsiella pneumonia*e showed that the lower adhesion value in experimental groups was (0.0 cell/mm²) for groups (PEG3:CHX4 and PEG4:CHX4), while the highest value was (453.33 cell/mm²) for the control group, and the mean values of adhesion of *Citrobacter farmeri* on denture base surface of control and experimental groups revealed that the lower adhesion value experimental groups were (0.0 cell/mm²) for groups (PEG4:CHX3 and PEG4:CHX4), while the highest value was (493.33 cell/mm²) for the control and PEG1 groups.

The mean values of adhesion of *Pseudomonas aeruginosa* revealed that the lower adhesion value in experimental groups was (0.0 cell/mm²) for groups (PEG4:CHX3 and PEG4:CHX4), while the highest value was (540.0 cell/mm²) for the control group.

The analysis of variance (Kruskal-Wallis) of *Candida albicans, Staphylococcus warneri, Klebsiella oxytoca, Klebsiella pneumoniae, Citrobacter farmeri and Pseudomonas aeruginosa* adherence assay of control and experimental groups was shown in table (5) which demonstrated that there were statistically significant differences between study groups at ($p \leq 0.05$). The Post Hoc test on study group samples (control and experimental groups) was shown in Figures (2 and 3) which explained that there were statistically significant differences between the control group and experimental groups. As increasing the concentrations of PEG and CHX significantly results in decreasing the adherence of tested microorganisms, the combination of PEG and CHX significantly enhanced the anti-adherence effect. The results revealed that the addition of PEG and CHX to denture base resin significantly decreased the adhesion of selected microorganisms compared with control groups. Except for experimental groups PEG1, PEG1:CHX1, and PEG1:CHX2 for *Citrobacter farmeri*, there were no significant differences with the control group.

Table (4): Means (cell/mm^2) of adherence for tested microorganisms on experimental groups.

Group	N	*Candida albicans*	*Staphylococcus warneri*	*Klebsiella Oxytoca*	*Klebsiella pneumoniae*	*Citrobacter farmeri*	*Pseudomonas aeruginosa*
Control	3	73.33	353.33	93.33	453.33	493.33	540.00
PEG1(5%)	3	9.33	106.66	73.33	386.66	493.33	440.00
PEG2(10%)	3	8.00	93.33	53.33	360.00	380.00	393.33
PEG3(15%)	3	2.66	73.33	32.00	142.66	200.00	120.00
PEG4(20%)	3	0	53.33	29.33	100.00	186.66	65.33
CHX1(78 µl)	3	6.66	93.33	56.00	50.66	333.33	266.66
CHX2(156 µl)	3	4.00	60.00	49.33	50.66	166.66	206.66
CHX3(312 µl)	3	0	46.66	42.66	37.33	106.66	153.33
CHX4(625µl)	3	0	18.66	14.66	29.33	106.66	69.33
PEG1:CHX1	3	61.33	233.33	85.33	108.00	473.33	208.00
PEG1:CHX2	3	60.00	160.00	78.66	54.66	466.66	140.00
PEG1:CHX3	3	36.00	134.66	21.33	28.00	153.33	126.66
PEG1:CHX4	3	6.66	138.66	25.33	2.66	57.33	85.33
PEG2:CHX1	3	64.00	137.33	72.00	78.66	80.00	205.33
PEG2:CHX2	3	61.33	135.33	70.66	78.66	34.66	204.00
PEG2:CHX3	3	37.33	130.66	68.00	8.00	12.00	77.33
PEG2:CHX4	3	2.66	132.00	16.00	1.33	6.66	76.00
PEG3:CHX1	3	32.00	117.33	53.33	60.00	13.33	120.00
PEG3:CHX2	3	12.00	49.33	50.66	49.33	13.33	120.00
PEG3:CHX3	3	2.66	18.66	40.00	1.33	5.33	78.66
PEG3:CHX4	3	1.33	2.66	4.00	.00	2.66	42.66
PEG4:CHX1	3	4.00	12.00	26.66	84.00	36.00	101.33
PEG4:CHX2	3	2.66	6.66	24.00	37.33	6.66	100.00
PEG4:CHX3	3	1.33	5.33	17.33	1.33	.00	61.33
PEG4:CHX4	3	.00	.00	2.66	.00	.00	40.00
N= number of samples							

Table (5): (Kruskal-Wallis) test for adherence of tested microorganisms on experimental groups.

Group		Adherence
Staphylococcus warneri	Kruskal-Wallis H	71.293
	Df	24
	Asymp. Sig.	.000
Citrobacter farmeri	Kruskal-Wallis H	72.852
	Df	24
	Asymp. Sig.	.000
Klebsiella pneumoniae	Kruskal-Wallis H	72.295
	Df	24
	Asymp. Sig.	.000
Pseudomonas aeruginosa	Kruskal-Wallis H	72.356
	Df	24
	Asymp. Sig.	.000
Klebsiella oxytoca	Kruskal-Wallis H	69.203
	Df	24
	Asymp. Sig.	.000
Candida albicans	Kruskal-Wallis H	66.697
	Df	24
	Asymp. Sig.	.000

Df = degree of freedom, Asymp. Sig. = asymptotic significance (P-value)

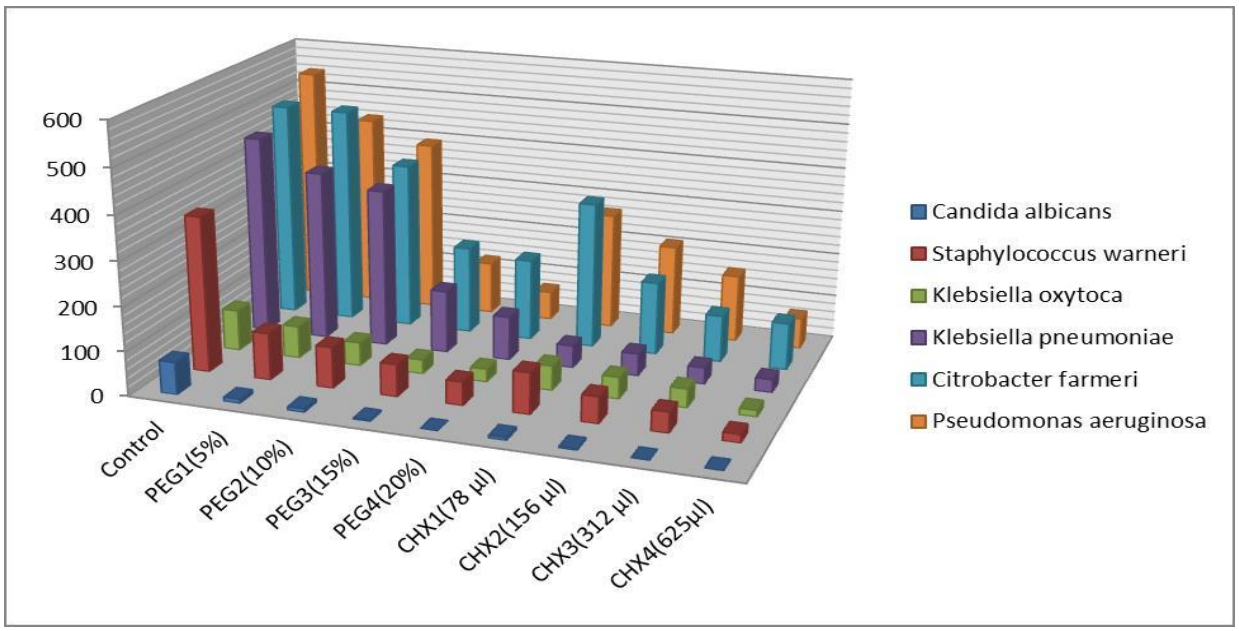

Figure (2): Post Hoc test for adherence of tested microorganisms on experimental groups (control, PEG and CHX).

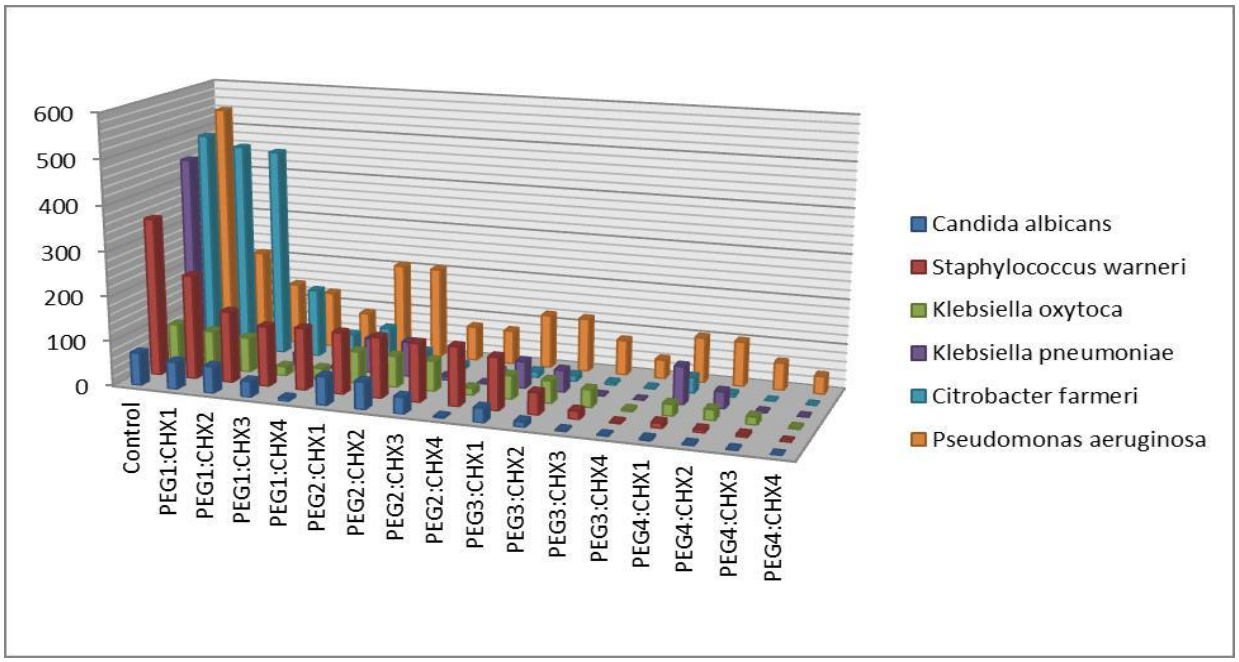

Figure (3): Post Hoc test for adherence of tested microorganisms on experimental groups (control, mixed PEG: CHX).

Discussion:

The identification of microorganisms in this research detected the occurrence of various species capable of forming biofilms. *Klebsiella pneumoniae* was the most common, accounting for 20% of all detected bacteria. This species is known to be an opportunistic pathogen that can create strong biofilms, enhancing its ability to colonize and stay in the host environment [24,25] It is considered one of the commonly isolated respiratory pathogens from removable prostheses [26]. *Klebsiella oxytoca*, another *Klebsiella* species, was also widely represented, accounting for 15% of all isolates. *K. oxytoca*, like *K. pneumoniae*, may produce biofilms on the denture base surface [27]. *Citrobacter farmeri* and *Pseudomonas aeruginosa* each accounted for 6.6% of the detected bacteria. Both may produce moderate to strong biofilms. *Pseudomonas aeruginosa*, in particular, is a major nosocomial pathogen recognized for its ability to thrive in biofilm communities [28,29]. *Stenotrophomonas maltophilia* accounted for an additional 6.6% of the isolates, however, its biofilm-forming ability was only moderate when compared to other species. This opportunistic pathogen is a growing issue in hospital settings [30]. *Candida albicans*, a fungus species, accounted for 10% of the detected microorganisms. It demonstrated a lower biofilm-forming potential than the bacterial species. However, *Candida albicans* is considered the major oral cavity isolate [31,32]. Biofilm formed on dentures is very similar to oral biofilms that form on teeth, except that dentures are much more likely to contain both respiratory pathogens and Candida species within the biofilm [33]. The denture biofilms can induce local infections such as denture stomatitis and systemic infections regarding respiratory infections [34].

The high occurrence of bacteria with strong to moderate biofilm-forming ability is a problematic issue since biofilms may increase persistence, antibiotic resistance, and overall pathogenicity. This confirms the need for biofilm prevention and disruption maneuvers, such as enhanced infection control measures, biofilm-targeting medicines, and

the development of anti-biofilm agents. More realization of the exact mechanisms and therapeutic applications of these biofilm-producing species would be beneficial [35]. In dealing with biofilm-forming microorganisms it was a valuable method to perform adequate therapeutic means by dividing biofilm formers as non, weak, moderate, and strong formers [36]. The study evaluated the anti-adhesion properties of incorporating polyethylene glycol (PEG) and chlorhexidine (CHX) into acrylic resin denture base materials. Several microorganisms were selected to assess the adherence, including *Staphylococcus warneri* (a gram-positive, weak biofilm-forming bacterium), *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Citrobacter farmeri*, *and Pseudomonas aeruginosa* (gram-negative, moderate to strong biofilm-forming bacteria), as well as *Candida albicans* (a fungal species). The results of the adherence test revealed that the experimental groups with greater concentrations of PEG and/or CHX had considerably lower adhesion values than the control group for all tested microorganisms. Specifically: *Candida albicans* had the lowest adherence (0 cells/mm²) in the PEG4, CHX3, CHX4, and PEG4:CHX4 groups, whereas the control group had the greatest adhesion (73.33 cells/mm²). In the PEG4:CHX4 group, *Staphylococcus warneri* had the lowest adherence (0 cells/mm²), whereas the control group had the highest at 353.33 cells/mm². *Klebsiella oxytoca* had the lowest adhesion (2.67 cells/mm²) in the PEG4:CHX4 group, whereas the control group had the highest value (93.33 cells/mm²). *Klebsiella pneumoniae* showed the lowest adhesion (0 cells/mm²) in the PEG3:CHX4 and PEG4:CHX4 groups, whereas the control group had the highest value of 453.33 cells/mm². *Citrobacter farmeri* and *Pseudomonas aeruginosa* exhibited the lowest adhesion (0 cells/mm²) in the PEG4:CHX3 and PEG4:CHX4 groups, whereas the control and PEG1 groups had the highest values (493.33 cells/mm²).

The Kruskal-Wallis test revealed significant differences in adhesion between control and experimental groups at ($p \leq 0.05$). The Post-Hoc test also demonstrated that increasing PEG and CHX concentrations induced a substantial reduction in the adhesion of the examined microorganisms. These results indicate that adding PEG and CHX to acrylic resin denture base materials may successfully limit the adherence of troublesome bacteria, even those with significant biofilm-forming abilities. This might have significant consequences for the prevention and treatment of denture-associated diseases, as well as the production of biofilms on denture surfaces. As increasing the concentrations of PEG and CHX significantly results in decreasing the adherence of tested microorganisms, the combination of PEG and CHX significantly enhanced the anti-adherence effect due to the added antifouling and antibacterial effect, especially at higher concentrations [8]. The acrylic resin's hydrophilic properties were improved by the addition of PEG, which also enhanced the material's adhesion-inhibiting capabilities [37]. Because of its high hydrophilicity, which creates a film of water on the surface, PEG can lessen the adherence of germs, platelets, and proteins [38,39]. Related to its reduced surface energy, PEG is a widely known bacterial-resistant substance with little protein binding. The creation of the hydration layer and PEG chain compression, resulting in an elastic repulsive force, during protein adsorption and migration towards a substrate surface, is thought to be the two potential mechanisms underlying the protein repellence of PEG-based materials [40,41]. The flexible polymer PEG with a repeating unit of (-CH2-CH2-O-), allows PEG to form hydrogen bonds with water molecules and form the steric repulsion property that keeps proteins and bacteria from invading [42,43]. Reducing the area of contact between protein and bacteria on substrate surfaces is an efficient way to prevent their absorption. The water barrier effect of hydrophilic materials may be useful in this regard [40,44].

Since the bacterial wall is negatively charged and the CHX wall is positively charged, they are drawn to one another. The phosphate-containing compounds adsorb strongly onto CHX on the bacterial wall surface. When CHX enters the bacterial wall, it damages the cell and prevents the cytoplasmic membrane-related enzymes from functioning. It also creates an overabundance of low-molecular-weight cytoplasmic elements, like potassium ions [45]. The action of antimicrobial product proceeds to the bactericidal phase, when coagulation of cytoplasm, and precipitation take place and compounds like adenosine triphosphate and nucleic acids are generated. If CHX is eliminated, the bacteriostatic stage can be reversed. Because oral surfaces have a negative charge, cationic molecules like CHX stick to them well and effectively inhibit bacterial adherence [46]. CHX possesses antifungal, antimicrobial, and antiviral properties against RNA, DNA, and lipophilic-enveloped viruses as well as anaerobes and aerobes [47]. Fathy *et al.* (2023) [48] revealed that regardless of surface roughness after coating the denture base resins with CHX nanoparticles, the anti-microbial and biofilm formation propensity of the resins was significantly enhanced by treating them with CHX NPs colloidal suspensions. Significant antibacterial effects persisted even after the specimens were stored and the NPs were released for 19 days. Maluf *et al*. (2020) [49] stated that the addition of CHX at 0.5 to 2 wt% to PMMA denture base resin significantly decreases *Candida albicans* adhesion.A drug delivery system based on hard reline acrylic resin loaded with chlorhexidine for prevention and treatment of denture stomatitis associated with *Candida albicans*. The reline resin demonstrated antibacterial action against *S. oralis* and *C. albicans* [50]. CHX has outstanding antibacterial qualities. Its wide range of antibacterial activity can be viewed as advantageous for general oral health. Its use in treating denture stomatitis in various forms is supported by a multitude of studies. It produces some cellular changes that lead to the fungicidal impact, including the cytoplasm's degeneration and the cell wall's fragmentation [51].

Conclusion: This in vitro study revealed that modifying denture base resin by the addition of PEG as an antifouling substance and CHX as an antibacterial and antifungal agent significantly enhances the anti-adherence property. The combination of PEG and CHX had a synergistic effect on anti-adherence properties, especially at high concentrations.

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