Comparison of microbial adhesion and biofilm formation on different orthodontic aligners

Aylin Pasaoglu Bozkurt,[a] Mehmet Demirci,[b] Pelin Erdogan,[a] and Emre Kayalar[c]
Istanbul and Kirklareli, Turkey, and Sydney, Australia

Introduction: This study aimed to compare and evaluate time-dependent biofilm formation and microbial adhesion on 6 different clear aligner systems: Invisalign (Align Technology, San Jose, Calif), Clarity (3M ESPE Maplewood, Minn), ClearCorrect (Institut Straumann AG, Basel, Switzerland), Smartee (Smartee Denti-Technology, Shanghai, China), Orthero (Orthero, Istanbul, Turkey) and Graphy (Graphy Inc, Seoul, South Korea). **Methods:** *Streptococcus mutans* (ATCC 25175) and *Lactobacillus acidophilus* (ATCC 4356) were used to evaluate the microbial adhesion and biofilm formation on orthodontic clear aligners at the 0, 24, 48, 72, 96, 120, 168, and 240 hours. Two-way repeated measures analysis of variance (Greenhouse-Geisser) test and post-hoc Bonferroni T2 tests were used for statistical evaluation. The statistical significance level was set at $P < 0.05$. **Results:** It was found that more bacterial formation occurred on ClearCorrect than on Smartee at 120, 168, and 240 hours ($P < 0.05$). It was observed more biofilm formation at 168 hours on Graphy than on Smartee ($P < 0.05$). It was found that *S mutans* + *L acidophilus* formed more biofilm at 120 and 168 hours on Graphy than on Invisalign ($P < 0.05$). **Conclusions:** Elevated biofilm formation across all materials carries substantial clinical implications. Orthodontists and patients should remain aware of the increased risk of microbial colonization with extended aligner usage. (Am J Orthod Dentofacial Orthop 2024;■:■-■)

Orthodontic treatment is a long and difficult treatment in which esthetic concerns are at the forefront. When we evaluate from past to present, we see that the devices used in orthodontic treatment have changed over time, from larger and more visible to smaller and more invisible.[1] Although the use of intraoral appliances and miniscrews instead of extraoral appliances is becoming more common, plastic and ceramic brackets are seen as an esthetic alternative instead of metal brackets. However, the search for esthetics has always continued, and the recent pandemic and economic reasons have directed patients to a different alternative. Finally, the most popular and esthetic form of orthodontic treatment is clear aligner treatment (CAT).[2-4]

CAT occurs by moving the teeth into a certain position step by step with a series of aligners created in the laboratory based on the impression and digital scan obtained from the patient. In this type of treatment, consecutive aligners are used by the patient for 20-22 h/d for varying periods of 7-10 days. The composition of the material used to manufacture an aligner affects the clinical performance in every aspect.[5] There are 2 main production methods in the preparation of aligners: vacuum thermoforming method with thermoplastic material molding in traditional physical models and direct 3-dimensional (3D) printing without intermediate physical models. Thermoplastic polymers, polymer blends, 3D printed aligner materials, and bioactive materials are used in the production of aligners. The most preferred materials are thermoplastic materials shaped by heat. The polymers most commonly used (individually or blended) in the production of commercial aligners are polyester, polyurethane or copolyester, polypropylene, polycarbonate, ethylene vinyl acetate, and polyvinyl chloride.[3,6,7]

[a]Department of Orthodontics, Faculty of Dentistry, Istanbul Aydin University, Istanbul, Turkey.
[b]Department of Medical Microbiology, Kirklareli University, Faculty of Medicine, Kirklareli, Turkey.
[c]Discipline of Orthodontics and Paediatric Dentistry, School of Dentistry, The University of Sydney, Sydney, Australia.
All authors have completed and submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest, and none were reported.
Address correspondence to: Emre Kayalar, Discipline of Orthodontics and Paediatric Dentistry, School of Dentistry, The University of Sydney, Sydney Dental Hospital, 2 Chalmers St, Surry Hills, NSW, 2010, Australia; e-mail, emre.kayalar@sydney.edu.au or Address correspondence to: Pelin Erdogan, Department of Orthodontics, Faculty of Dentistry, Istanbul Aydin University, 34295, Istanbul, Turkey; e-mail, dtpelinerdogan@gmail.com.
Submitted, February 2024; revised and accepted, August 2024.
0889-5406/$36.00

https://doi.org/10.1016/j.ajodo.2024.08.010

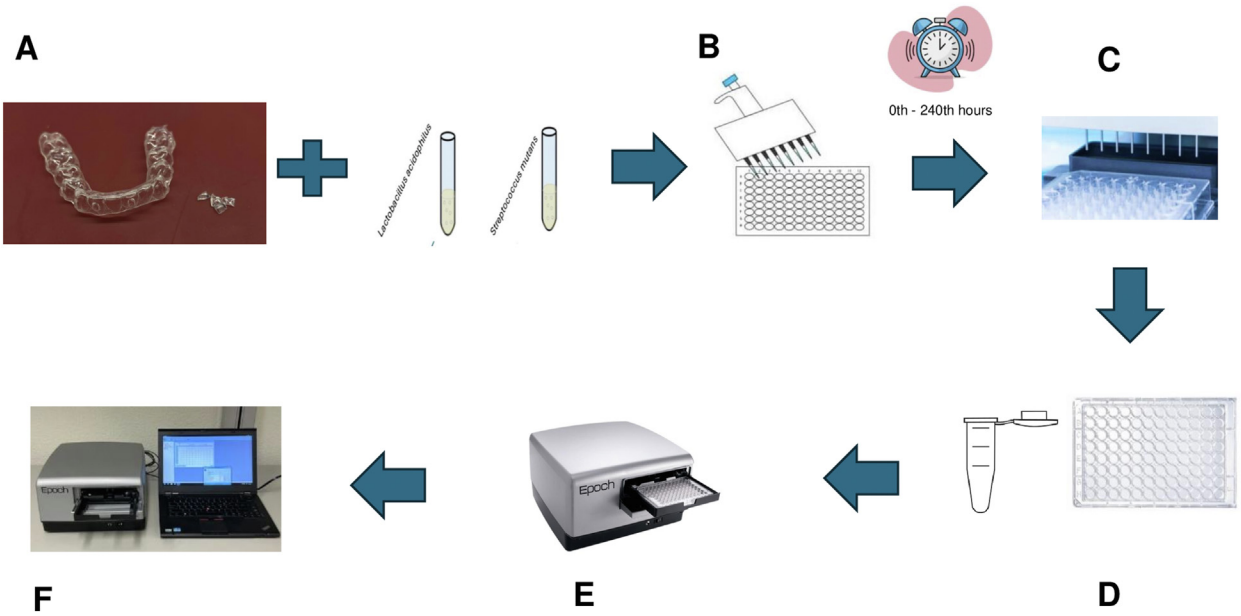

Fig 1. A, Part of the orthodontic aligner and microbial sample were combined; **B,** Plates were incubated at 37°C in a carbon dioxide incubator for various durations: 0, 24, 48, 72, 96, 120, 168, and 240 hours; **C,** Postincubation, liquid in the wells was removed; **D,** Wells were stained with 100 μL of 0.4% crystal violet for 15 minutes; **E,** at 540 nm (OD540) was measured using an Epoch spectrophotometer; **F,** Result analysis was performed.

Clear aligner systems are widely used today, and there are many commercial clear aligner systems on the market. Invisalign (Align Technology, San Jose, Calif) is produced by SmartTrack material (Align Technology): multilayer aromatic thermoplastic polyurethane from methylene diphenyl diisocyanate and 1,6-hexanediol plus additives.[8] 3M company (3M ESPE Maplewood, Minn) introduced their clear aligner system called Clarity, made from durable rigid material proprietary 5-layer copolymer blend.[9] ClearCorrect aligners (Institut Straumann AG, Basel, Switzerland) are produced from ClearQuartz (Institut Straumann AG) material: Zendura Polyurethane Tri-layer proprietary material.[10] The Smartee aligner (Smartee Denti-Technology, Shanghai, China) is manufactured with Erkodur foil (Erkodent Erich Kopp GmbH, Pfalzgrafenweiler, Germany), which is composed of polyethylene terephthalate glycol (PETG) based material. PETG is a noncrystallising amorphous copolymer of polyethylene terephthalate and is a relatively hard material with good mechanical properties, formability, fatigue resistance, and dimensional stability.[11] Orthero (Orthero, Istanbul, Turkey) is a clear aligner system that uses ABA 3-layer film consisting of copolyester (A), thermoplastic elastomer (B), and again copolyester (A) materials to produce aligners.[12] Graphy is a transparent and elastic resin-based material that can be directly printed as an aligner. Direct printed aligners were directly 3D-printed with Tera Harz TC-85 (Graphy Inc, Seoul, South Korea); clear photocurable resin in 100-μm layers showed up in 2019 and gradually gained ground in the field of aligner production.[13]

The human oral cavity contains a rich habitat with different anatomic features such as teeth, gingiva, tongue, and soft and hard palate. Oral microbiota is a community of microorganisms consisting of bacteria, fungi, and viruses that settle in these habitats. More than 700 different microbial species exist, including aerobic and anaerobic bacteria.[14-17] Oral biofilm attaches mainly to tooth surfaces or gingival epithelial cells. Adherence is the first condition for infection. There are many surfaces that bacteria can attach to in the mouth. Keratinized epithelium, nonkeratinized epithelium, hydroxyl apatite surfaces, and all material surfaces used in orthodontic treatment in the mouth can be given as examples.[18-23] Orthodontic devices can affect the composition of the oral microbiota by causing plaque accumulation and inability to perform oral hygiene.[16,24-27] The fact that there are fewer attachment surfaces compared with fixed orthodontic treatment may suggest that CAT causes less caries and periodontal disease, but for CAT to be successful, it is recommended for patients to use their aligners for 20-

Table I. Comparison of the amount of biofilm formation after interaction of SM and different orthodontic aligners in vitro environment over time

Material	*Range*	*Mean ± SD*	*Median*
0 h			
ClearCorrect	1.27-1.52	1.37 ± 0.13	1.31
Smartee	1.43-1.89	1.63 ± 0.23	1.58
Orthero	0.67-0.81	0.73 ± 0.07	0.71
Clarity	0.92-1.23	1.03 ± 0.18	0.93
Graphy	0.82-1.21	0.99 ± 0.20	0.94
Invisalign	0.98-1.36	1.18 ± 0.19	1.21
24 h			
ClearCorrect	1.64-1.76	1.71 ± 0.06	1.72
Smartee	1.78-1.92	1.84 ± 0.07	1.81
Orthero	0.94-1.13	1.03 ± 0.10	1.02
Clarity	1.12-1.25	1.17 ± 0.07	1.14
Graphy	0.99-1.25	1.13 ± 0.13	1.14
Invisalign	1.28-1.44	1.38 ± 0.09	1.41
48 h			
ClearCorrect	1.76-2.02	1.89 ± 0.13	1.89
Smartee	2.39-2.51	2.46 ± 0.06	2.48
Orthero	1.58-1.98	1.81 ± 0.21	1.87
Clarity	1.64-2.01	1.85 ± 0.19	1.89
Graphy	2.04-2.12	2.08 ± 0.04	2.08
Invisalign	2.02-2.41	2.26 ± 0.21	2.35
72 h			
ClearCorrect	3.96-4.34	4.16 ± 0.19	4.18
Smartee	4.24-5.01	4.71 ± 0.41	4.87
Orthero	3.76-4.21	3.98 ± 0.23	3.98
Clarity	4.24-5.02	4.71 ± 0.41	4.87
Graphy	5.20-5.42	5.28 ± 0.12	5.23
Invisalign	5.12-5.67	5.33 ± 0.30	5.20
96 h			
ClearCorrect	7.84-8.01	7.92 ± 0.09	7.92
Smartee	6.14-6.91	6.43 ± 0.42	6.23
Orthero	7.68-8.42	8.07 ± 0.37	8.12
Clarity	8.35-8.84	8.53 ± 0.27	8.39
Graphy	7.96-8.95	8.42 ± 0.50	8.34
Invisalign	7.93-8.56	8.35 ± 0.36	8.56
120 h			
ClearCorrect	12.71-13.23	12.94 ± 0.27	12.87
Smartee	10.36-10.91	10.57 ± 0.30	10.43
Orthero	12.45-13.05	12.68 ± 0.33	12.53
Clarity	13.50-14.63	13.98 ± 0.58	13.81
Graphy	13.36-14.84	14.27 ± 0.80	14.61
Invisalign	11.08-11.66	11.33 ± 0.30	11.25
168 h			
ClearCorrect	16.71-17.93	17.36 ± 0.61	17.43
Smartee	13.32-14.54	14.03 ± 0.64	14.24
Orthero	15.24-15.63	15.37 ± 0.23	15.24
Clarity	15.36-16.61	16.12 ± 0.67	16.39
Graphy	16.30-16.82	16.55 ± 0.26	16.52
Invisalign	13.91-15.94	14.79 ± 1.04	14.53
240 h			
ClearCorrect	20.21-20.86	20.57 ± 0.33	20.65
Smartee	19.81-21.12	20.52 ± 0.66	20.64
Orthero	17.91-18.59	18.35 ± 0.38	18.54
Clarity	17.89-18.91	18.39 ± 0.51	18.36
Graphy	19.37-20.05	19.61 ± 0.38	19.41
Invisalign	20.43-21.25	20.85 ± 0.41	20.88

Table I. Continued

Source	*Type 3 SS*	*DF*	*MS*	F *value*	P *value*
Time	6386.041	3.008	2122.877	6121.984	<0.001*
Material × time	76.870	15.041	5.111	14.738	<0.001*
Error (time)	12.518	36.098	0.347		

SD, standard deviation; *SS*, sum of squares; *DF*, degrees of freedom; *MS*, mean squares.
*Statistical significance ($P < 0.05$).

22 h/d and to remove it only for eating and maintaining oral hygiene. This means that during this period, the tooth and keratinized gingival tissues are in contact and covered by the aligners. It is very important that the physical, mechanical, and material properties of the aligners, which are exposed to constantly changing heat, pressure, functional and parafunctional forces in the oral cavity, remain stable. Both the design and material structure of aligners have a significant impact on the oral microenvironment and can affect bacterial adhesion.[28,29] Little data are available regarding biofilm formation and bacterial adhesion on aligner materials. It is of clinical importance to examine this issue in detail.

Therefore, this study aimed to compare and evaluate time-dependent biofilm formation and microbial adhesion on 6 different clear aligner systems: Invisalign, Clarity, ClearCorrect, Smartee, Orthero and Graphy.

MATERIAL AND METHODS

This study was exempt from ethics committee approval by the Faculty of Dentistry Research Ethics Committee. Six orthodontic clear aligner systems were selected: Invisalign, Clarity, ClearCorrect, Smartee, Orthero, and Graphy. These clear aligner products were supplied. These materials were adjusted into the laboratory environment to sizes that could be placed in the microwells of 96-well plates. A coding system was used to make a blind evaluation, and all analyses were made with this system. In the in vitro environment, *Streptococcus mutans* (SM) (ATCC 25175) and *Lactobacillus acidophilus* (LM) (ATCC 4356) standard strains were used to examine the amount of biofilm formation over time after their interaction with clear aligner materials. Bacterial suspensions were prepared using tryptic soy

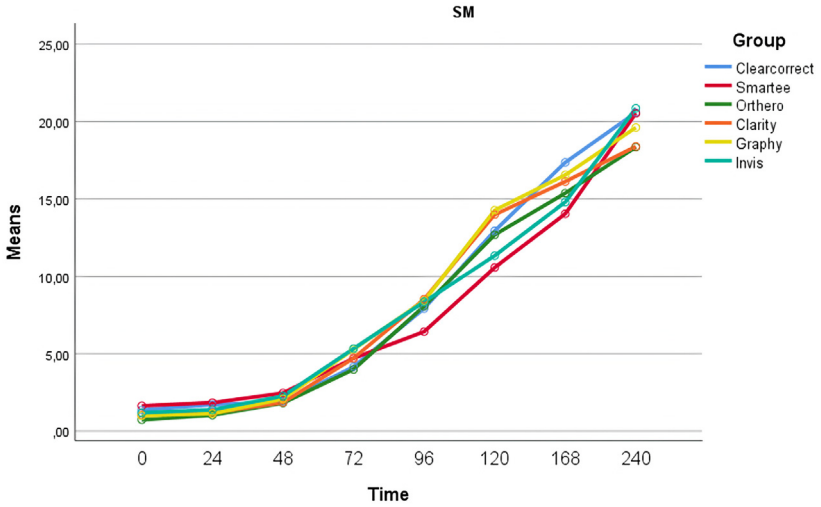

Fig 2. Line chart of measurements in the SM group according to different times and aligners.

broth (Oxoid, Basingstoke, United Kingdom) medium, and the optical density (OD) was prepared to be 0.5 McFarland turbidity standard (1.5×10^8 colony-forming units/mL) at 600 nm. Clear aligner materials were adjusted sterilely in a laboratory environment according to the size of the microwells in 96-well plates and added into the wells, and 10 uL of bacterial suspensions and tryptic soy broth were added to it with an OD of 0.5 McFarland turbidity standard (1.5×10^8 colony-forming units/mL) at 600 nm. The crystal violet staining method was used to check the effects of SM and LA standard strains on biofilm activities after their interaction with clear aligner materials; 90 uL of (Oxoid) liquid medium was added. After these procedures were performed on different plates, the microwell plates were incubated at 37°C in a carbon dioxide oven for up to 240 hours, and the data for the amount of biofilm was recorded at 0, 24, 48, 72, 96, 120, 168 and 240 hours spectrophotometrically. After the incubations, the liquid in the microwells was drained, and 100 µL of 0.4% crystal violet was added. It was incubated for 15 minutes. The microwells were washed twice, and 95% alcohol was added to the microwells to dissolve the biofilm formed after bacterial interactions. The 96-microwell plates were scanned at 540 nm wavelength (OD 540) using an Epoch spectrophotometer (BioTek Instruments, Winooski, Vt), and the turbidity in the microwells was measured and compared with 0-hour results.[30] All tests were repeated 3 times. All operations performed are shown in detail in Figure 1.

Statistical analysis

SPSS software (version 25, IBM, Armonk, NY) was used for all statistical evaluations. Statistical significance was set at $P < 0.05$. In this study, the mean, standard deviation, minimum, maximum, and median values are given as descriptive statistics data. As the first step of the statistical analysis, the assumption of normality was checked with the Shapiro-Wilk test. To decide on the statistic to be considered, homogeneity of variance was checked with Levene's test, and the assumption of sphericity was checked with Mauchly's W test. Two-way repeated measures analysis of variance (Greenhouse-Geisser) test was performed to examine the difference among the averages of the 3 dependent groups, in which the normality assumption was met, with the interaction effect. Post-hoc Bonferroni T2 tests were used to reveal the group or groups that made the difference.

RESULTS

Intraclass correlation coefficients were calculated as a weighted kappa score of 0.90 (range, 0.91-0.88). When comparing the SM biofilm formation on different orthodontic aligners during the 0-240 hour observation period, in the tests performed for ClearCorrect, Smartee, Orthero, Clarity, Graphy, and Invisalign materials, statistically significant differences were detected among the measurements obtained at all times, except for the difference between the 0 and 24-hour measurements

Table II. Bonferroni test results for comparison of measurements over time for aligner materials in the SM group

Time	*0 h*	*24 h*	*48 h*	*72 h*	*96 h*	*120 h*	*168 h*
ClearCorrect							
24 h	0.422						
48 h	0.027	1.000					
72 h	<0.001	<0.001	<0.001				
96 h	<0.001	<0.001	<0.001	<0.001			
120 h	<0.001	<0.001	<0.001	<0.001	<0.001		
168 h	<0.001	<0.001	<0.001	<0.001	<0.001		
240 h	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	
Smartee							
24 h	1.000						
48 h	0.001	0.001					
72 h	<0.001	<0.001	<0.001				
96 h	<0.001	<0.001	<0.001	<0.001			
120 h	<0.001	<0.001	<0.001	<0.001	<0.001		
168 h	<0.001	<0.001	<0.001	<0.001	<0.001	0.001	
240 h	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Orthero							
24 h	0.781						
48 h	<0.001	<0.001					
72 h	<0.001	<0.001	<0.001				
96 h	<0.001	<0.001	<0.001	<0.001			
120 h	<0.001	<0.001	<0.001	<0.001	<0.001		
168 h	<0.001	<0.001	<0.001	<0.001	<0.001	0.005	
240 h	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.003
Clarity							
24 h	1.000						
48 h	0.001	<0.001					
72 h	<0.001	<0.001	<0.001				
96 h	<0.001	<0.001	<0.001	<0.001			
120 h	<0.001	<0.001	<0.001	<0.001	<0.001		
168 h	<0.001	<0.001	<0.001	<0.001	<0.001	0.035	
240 h	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.030
Graphy							
24 h	1.000						
48 h	<0.001	<0.001					
72 h	<0.001	<0.001	<0.001				
96 h	<0.001	<0.001	<0.001	<0.001			
120 h	<0.001	<0.001	<0.001	<0.001	<0.001		
168 h	<0.001	<0.001	<0.001	<0.001	<0.001	0.022	
240 h	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.002
Invisalign							
24 h	1.000						
48 h	<0.001	<0.001					
72 h	<0.001	<0.001	<0.001				
96 h	<0.001	<0.001	<0.001	<0.001			
120 h	<0.001	<0.001	<0.001	<0.001	<0.001		
168 h	<0.001	<0.001	<0.001	<0.001	<0.001	0.001	
240 h	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

(P <0.05). In all materials, measurement averages increase as time progresses. Statistically significant differences were observed between the Graphy and Invisalign groups at 24 hours (P <0.05). It was found that the SM formed statistically significantly more biofilm on ClearCorrect than Smartee, Orthero, and Invisalign at 168 hours (P <0.05). It was found that the SM formed significantly more biofilm on Invisalign than on Clarity and Orthero at 240 hours (P <0.05). A comparison of the amount of biofilm formation after the interaction of SM and different orthodontic aligners in an in vitro environment over time is shown in Table I and Figure 2. Post-hoc Bonferroni test results applied to compare measurements over time for aligners in the SM group are given in Table II. Post-hoc Bonferroni test results applied to compare measurements according to aligners

Table III. Bonferroni test results for comparison of measurements according to materials for different times in the SM group

Material	*ClearCorrect*	*Smartee*	*Orthero*	*Clarity*	*Graphy*
0 h					
Smartee	1.000				
Orthero	0.012	0.001			
Clarity	0.535	0.018			
Graphy	0.335	0.011	1.000		
Invisalign	1.000	0.130	0.125	1.000	1.000
24 h					
Smartee	1.000				
Orthero	<0.001	<0.001			
Clarity	<0.001	<0.001	1.000		
Graphy	<0.001	<0.001	1.000	1.000	
Invisalign	0.010	0.001	0.007	0.221	0.073
48 h					
Smartee	0.011				
Orthero	1.000	0.004			
Clarity	1.000	0.006	1.000		
Graphy	1.000	0.167	0.821	1.000	
Invisalign	0.194	1.000	0.060	0.103	1.000
72 h					
Smartee	0.654				
Orthero	1.000	0.171			
Clarity	0.638	1.000	0.167		
Graphy	0.009	0.523	0.003	0.536	
Invisalign	0.006	0.368	0.002	0.377	1.000
96 h					
Smartee	0.004				
Orthero	1.000	0.002			
Clarity	0.941	<0.001	1.000		
Graphy	1.000	<0.001	1.000	1.000	
Invisalign	1.000	<0.001	1.000	1.000	1.000
120 h					
Smartee	0.001				
Orthero	1.000	0.002			
Clarity	0.283	<0.001	0.081		
Graphy	0.070	<0.001	0.020	1.000	
Invisalign	0.019	1.000	0.065	<0.001	<0.001
168 h					
Smartee	0.001				
Orthero	0.036	0.365			
Clarity	0.520	0.026	1.000		
Graphy	1.000	0.006	0.641	1.000	
Invisalign	0.005	1.000	1.000	0.379	0.083
240 h					
Smartee	1.000				
Orthero	0.001	0.001			
Clarity	0.001	0.002	1.000		
Graphy	0.370	0.473	0.084	0.103	
Invisalign	1.000	1.000	<0.001	<0.001	0.093

for different times in the SM group are given in Table III. When the LA biofilm formation on different aligners was compared over the 0-240 hour observation period, statistically significant differences were found between

Table IV. Comparison of the amount of biofilm formation after interaction of LA and different orthodontic aligners in vitro environment over time

Material	*Range*	*Mean ± SD*	*Median*
0 h			
ClearCorrect	1.21-1.56	1.33 ± 0.20	1.23
Smartee	1.51-1.75	1.59 ± 0.14	1.52
Orthero	0.94-1.32	1.13 ± 0.19	1.14
Clarity	0.94-1.25	1.07 ± 0.16	1.01
Graphy	0.71-0.99	0.87 ± 0.14	0.91
Invisalign	1.11-1.42	1.22 ± 0.18	1.12
24 h			
ClearCorrect	1.53-1.78	1.69 ± 0.14	1.76
Smartee	1.77-2.01	1.87 ± 0.12	1.84
Orthero	1.24-1.39	1.30 ± 0.08	1.28
Clarity	1.19-1.31	1.26 ± 0.06	1.28
Graphy	0.91-1.23	1.11 ± 0.17	1.18
Invisalign	1.28-1.41	1.34 ± 0.07	1.34
48 h			
ClearCorrect	1.84-2.12	1.96 ± 0.15	1.91
Smartee	2.39-2.51	2.46 ± 0.06	2.47
Orthero	1.64-1.82	1.74 ± 0.09	1.75
Clarity	1.72-2.02	1.89 ± 0.15	1.93
Graphy	1.73-1.81	1.77 ± 0.04	1.78
Invisalign	2.09-2.23	2.15 ± 0.07	2.12
72 h			
Clearcorrect	3.87-4.24	4.01 ± 0.20	3.91
Smartee	4.12-4.74	4.49 ± 0.33	4.62
Orthero	3.16-4.42	3.98 ± 0.71	4.36
Clarity	4.15-4.40	4.29 ± 0.13	4.32
Graphy	4.78-4.91	4.86 ± 0.07	4.89
Invisalign	5.14-5.43	5.32 ± 0.16	5.40
96 h			
ClearCorrect	6.98-7.12	7.04 ± 0.07	7.01
Smartee	6.24-6.83	6.48 ± 0.31	6.36
Orthero	7.36-7.78	7.56 ± 0.21	7.54
Clarity	7.93-8.35	8.13 ± 0.21	8.12
Graphy	7.99-8.34	8.15 ± 0.18	8.12
Invisalign	8.14-8.43	8.31 ± 0.15	8.37
120 h			
ClearCorrect	12.01-12.53	12.32 ± 0.27	12.41
Smartee	11.20-11.84	11.46 ± 0.34	11.34
Orthero	12.42-12.95	12.73 ± 0.28	12.82
Clarity	12.50-13.05	12.82 ± 0.29	12.91
Graphy	12.40-14.60	13.57 ± 1.11	13.70
Invisalign	11.27-13.35	12.06 ± 1.12	11.57
168 h			
ClearCorrect	15.98-16.48	16.30 ± 0.28	16.43
Smartee	12.95-13.92	13.49 ± 0.49	13.59
Orthero	14.95-15.52	15.22 ± 0.29	15.20
Clarity	14.09-15.40	14.90 ± 0.71	15.21
Graphy	15.09-16.45	15.91 ± 0.72	16.20
Invisalign	14.05-14.55	14.37 ± 0.28	14.51
240 h			
ClearCorrect	19.71-20.72	20.08 ± 0.55	19.82
Smartee	18.71-20.19	19.60 ± 0.78	19.89
Orthero	18.20-19.52	18.76 ± 0.68	18.55
Clarity	17.84-18.12	18.02 ± 0.15	18.09

Table IV. Continued

Material	*Range*		*Mean ± SD*		*Median*
Graphy	18.79-20.43		19.65 ± 0.82		19.73
Invisalign	20.34-20.75		20.53 ± 0.21		20.50
Source	*Type 3 SS*	*DF*	*MS*	F *value*	P *value*
Time	6090.895	2.639	2308.452	5189.814	<0.001*
Material × time	45.101	13.193	3.419	7.686	<0.001*
Error (time)	14.084	31.662	0.445		

SD, standard deviation; *SS*, sum of squares; *DF*, degrees of freedom; *MS*, mean squares.
*Statistical significance (P <0.05).

the measurements obtained at all times except at 24 and 48 hours in the ClearCorrect group (P <0.05). In all materials, measurement averages increase as time progresses. It was found that LA bacteria formed statistically significantly more biofilm on Invisalign than on Clarity and Orthero at 240 hours (P <0.05). The effects of aligners on LA biofilm formation are shown in Table IV and Figure 3.

Post-hoc Bonferroni test results applied to compare measurements over time for aligners in the LA group are given in Table V. Post-hoc Bonferroni test results applied to compare measurements according to aligners for different times in the LA group are given in Table VI. Considering the ability of SM and LA to form biofilms together on different aligners, more bacterial formation occurred on ClearCorrect than on Smartee at 120, 168, and 240 hours (P <0.05). It was found that more biofilm was formed at 168 hours on Graphy than on Smartee (P <0.05). It was found that SM + LA formed more biofilm at 120 and 168 hours on Graphy than on Invisalign (P <0.05). The effects of all aligners on the biofilm formation of SM and LA together are shown in Table VII and Figure 4. Post Hoc Bonferroni test results applied to compare measurements over time for aligners in the SM + LA group are given in Table VIII. Post-hoc Bonferroni test results applied to compare measurements according to aligners for different times in the SM + LA group are given in Table IX.

DISCUSSION

Orthodontic treatment has undergone significant transformations, with an increasing focus on esthetic alternatives. CAT has gained popularity as a discreet orthodontic option involving the use of clear aligners with diverse material compositions.[7] This study aimed to compare and evaluate the time-dependent biofilm formation and microbial adhesion on 6 different clear aligner systems, namely Invisalign, Clarity, ClearCorrect, Smartee, Orthero, and Graphy. The results revealed significant differences in microbial adhesion and biofilm formation among these aligner systems, shedding light on the potential impact of aligner material and design.

The choice of aligner material is crucial in orthodontic treatment, as it directly influences clinical performance and patient outcomes. The materials used in clear aligners vary, including thermoplastic polymers, polymer blends, 3D-printed aligner materials, and bioactive materials. Each material has distinct properties that may affect its susceptibility to microbial adhesion and biofilm formation.[16,24-27] Understanding these differences is essential for orthodontists and patients when selecting the most suitable aligner system. The observed differences highlight the importance of considering not only the material but also the design and production method of clear aligners. For instance, direct printed aligners, such as Graphy, have gained popularity in recent years. The unique properties of Graphy aligners, being transparent and elastic resin-based materials printed in 100-μm layers, may contribute to differences in bacterial adhesion compared with aligners produced using traditional methods.[13]

The formation of biofilm, which has a microbially cosmopolitan structure, is a complex process, and often, >1 species and a number of microorganisms are involved in this formation process.[21,27] The study used 2 standard bacterial strains, SM and LA, to assess microbial adhesion and biofilm formation. These bacteria are commonly found in the oral cavity and are associated with dental caries and plaque formation.[18-23] Although SM and other species are no longer recommended to be used individually for the assessment of biofilm and microbial colonization, oral *streptococci*, including SM, are one of the earliest oral colonizing species that can be acquired soon after birth. It is a bacterium with multiple virulence factors that can contribute to dental caries and biofilm formation, and *in vitro* or clinical study data on biofilm production by this bacterium is valuable, as it can adhere to *Candida albicans*, enabling it to enhance the production of extracellular polysaccharides-rich matrix in biofilms with *C*

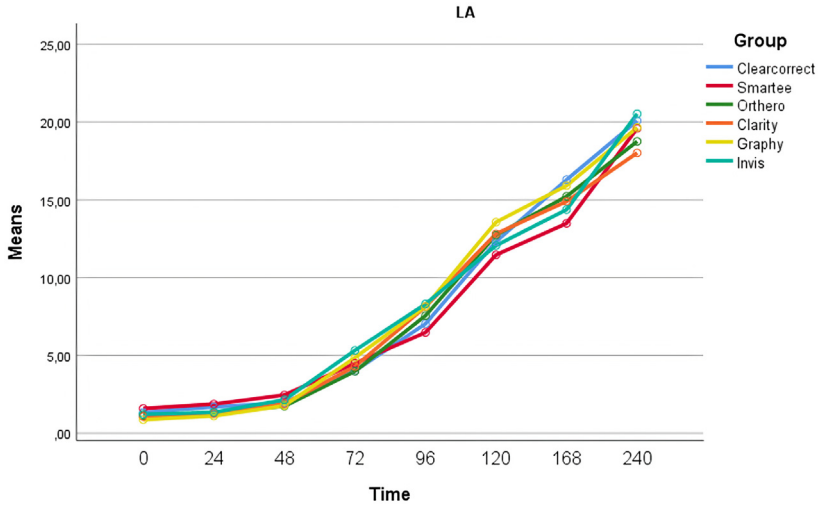

Fig 3. Line chart of measurements in the LA group according to different times and aligners.

albicans.[31] ClearCorrect showed higher bacterial formation than Smartee at certain time points, and Graphy exhibited increased biofilm formation than Smartee at 168 hours. This study's results also demonstrated that the combination of SM and LA led to increased biofilm formation on ClearCorrect than on Smartee at certain time points. Similarly, Graphy showed higher biofilm formation than Invisalign at specific hours when both bacteria were present. This finding emphasizes the importance of studying biofilm formation with multiple bacterial strains, as the oral environment is a complex ecosystem with various microbial species.[21]

Although the contact with the tissue is interrupted when the aligners, which are recommended to be used for 20-22 h/d for 7-10 days, are removed, the attachments remain on the tooth surface continuously, causing the formation of an extra adhesion surface. Therefore, in our study, biofilm formation and microbial adhesion were evaluated for 240 hours. In a study, the effect of SM on surface adhesion was not significantly affected by the coating of the surface with saliva, but it was found that there was a significant correlation between surface roughness and free surface energy properties. Because previous studies have shown that the adhesion patterns of SM and LA are not significantly affected by coating with saliva, saliva was not used to coat the samples in our study.[32-34]

Notably, measurement averages consistently increased over time for all materials. This temporal pattern underscores a crucial point—the prolonged use of any aligner may contribute to increased biofilm formation. Therefore, the study findings imply that, over time, the microbial load on aligner surfaces tends to rise, suggesting potential implications for long-term aligner treatment.[30] Understanding the temporal increase in biofilm formation across all materials has significant clinical implications. Aligners can deteriorate over time. After the incubation period, deformation of the aligners may occur. Anecdotal evidence has shown that aligners tend to deteriorate and deform after regular use. This can result in increased surface porosity or microscopic valleys and grooves. Bacteria settle into and adhere to materials that are better able to maintain their physical shape. Orthodontists and patients need to be cognizant of the potential for elevated microbial colonization with prolonged aligner use. Emphasizing meticulous oral hygiene practices and regular checkups becomes paramount in mitigating these effects.[35]

There are some limitations in our study. These 2 microbial structures, which are one of the important

Table V. Bonferroni test results for comparison of measurements over time for aligner materials in the LA group

Time	*0 h*	*24 h*	*48 h*	*72 h*	*96 h*	*120 h*	*168 h*
ClearCorrect							
24 h	0.121						
48 h	0.004	0.342					
72 h	<0.001	<0.001	<0.001				
96 h	<0.001	<0.001	<0.001	<0.001			
120 h	<0.001	<0.001	<0.001	<0.001	<0.001		
168 h	<0.001	<0.001	<0.001	<0.001	<0.001	0.001	
240 h	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Smartee							
24 h	0.490						
48 h	<0.001	0.001					
72 h	<0.001	<0.001	<0.001				
96 h	<0.001	<0.001	<0.001	<0.001			
120 h	<0.001	<0.001	<0.001	<0.001	<0.001		
168 h	<0.001	<0.001	<0.001	<0.001	<0.001	0.143	
240 h	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Orthero							
24 h	1.000						
48 h	0.005	0.012					
72 h	<0.001	<0.001	<0.001				
96 h	<0.001	<0.001	<0.001	<0.001			
120 h	<0.001	<0.001	<0.001	<0.001	<0.001		
168 h	<0.001	<0.001	<0.001	<0.001	<0.001	0.034	
240 h	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Clarity							
24 h	1.000						
48 h	<0.001	<0.001					
72 h	<0.001	<0.001	<0.001				
96 h	<0.001	<0.001	<0.001	<0.001			
120 h	<0.001	<0.001	<0.001	<0.001	<0.001		
168 h	<0.001	<0.001	<0.001	<0.001	<0.001	0.121	
240 h	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Graphy							
24 h	1.000						
48 h	<0.001	<0.001					
72 h	<0.001	<0.001	<0.001				
96 h	<0.001	<0.001	<0.001	<0.001			
120 h	<0.001	<0.001	<0.001	<0.001	<0.001		
168 h	<0.001	<0.001	<0.001	<0.001	<0.001	0.053	
240 h	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Invisalign							
24 h	1.000						
48 h	<0.001	<0.001					
72 h	<0.001	<0.001	<0.001				
96 h	<0.001	<0.001	<0.001	<0.001			
120 h	<0.001	<0.001	<0.001	<0.001	<0.001		
168 h	<0.001	<0.001	<0.001	<0.001	<0.001	0.060	
240 h	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Table VI. Bonferroni test results for comparison of measurements according to materials for different times in the LA group

Material	*ClearCorrect*	*Smartee*	*Orthero*	*Clarity*	*Graphy*
0 h					
Smartee	1.000				
Orthero	1.000	0.090			
Clarity	1.000	0.037	1.000		
Graphy	0.086	0.003	1.000	1.000	
Invisalign	1.000	0.275	1.000	1.000	0.410
24 h					
Smartee	1.000				
Orthero	0.020	0.001			
Clarity	0.009	<0.001	1.000		
Graphy	0.001	<0.001	0.851	1.000	
Invisalign	0.044	0.002	1.000	1.000	0.391
48 h					
Smartee	0.001				
Orthero	0.342	<0.001			
Clarity	1.000	<0.001	1.000		
Graphy	0.756	<0.001	1.000	1.000	
Invisalign	0.656	0.047	0.006	0.153	0.012
72 h					
Smartee	1.000				
Orthero	1.000	1.000			
Clarity	1.000	1.000	1.000		
Graphy	0.149	1.000	0.125	0.956	
Invisalign	0.007	0.174	0.006	0.045	1.000
96 h					
Smartee	0.081				
Orthero	0.122	<0.001			
Clarity	<0.001	<0.001	0.070		
Graphy	<0.001	<0.001	0.058	1.000	
Invisalign	<0.001	<0.001	0.010	1.000	1.000
120 h					
Smartee	1.000				
Orthero	1.000	0.644			
Clarity	1.000	0.481	1.000		
Graphy	0.687	0.041	1.000	1.000	
Invisalign	1.000	1.000	1.000	1.000	0.301
168 h					
Smartee	<0.001				
Orthero	0.332	0.017			
Clarity	0.077	0.071	1.000		
Graphy	1.000	0.001	1.000	0.435	
Invis	0.008	0.774	0.882	1.000	0.040
240 h					
Smartee	1.000				
Orthero	0.276	1.000			
Clarity	0.017	0.105	1.000		
Graphy	1.000	1.000	1.000	0.086	
Invisalign	1.000	1.000	0.050	0.004	1.000

Table VII. Comparison of the amount of biofilm formation after interaction with SM + LA strains with different orthodontic aligners in vitro environment over time

Material	*Range*	Mean ± SD	*Median*
0 h			
ClearCorrect	1.12-1.51	1.37 ± 0.22	1.49
Smartee	1.63-1.97	1.80 ± 0.17	1.79
Orthero	1.09-1.34	1.23 ± 0.13	1.25
Clarity	1.01-1.31	1.15 ± 0.15	1.12
Graphy	0.87-1.02	0.95 ± 0.08	0.96
Invisalign	0.72-1.34	1.10 ± 0.34	1.25
24 h			
ClearCorrect	1.59-1.73	1.65 ± 0.07	1.63
Smartee	1.87-2.04	1.96 ± 0.09	1.98
Orthero	1.14-1.41	1.30 ± 0.14	1.36
Clarity	1.21-1.36	1.27 ± 0.08	1.24
Graphy	1.07-1.24	1.14 ± 0.09	1.12
Invisalign	1.09-1.26	1.16 ± 0.09	1.14
48 h			
ClearCorrect	1.85-2.03	1.96 ± 0.10	2.01
Smartee	2.41-2.83	2.62 ± 0.21	2.61
Orthero	1.49-1.71	1.61 ± 0.11	1.62
Clarity	1.59-2.03	1.82 ± 0.22	1.85
Graphy	1.89-2.01	1.95 ± 0.06	1.96
Invisalign	2.06-2.32	2.17 ± 0.13	2.14
72 h			
ClearCorrect	3.69-3.81	3.75 ± 0.06	3.76
Smartee	4.23-4.61	4.45 ± 0.20	4.52
Orthero	3.17-3.81	3.54 ± 0.33	3.64
Clarity	4.02-4.58	4.24 ± 0.30	4.12
Graphy	4.69-5.01	4.86 ± 0.16	4.87
Invisalign	5.13-5.47	5.30 ± 0.17	5.3
96 h			
ClearCorrect	7.30-7.81	7.48 ± 0.28	7.34
Smartee	6.01-6.43	6.19 ± 0.22	6.12
Orthero	7.46-7.98	7.66 ± 0.28	7.54
Clarity	7.86-8.34	8.12 ± 0.24	8.17
Graphy	8.41-8.64	8.49 ± 0.13	8.43
Invisalign	8.13-8.99	8.49 ± 0.45	8.35
120 h			
ClearCorrect	11.99-12.86	12.50 ± 0.45	12.65
Smartee	10.76-11.12	10.95 ± 0.18	10.98
Orthero	12.58-13.64	13.10 ± 0.53	13.08
Clarity	12.57-13.01	12.80 ± 0.22	12.81
Graphy	14.31-14.60	14.48 ± 0.15	14.52
Invisalign	11.07-12.18	11.68 ± 0.56	11.78
168 h			
ClearCorrect	15.82-16.73	16.36 ± 0.48	16.54
Smartee	13.54-14.52	13.88 ± 0.55	13.59
Orthero	14.69-15.39	15.13 ± 0.39	15.32
Clarity	14.83-16.01	15.46 ± 0.59	15.55
Graphy	15.55-17.39	16.29 ± 0.97	15.93
Invisalign	14.53-15.42	15.06 ± 0.47	15.24
240 h			
ClearCorrect	19.81-21.09	20.35 ± 0.66	20.16
Smartee	18.67-20.19	19.55 ± 0.79	19.78
Orthero	18.51-19.24	18.87 ± 0.37	18.86
Clarity	17.69-18.64	18.12 ± 0.48	18.03

Table VII. Continued

Material	*Range*		Mean ± SD		*Median*
Graphy	18.93-20.10		19.46 ± 0.59		19.35
Invisalign	19.31-19.41		19.35 ± 0.05		19.34
Source	*Type 3 SS*	*DF*	*MS*	F *value*	P *value*
Time	6126.529	3.354	1826.422	7221.226	<0.001*
Material × time	56.049	16.772	3.342	13.213	<0.001*
Error (time)	10.181	40.253	0.253		

SD, standard deviation; *SS*, sum of squares; *DF*, degrees of freedom; *MS*, mean squares.
*Statistical significance (P <0.05).

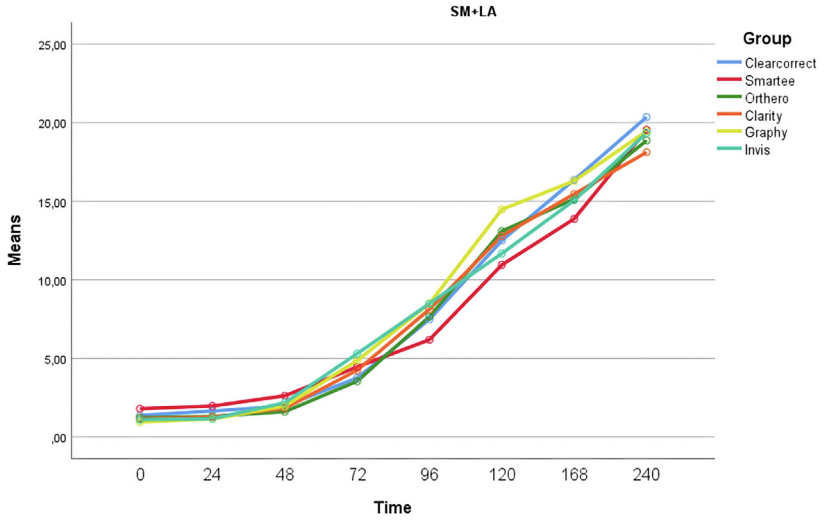

Fig 4. Line chart of measurements in the SM + LA group according to different times and aligners.

factors in the formation of caries and periodontal problems, were evaluated both separately and together. One limitation is the use of standard bacterial strains instead of the entire oral microbiota. Although it is known that the addition of saliva does not produce significant binding results when adhesion studies are evaluated, this study conducted *in vitro* may not fully reflect the dynamic and complex conditions of the oral cavity. Another limitation is that clinical factors such as the patient's oral hygiene practices and diet were not taken into account in this experimental setup. Another limitation to consider is the surface roughness and texture of the aligners. Future studies should examine the surface roughness and texturing of

Table VIII. Bonferroni test results for comparison of measurements over time for aligner materials in the SM + LA group

Time	*0 h*	*24 h*	*48 h*	*72 h*	*96 h*	*120 h*	*168 h*
ClearCorrect							
24 h	0.753						
48 h	0.048	<0.001					
72 h	<0.001	<0.001	<0.001				
96 h	<0.001	<0.001	<0.001	<0.001			
120 h	<0.001	<0.001	<0.001	<0.001	<0.001		
168 h	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	
240 h	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Smartee							
24 h	1.000						
48 h	0.003	<0.001					
72 h	<0.001	<0.001	<0.001				
96 h	<0.001	<0.001	<0.001	<0.001			
120 h	<0.001	<0.001	<0.001	<0.001	<0.001		
168 h	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	
240 h	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Orthero							
24 h	1.000						
48 h	0.664	0.003					
72 h	<0.001	<0.001	<0.001				
96 h	<0.001	<0.001	<0.001	<0.001			
120 h	<0.001	<0.001	<0.001	<0.001	<0.001		
168 h	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	
240 h	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Clarity							
24 h	1.000						
48 h	0.001	<0.001					
72 h	<0.001	<0.001	<0.001				
96 h	<0.001	<0.001	<0.001	<0.001			
120 h	<0.001	<0.001	<0.001	<0.001	<0.001		
168 h	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	
240 h	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Graphy							
24 h	1.000						
48 h	0.001	<0.001					
72 h	<0.001	<0.001	<0.001				
96 h	<0.001	<0.001	<0.001	<0.001			
120 h	<0.001	<0.001	<0.001	<0.001	<0.001		
168 h	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	
240 h	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Invisalign							
24 h	1.000						
48 h	<0.001	<0.001					
72 h	<0.001	<0.001	<0.001				
96 h	<0.001	<0.001	<0.001	<0.001			
120 h	<0.001	<0.001	<0.001	<0.001	<0.001		
168 h	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	
240 h	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Table IX. Bonferroni test results for comparison of measurements according to materials for different times in the SM + LA group

Material	*Clearcorrect*	*Smartee*	*Orthero*	*Clarity*	*Graphy*
0 h					
Smartee	0.333				
Orthero	1.000	0.062			
Clarity	1.000	0.025	1.000		
Graphy	0.333	0.003	1.000	1.000	
Invisalign	1.000	0.016	1.000	1.000	1.000
24 h					
Smartee	0.026				
Orthero	0.012	<0.001			
Clarity	0.006	<0.001	1.000		
Graphy	<0.001	<0.001	0.943	1.000	
Invisalign	0.001	<0.001	1.000	1.000	1.000
48 h					
Smartee	0.003				
Orthero	0.201	<0.001			
Clarity	1.000	<0.001	1.000		
Graphy	1.000	0.002	0.234	1.000	
Invisalign	1.000	0.055	0.009	0.222	1.000
72 h					
Smartee	0.034				
Orthero	1.000	0.004			
Clarity	0.301	1.000	0.034		
Graphy	0.001	0.695	<0.001	0.080	
Invisalign	<0.001	0.008	<0.001	0.001	0.466
96 h					
Smartee	0.002				
Orthero	1.000	0.001			
Clarity	0.256	<0.001	1.000		
Graphy	0.014	<0.001	0.054	1.000	
Invisalign	0.014	<0.001	0.056	1.000	1.000
120 h					
Smartee	0.006				
Orthero	1.000	<0.001			
Clarity	1.000	0.001	1.000		
Graphy	0.001	<0.001	0.015	0.003	
Invisalign	0.353	0.627	0.011	0.062	<0.001
168 h					
Smartee	0.005				
Orthero	0.429	0.399			
Clarity	1.000	0.116	1.000		
Graphy	1.000	0.006	0.563	1.000	
Invisalign	0.331	0.516	1.000	1.000	0.435
240 h					
Smartee	1.000				
Orthero	0.088	1.000			
Clarity	0.004	0.112	1.000		
Graphy	1.000	1.000	1.000	0.161	
Invisalign	0.658	1.000	1.000	0.251	1.000

aligners, as these factors vary among different brands and may play a role in microbial adhesion and biofilm formation.

Despite all this, we hope that evaluating the aligner materials, which are frequently used in our clinical practice under the product name in terms of their effects on microbial adhesion and plaque formation, will contribute to our clinical practice.

CONCLUSIONS

In conclusion, this comparative study provides valuable insights into the biofilm formation and microbial adhesion on different clear aligner systems. The results suggest that the choice of aligner material and design may influence the oral microenvironment and bacterial adhesion. However, the temporal increase in measurement averages across all materials highlights the importance of considering the potential for increased biofilm with prolonged aligner use, and further research is needed to explore the clinical implications of these findings.

AUTHOR CREDIT STATEMENT

Aylin Pasaoglu Bozkurt contributed to conceptualization, investigation, methodology, project administration, resources, software, supervision, validation, visualization, original draft preparation, and manuscript review and editing; Mehmet Demirci contributed to conceptualization, data curation, formal analysis, methodology, software, visualization, original draft preparation, and manuscript review and editing; Pelin Erdogan contributed to conceptualization, investigation, methodology, resources, software, visualization, original draft preparation, and manuscript review and editing; and Emre Kayalar contributed to conceptualization, investigation, methodology, resources, software, visualization, PhD supervision, original draft preparation, and manuscript review and editing.

REFERENCES

1. Kaklamanos EG, Makrygiannakis MA, Athanasiou AE. Oral Health-related quality of life throughout treatment with clear aligners in comparison to conventional metal fixed orthodontic appliances: a systematic review. Int J Environ Res Public Health 2023;20:3537.
2. Iliadi A, Koletsi D, Papageorgiou SN, Eliades T. Safety considerations for thermoplastic-type appliances used as orthodontic aligners or retainers. A systematic review and meta-analysis of clinical and in-vitro research. Materials (Basel) 2020;13:1843.
3. Bichu YM, Alwafi A, Liu X, Andrews J, Ludwig B, Bichu AY, et al. Advances in orthodontic clear aligner materials. Bioact Mater 2023;22:384-403.
4. Macrì M, Murmura G, Varvara G, Traini T, Festa F. Clinical performances and biological features of clear aligners materials in orthodontics. Front Mater 2022;9:819121.
5. Kundal S, Shokeen T. Aligners: the science of clear orthodontics. Int J Dent Med Spec 2020;7:38-42.
6. Zheng M, Liu R, Ni Z, Yu Z. Efficiency, effectiveness and treatment stability of clear aligners: A systematic review and meta-analysis. Orthod Craniofac Res 2017;20:127-33.
7. Gold BP, Siva S, Duraisamy S, Idaayath A, Kannan R. Properties of orthodontic clear aligner materials – a review. J Evol Med Dent Sci 2021;10:3288-94.
8. Invisalign. Available at: https://www.aligntech.com/solutions. Accessed December 12, 2023.
9. Clarity. Available at: https://www.3m.com/3M/en_US/orthodontics-us/featured-products/clarity-eos/clear-aligners/. Accessed November 5, 2023.
10. Clear Correct. Available at: https://www.straumann.Com/Clearcorrect/En/Patients/about-the-aligners.HTML. Accessed December 26, 2023.
11. Smartee. Available at: https://www.smartee.uk/technology. Accessed November 24, 2023.
12. Orthero. Available at: https://orthero.com.tr/doktor/. Accessed December 17, 2023.
13. Graphy. Available at: http://www.itgraphy.com/ENG/. Accessed November 9, 2023.
14. Rossini G, Parrini S, Castroflorio T, Deregibus A, Debernardi CL. Periodontal health during clear aligners treatment: a systematic review. Eur J Orthod 2015;37:539-43.
15. Crego-Ruiz M, Jorba-García A. Assessment of the periodontal health status and gingival recession during orthodontic treatment with clear aligners and fixed appliances: a systematic review and meta-analysis. Med Oral Patol Oral Cir Bucal 2023;28:e330-40.
16. Santonocito S, Polizzi A. Oral microbiota changes during orthodontic treatment. Front Biosci (Elite Ed) 2022;14:19.
17. Kilian M, Chapple IL, Hannig M, Marsh PD, Meuric V, Pedersen AM, et al. The oral microbiome – an update for oral healthcare professionals. Br Dent J 2016;221:657-66.
18. Wang Q, Ma JB, Wang B, Zhang X, Yin YL, Bai H. Alterations of the oral microbiome in patients treated with the Invisalign system or with fixed appliances. Am J Orthod Dentofacial Orthop 2019;156:633-40.
19. Lucchese A, Bondemark L, Marcolina M, Manuelli M. Changes in oral microbiota due to orthodontic appliances: a systematic review. J Oral Microbiol 2018;10:1476645.
20. Papageorgiou SN, Xavier GM, Cobourne MT, Eliades T. Effect of orthodontic treatment on the subgingival microbiota: a systematic review and meta-analysis. Orthodo Craniofac Res 2018;21:175-85.
21. Tektas S, Thurnheer T, Eliades T, Attin T, Karygianni L. Initial bacterial adhesion and biofilm formation on aligner materials. Antibiotics (Basel) 2020;9:908.
22. Thurnheer T, Bostanci N, Belibasakis GN. Microbial dynamics during conversion from supragingival to subgingival biofilms in an in vitro model. Mol Oral Microbiol 2016;31:125-35.
23. Eliades T, Eliades G, Brantley WA. Microbial attachment on orthodontic appliances: I. Wettability and early pellicle formation on bracket materials. Am J Orthod Dentofacial Orthop 1995;108:351-60.
24. Low B, Lee W, Seneviratne CJ, Samaranayake LP, Hägg U. Ultrastructure and morphology of biofilms on thermoplastic

orthodontic appliances in 'fast' and 'slow' plaque formers. Eur J Orthod 2011;33:577-83.
25. Magno AFF, Enoki C, Ito IY, Matsumoto MAN, Faria G, Nelso-Filho P. In-vivo evaluation of the contamination of super sick elastometic rings by streptococcus mutans in orthodontic patients. Am J Orthod Dentofacial Orthop 2008;133:104.e9.
26. Bozkurt AP, Ünlü Ö, Demirci M. Comparison of microbial adhesion and biofilm formation on orthodontic wax materials; an in vitro study. J Dent Sci 2020;15:493-9.
27. Baybekov O, Stanishevskiy Y, Sachivkina N, Bobunova A, Zhabo N, Avdonina M. Isolation of clinical microbial isolates during orthodontic aligner therapy and their ability to form biofilm. Dent J (Basel) 2023;11:13.
28. Xu X, He J, Xue J, Wang Y, Li K, Zhang K, et al. Oral cavity contains distinct niches with dynamic microbial communities. Environ Microbiol 2015;17:699-710.
29. Jabur SF. Influence of removable orthodontic appliance on oral microbiological status. JFacMedBagdad 2008;50:199-202.
30. Sun Y, Chen S, Zhang C, Liu Y, Ma L, Zhang X. Effects of subminimum inhibitory concentrations of lemon essential oil on the acid tolerance and biofilm formation of streptococcus mutans. Arch Oral Biol 2018;87:235-41.
31. Liu T, Liu J, Liu J, Yang R, Lu X, He X, et al. Interspecies interactions between streptococcus mutans and streptococcus agalactiae in vitro. Front Cell Infect Microbiol 2020;10:344.
32. Lee SP, Lee SJ, Lim BS, Ahn SJ. Surface characteristics of or- thodontic materials and their effects on adhesion of mutans streptococci. Angle Orthod 2009;79:353.e60.
33. Ahn SJ, Lim BS, Lee SJ. Surface characteristics of orthodontic adhesives and effects on streptococcal adhesion. Am J Orthod Dentofacial Orthop 2010;137:489-95.
34. Velazquez-Enriquez U, Scougall-Vilchis RJ, Contreras-Bulnes R, Flores-Estrada J, Uematsu S, Yamaguchi R. Quantitative analysis of S. mutans and S. sobrinus cultivated independently and adhered to polished orthodontic composite resins. J Appl Oral Sci 2012;20:544-9.
35. Ihssen BA, Willmann JH, Nimer A, Drescher D. Effect of in vitro aging by water immersion and thermocycling on the mechanical properties of PETG aligner material. J Orofac Orthop 2019;80:292-303.