Original Article

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Evaluation of biofilm formation on different clear orthodontic retainer materials

Suhad M. Hamdoon, Saeed AlSamak[1], Mahmood Kh. Ahmed[1] and Saad Gasgoos[1]

Abstract

Aim: To assess the chemical composition and oral biofilm formation on different types of commercially available clear orthodontic retainer materials (CORM).

Materials and Methods: Four types of CORM commercially available were used (Clear advantage series I (CAS1), Clear advantage series II (CAS2), Endure (ES), and CENTRI FORM-clear rigid material (CFCRM)). Circular samples (12 mm diameter) of each CORM were prepared for (n = 40). Unstimulated saliva from twenty volunteers was collected. Fourier Transformation Infrared Spectroscopy (FTIR) was used for the evaluation of the chemical composition of CORM. For the quantitative assessment of oral biofilm formation, samples of each CORM were incubated for twenty-four hours, and crystal violet assay (CVA) was utilized. The degree of absorbance was measured using a spectrophotometer at 570 nm. For qualitative evaluation of oral formation, the samples of each CORM were incubated for 24 hours, and viable biofilm cells stained by acridine orange were examined under a fluorescent microscope.

Results: FTIR findings showed that CAS2 was made of polypropylene and ES is made of polyvinyl chloride, while others were made of co-polyester. CVA results confirmed that CAS2 showed the lowest biofilm formation, which differs significantly compared to CAS1, CFCRM, and ES. No significant difference in biofilm formation was detected between CAS1, CFCRM, and ES. Viable biofilm cells staining by acridine orange showed that CAS2 demonstrated smaller microcolonies of viable biofilm cells compared with CAS1, CFCRM, and ES, which confirmed the result obtained by CVA.

Conclusions: CAS2 showed anti-microbial activities with a decrease the *in vitro* biofilm formation, which may be related to its chemical composition.

Keywords:

Acridine orange, clear orthodontic retainer materials, Essex, oral biofilm

Introduction

In recent years, increased esthetic demands put a great need on dental therapy. One of the significant problems that affect esthetics is malocclusion. Orthodontic treatment offers an excellent treatment option for various degrees of malocclusion complexity. Orthodontic treatment can be achieved with a fix or removable appliances). One of the recent advancements in orthodontic treatment is clear aligner therapy.[1] In addition, the results obtained by orthodontic treatment should be maintained by proper retention time and appliance. Different protocols were used for retention, including fix (permanent bonded retainer) and removable retainers (Hawley Retainer and Essix Retainer). Every type of retainer has its advantage and disadvantage.[2]

Clear aligner therapy and Essex retainer are constructed from clear orthodontic retainer materials (CORM). The main advantage includes less chair time, invisibility, ease to put and to remove, and good patient compliance. However, drawbacks include

Departments of Basic Science and [1]Pedodontics Orthodontics and Preventive Dentistry, College of Dentistry, University of Mosul, Mosul, Iraq

Address for correspondence:
Dr. Saeed AlSamak, Department of Pedodontics, Orthodontics and Preventive Dentistry, University of Mosul, Mosul, Iraq.
E-mail: saaid_sammak@uomosul.edu.iq

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patient cooperation, appliance loss, wearing, loosening over time, discoloration, and accumulation of biofilm.[3-5]

The oral biofilm consists of adhering bacteria embedded in a complex extracellular matrix which facilitates bacterial adherence and protection of bacterial colonization.[6] Many studies revealed that the placement of an orthodontic appliance in a patient's mouth changes the bacterial structure of oral biofilm, which may increase the occurrence of bacterial species over other species that may be considered as cariogenic and periodontal pathogens.[4,5,7] The biofilm formation and adherence depend on surface characteristics, surface area, and chemical composition.[8] On the other hand, the clear orthodontic appliances themselves decrease salivary wash and buffering capacity on dental and periodontal structures. In addition, orthodontic appliances are factors that act as new niches to which microorganisms can adhere and result in biofilm.[9]

Microbial adherence on the abiotic surface is the early step of biofilm development, particularly after applying orthodontic appliances or implants.[10,11] This step of biofilm formation can be affected by several chemical or physical factors like chemical composition, surface roughness, surface free energy, and surface tension, affecting wettability and salivary protein adhesion. Studies demonstrated that hydrophobic and electrostatic interactions are responsible for initial bacterial attachment to abiotic surfaces as different bracket materials due to their surface properties or even tissue surface.[12,13] The CORM used in orthodontics is a class of polymers with different characteristics, including polyethylene terephthalate and polyethylene terephthalate-polyethylene glycol (polyethylene) terephthalate-glycol), thermoplastic polyurethane, polyvinyl chloride, polycarbonate, polypropylene materials, and ethylene-vinyl acetate.[1,14-17]

The variation of the chemical composition of CORM will influence the mechanical properties, including stress release and relief, aging, water absorption, and abrasion resistance.[1] These variations may play a role in creating conditions favorable for bacterial colonization.[18] As far as is known, there is little data in the literature regarding the oral biofilm formation on CORM. The purpose of this research was to evaluate the effect of the chemical composition of four commercially available CORM on oral biofilm formation *in vitro*. The null hypothesis assumed that the chemical composition of clear thermoplastic retainer materials has no effects on oral biofilm formation and adherence.

Materials and Methods

Four brands of CORM were used in this study. Detailed information's were described in Table 1.

Preparation of samples

The sample size was determined according mean and standard deviation of former study of confidence interval of 90%.[8] Forty round samples (12 mm in diameter) of each type of CORM were cut by round hollow punch is made of stainless steel of 12 mm in diameter (Utoolmart, China) without heating to avoid any effect on the physical or chemical properties of the materials. A unique mark was added to each type of CORM tested to distinguish between samples of CORM. Sterilization was carried out by immersion in 2% glutaraldehyde for 30 minutes (Sasma BV, Zoetermeer, Netherlands). The glutaraldehyde not adsorb to the surface of thermoplastic material tested.[19] After sterilization, the specimens were air-dried inside a laminar flow cabinet under UV light (Diahann Labtech Com, Indonesia) and prepared for culturing. Twenty pieces of each type of CORM were used for crystal violet assay (CVA), and the other twenty pieces of each kind of CORM were used for viable cell account with acridine orange.

Fourier Transformation Infrared Spectroscopy (FTIR)

Fourier Transformation Infrared Spectroscopy (FTIR) (Platinum Atr, Bruker, Germany) was used to evaluate the chemical composition of four types of CORM at FTIR spectra wavelength range 400-4000 cm^{-1}. The FTIR spectra were generated and recorded.

Salivary samples collection

Unstimulated saliva was collected from 20 volunteers from male dental students, college of dentistry, University of Mosul, age range between 18 and 23 years. This research was ethically approved by the scientific committee of the basic science, college of dentistry, University of Mosul. Medical consents were taken from the volunteers, and research objectives were explained. Each volunteer's medical history was taken (non-smoking, no systemic disease, no syndrome, no medication, no radiation). A dental examination was carried out to exclude volunteers with dental caries and periodontal diseases. The volunteer had renounced eating, drinking, mouth wash, and brushing for at least 3 hours before collection.

Preparation of culture media

Brain heart infusion broth (BHIB) (Oxoid, England) is prepared by adding 37 g of powdered medium to 1-liter distilled water, supplemented with 0.5% yeast extract and 0.4% sodium carbonate to enhance bacterial adherence and biofilms formation, sterilized by autoclave (EMC-LAB, Duisburg, Germany) for 15 min at 15 PSI and 121°C.[20]

Preparation of biofilms cells

Saliva samples (100 µl) were added to a plastic collector containing 20 ml of (BHIB). After18 hours of incubation,

Table 1: List of clear orthodontic materials used in the study

Retainer materials	REF/LOT	Chemical composition	Manufacture	City, State, Country
Clear advantage series I (1 mm)-(CAS1)	#:7500-110/036191	Thermal forming splint/ Co-polyester	Orthotechnolgy ®	Lutz, Florida, USA
Clear advantage series II (1 mm)-(CAS2)	#:7500-125/010911	Thermal forming coping/polypropylene	Orthotechnolgy®	Lutz, Florida, USA
Endure® square (1 mm)-(ES)	025-046/053013	Rigid Polyvinyl Chloride Sheeting	Great Lakes Orthodontics, Ltd	Tonawanda, N.Y., USA
CENTRI™ FORM-Clear rigid material (CFCRM)	#562839/JL4143	Vivak VI Co-polyester	WHW Plastics	Yorkshire, Leeds, UK

the biofilms cells have adhered to the surfaces of the container. The broth was discarded, and the collector was filled with phosphate buffer saline (PBS, pH 7.3), vortexes with a vortex (Dragon Lab, Beijing China) to detach non-adherent cells biofilm cells that adhered to the collector's wall were scraped off with a sterile spatula. A broth culture (Oxoid Ltd, Basingstoke, United Kingdom) containing approximately 6.0 x 10^8 colony-forming unit (CFU) of scraped biofilms cells equal to tube 2 McFarland were used for subsequent inoculation.[21] To evaluate biofilm formation on each CORM, new collectors (n = 20) containing 20 ml sterile (BHIB) with eight pieces, two from each of the four CORM types, were incubated for 24 hours at 37 C°. This step was done for each of the 20 saliva samples. After incubation, biofilms formed on each piece were evaluated qualitatively and quantitatively.[22]

Crystal violet assay (CVA)

A CVA test was used for the quantitative assessment of microbial biofilms.[21] Eighty samples of CORM were collected from the twenty-saliva collectors. Each sample was put in a plastic Petri plate, stained with 1 ml of 0.1% crystal violet for 1 min, then washed with 1 µl phosphate buffer saline (7.3 pH) 2-3 times to remove the unbounded dye. The samples of CORM were treated with 1 ml of 99% ethanol to elute the dye bound to biofilms cells that remain adherent to each piece. The dye eluted solutions were double diluted by 99% ethanol then examined by spectrophotometer (Thermo Fisher Scientific, Waltham, Germany) to measure the absorbance at 570 nm.[20]

Viable biofilm cells staining by acridine orange

For qualitative assessment of microbial biofilms, viable biofilm cells staining by acridine orange was used. Eighty samples of CORM were collected from the twenty-saliva collectors. The samples of CORM were stained with 1 ml acridine orange acidic stain stock solution (ThermoFisher Scientific, Waltham, Germany). Acridine orange acidic stain was prepared by dissolving 50 mg of acridine orange in 10 ml of distilled water to prepare a reserve solution. To prepare a working solution, 1 ml of Acridine orange stock solution mixed with 0.5 ml of glacial acetic acid and 50 ml distilled water. The biofilm on the samples was fixed with methanol, dried, and stained with acridine orange staining working solution for 2 min. The samples were washed gently with water, dried, and then examined using a fluorescent microscope (Thermo Fisher Scientific, Waltham, Germany) under a high-power magnification oil lens (100*10x).

Statistical analysis

Statistical analysis was calculated using Statistical Package for the Social Sciences (version 26, SPSS Inc., Chicago, Illinois, USA). Descriptive statistics analysis, multiple comparisons using one way ANOVA, and post hoc tests were used to compare between means of absorptions measured in spectrophotometer of different CORM tested. The level of significance was recorded to be at $P < 0.05$.

Results

FTIR analysis: FTIR results of CAS1 [Figure 1] and CFCRM [Figure 2] showed identical transmission FTIR spectra in the functional group region and fingerprint region. In the functional group region, CH2– aliphatic stretching at 2927.80 cm^{-1} and 2855 cm^{-1}. In the fingerprint region, a sharp peck at 1712.48 cm^{-1} represents carbonyl –C = O stretching of ester group stretching confirmed co-polyester. The absorption 1200-1150 cm^{-1} (CO-O stretching) and 1115-1042 cm^{-1} (OCH2 stretch) are distinctive of central chain polyesters. The absorptions at 1371.06 and 1338.50 cm^{-1} arise from the ethylene glycol. FTIR results of CAS2 [Figure 3] showed that the present hydrogen binding functional groups of methyl (C-H) stretch at 2949 cm^{-1}, methylene (C-H) stretches at 2919 cm^{-1}, 2866 cm^{-1}, and 2837 cm^{-1}. Aldehydic (C-H) stretch at 2722 cm^{-1}. In fingerprint region showed the presence of asymmetric and symmetric in-plane C–H (–CH3) at 1453.58 and 1358.83 cm^{-1} proving that it is polypropylene. The stretch at 1375 cm^{-1} is related to the –CH3 group.

FTIR spectra of ES [Figure 4] at the functional group region showed a peck at 3025.12 cm^{-1} representing C-H stretch. FTIR spectra at a peck of 2916.87 cm^{-1} is the CH2 stretching vibration mode. In the fingerprint's region, the peaks at 1427.22 cm^{-1} are assigned to the Ch_2–Cl aliphatic bending bond. The peak at 1234.88 cm^{-1} is

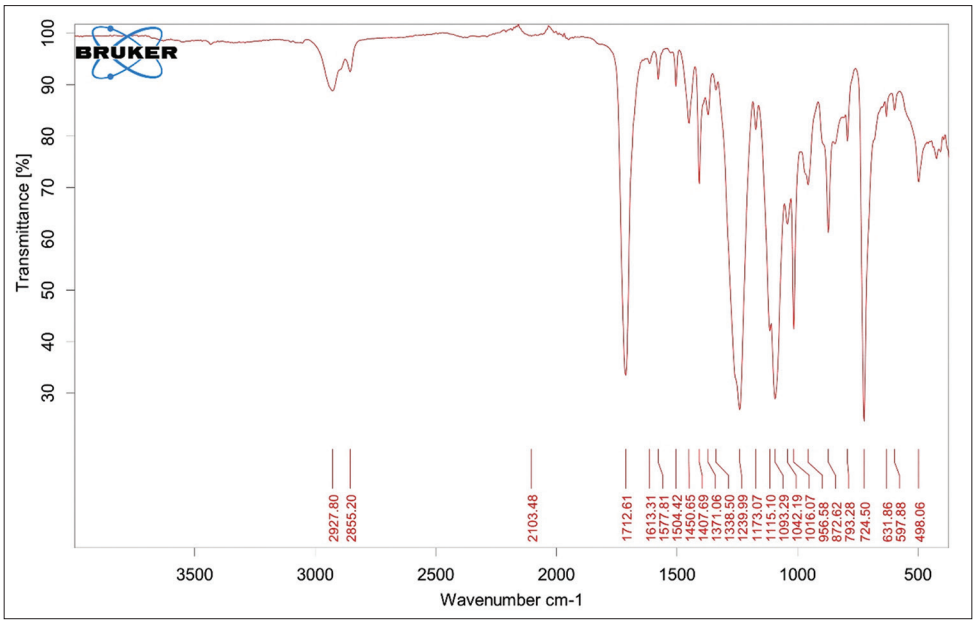

Figure 1: FTIR spectra of clear advantage series I (CAS1)

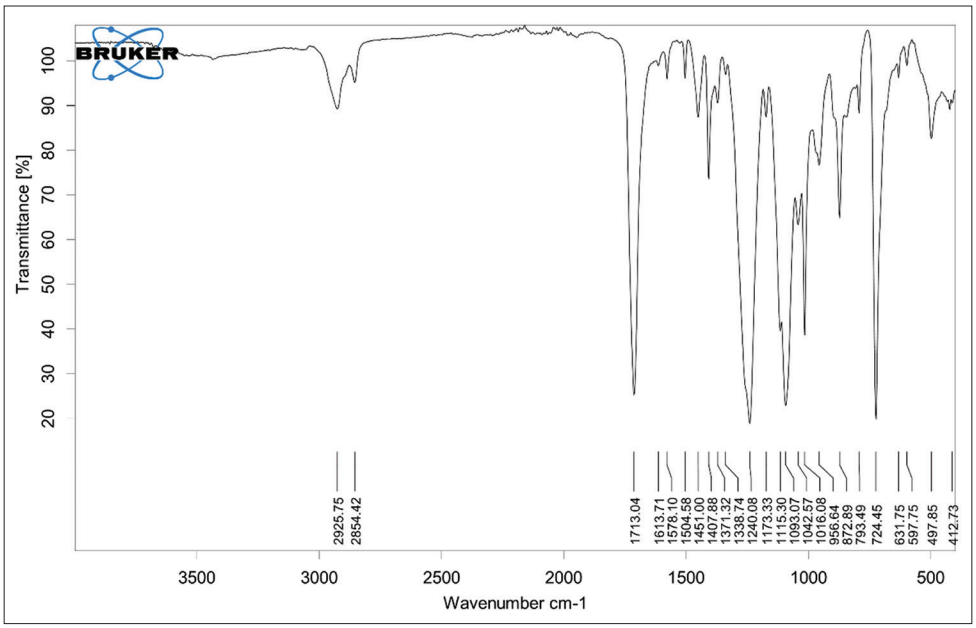

Figure 2: FTIR spectra of Centri Form-Clear rigid material CFCRM

attributed to the bending bond of CH – Cl. The C–C and C-H stretching presents at 1098.48 – 1024.79 cm^{-1}, C-Cl stretching at 835.74 cm^{-1}. FTIR spectra in the range of 698.47 – 611.11 cm^{-1} relate to the C – Cl gauche bond. The fingerprint spectra confirm the polyvinyl chloride.

One way ANOVA multiple comparisons and post hoc Duncan's test of the mean of absorbance of oral biofilm in spectrophotometer showed that the results of CAS2 showed lower significant differences in oral biofilm formation compared to CAS1, CFCRM, and ES. No significant differences were detected between CAS1, CFCRM, and ES [Table 2]. Viable biofilm cells staining by acridine orange showed that CAS2 demonstrated smaller microcolonies of viable biofilm cells [Figure 5] compared with CAS1, CFCRM, and ES, which confirmed the result obtained by CVA.

Discussion

Orthodontic retention is a complementary procedure that secures the teeth to their final position obtained by orthodontic treatment.[23] Retention can be maintained either by fixed or removable retainers. A clear retainer is

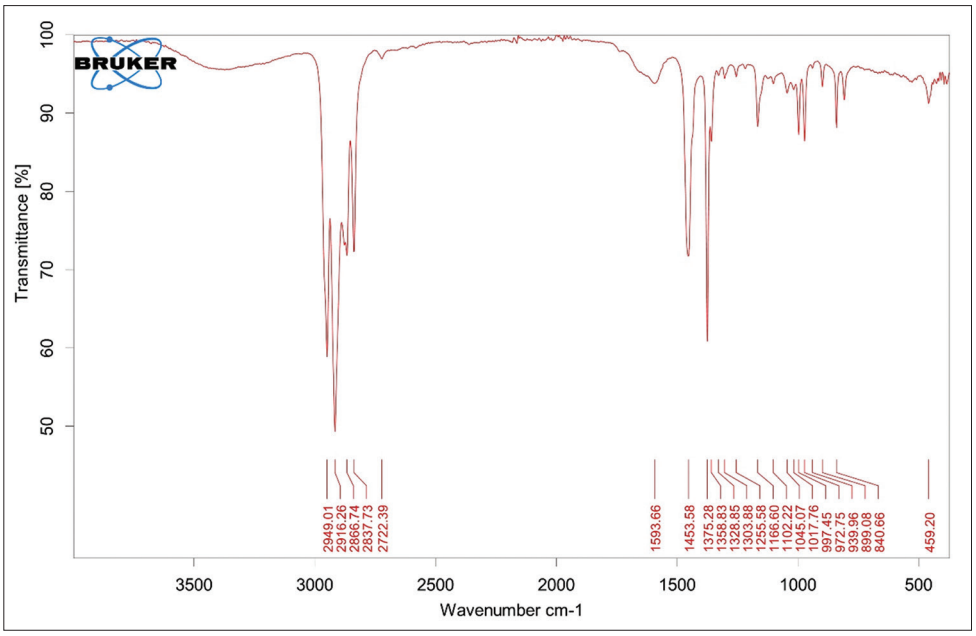

Figure 3: FTIR spectra of clear advantage series II (CAS2)

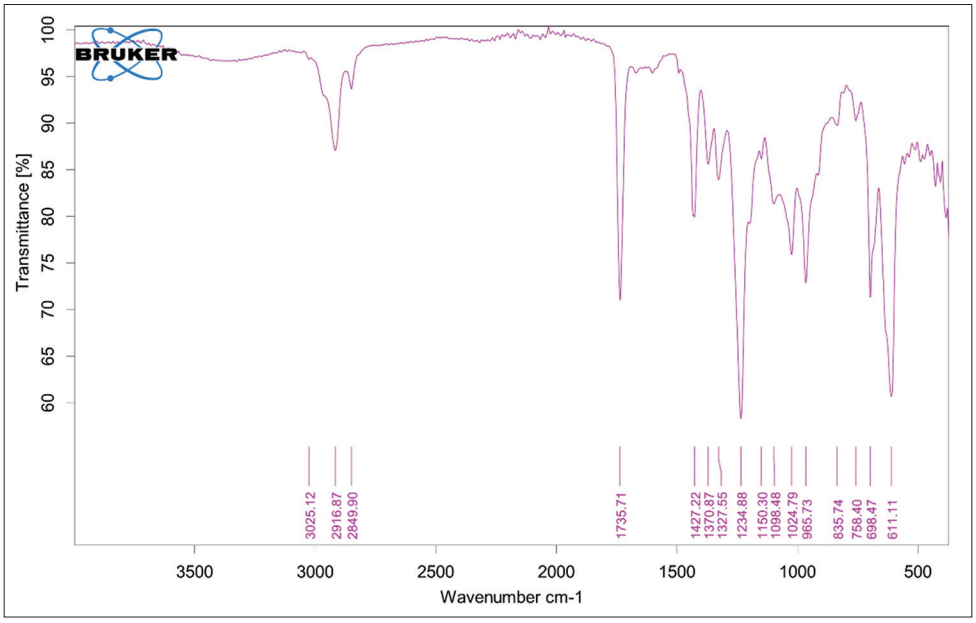

Figure 4: FTIR spectra of Endure square (ES)

a removable retainer that was applied in 1993 by Dr. John Sheridan.[24] It is an esthetically acceptable, comfortable, and inexpensive appliance. Many oral pathogens can adhere to retentive appliances and lead to biofilms formation in which biofilms cells are more resistant to anti-microbial agents. Once the biofilms are formed in the retainer, it is difficult to be eliminated and challenging to clear.[25,26] These events will lead to the formation of white spot lesions, dental caries, and periodontal diseases.[27]

This research evaluated quantitively the *in vitro* ability of oral biofilm to adhere to the surface of CORM using CVA. Although CVA method is the most accurate method for bacterial quantitative evaluation, but it offers an effective method for various components of living and dead bacterial cells and even extracellular material in which biofilm cells are embedded.[21,28] The qualitative assessment of viable cells of oral biofilm was accomplished using acridine orange staining in which dye can bind to the cellular matrix of viable cells.[29] The chemical composition of CORM was assessed through FTIR analysis. FTIR spectrometry is commonly utilized for polymer detection, which has been shown to give excellent results.[30] CORM is

Table 2: Multiple comparisons and post hoc Duncan's test of the mean of absorbance of oral biofilm in spectrophotometer formed on Clear advantage series I (CAS1), Clear advantage series II (CAS2), Endure square (ES), and Centri Form-Clear rigid material (CFCRM)

Retainer material	*n*	Range	Minimum	Maximum	Mean±SD	Significance	Duncan s test
Clear advantage series I (1 mm)-(CAS1)	20	0.48	0.11	0.58	0.29075±0.14288	0.064	B
Clear advantage series II (1 mm)-(CAS2)	20	0.11	0.10	0.21	0.14316±0.02964	0.000	A*
Endure® square (1 mm)-(ES)	20	0.34	0.11	0.45	0.23755±0.09522	0.064	B
CENTRI™ FORM-Clear rigid material (CFCRM)	20	0.27	0.11	0.38	0.22995±0.08588	0.064	B

The level of signicance is ≤0.05

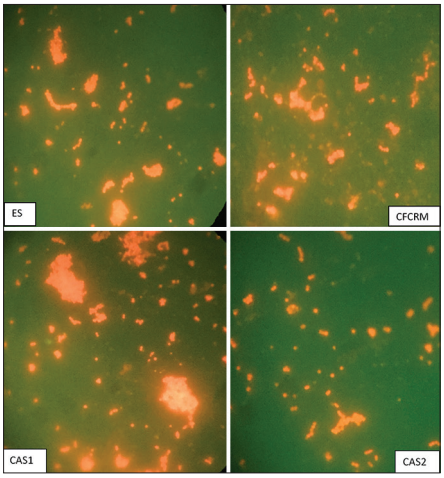

Figure 5: Biofilm staining by acridine orange examined by fluorescent microscope Clear advantage series I (CAS1), Clear advantage series II (CAS2), Endure square (ES), and Centri Form-Clear rigid material (CFCRM). CAS2 showed the most minuscule and scattered aggregation of biofilm cells among the other types of CORM tested

synthesized from different polymers using various preliminary chemical compounds or by the addition of other substances which exhibit different physical and chemical properties. The chemical composition of the polymer is responsible for its properties, which can be used as a reference for its analysis by FTIR spectroscopy.[31]

The null hypothesis tested was rejected. CAS2 showed statistically significant lowered biofilm formation compared to CAS1, CFCRM, and ES. The FTIR results showed that CAS2 is made of polypropylene which was confirmed by the manufactural data. The introduction of a removable retainer inside the oral cavity creates a condition that facilitates proliferation and adherence of oral biofilm to dental structures by preventing saliva washing from reaching dental structures.[8] Streptococcus mutans, lactobacilli, and gram-negative bacteria are the essential pathogens that increase with orthodontic treatment.[31] Türköz *et al*.[8] stated that the use of removable thermoplastic retainer creates a condition favorable to strepotococcus and lactobacillus proliferation. Therefore, using a retainer appliance with anti-microbial properties will be more beneficial. CAS2 has fewer functional groups that make it a chemically and physically inert substance since bacterial adherence requires different forces and bonds between the surface and microorganisms responsible for short-range and long-range forces.[31,32] Previous research showed the anti-microbial activities of polypropylene against oral biofilm bacteria.[33] These differences in chemical composition may influence their mechanical and clinical performance.[34,35] This is maybe related to surface free energy and chemical composition.[36] Previous research had stated that increased surface energy would increase bacterial adherence.[37] Although the CAS1 and CFCRM were made from co-polyester and ES from polyvinyl chloride, there were no significant differences in biofilm formation. Tektas *et al*.[17] studied the biofilm formation on four orthodontic retainer materials (CA-medium, co-polyester, Duran, and Erkodur). They found no significant differences in initial oral biofilm formation between the four types tested, deprived of addressing the chemical composition of retainer materials. The chemical composition modification of removable orthodontic retainer decreases biofilm formation significantly.[38] Lee *et al*.[39] studied the surface characteristics of orthodontic material and its relation to adhesion of streptococcus mutans, and they found that bacterial adhesion is related to increase surface roughness and surface energy. Further experimental studies can be conducted to evaluate CORM's mechanical and topographic characteristics.

In summary of the findings of this research, CAS2 made of polypropylene showed anti-microbial activities against viable and non-viable biofilm microorganisms. This may be attributed to lower functional groups of CAS2 which may interfere with bacterial adhesion. CAS1, and CFCRM were made from co-polyester, and ES from polyvinyl chloride demonstrated insignificant differences in biofilm formation.

Conclusions

Within the study's limitations, CAS2 showed anti-microbial activities that decreased the *in vitro* biofilm formation, which may be related to its chemical composition. CAS1, CFCRM, and ES demonstrated different chemical compositions with no significant effect on oral biofilm formation.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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