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Review Article

Dentistry Section

Microbial Adhesion and Biofilm Formation on Clear Aligners: A Literature Review

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ABSTRACT

Oral cavity is a host for a variety of microorganisms, these microbes tend to adhere to retentive areas in the oral cavity such as deep occlusal grooves on posterior teeth, interdental areas and form biofilm. This leads to the development of dental caries and gingivitis. Patients undergoing fixed appliance orthodontic treatment are more prone for enamel demineralisation caused by adherence of microbial colonies. Clear aligners, which are made of clear thermoplastic material are commonly used in clinical practice recently. Clear aligners are a series of plastic trays which have to be worn for 22 hours a day for effective tooth movement and have to be changed for every two weeks. These aligners cover the tooth completely, making it difficult for the antibacterial effect of saliva to act on the tooth surfaces. Hence, the present review aimed to assess the microbial adhesion and biofilm formation in patients undergoing clear aligner therapy and the effectiveness of various methods to clean aligners.

Keywords: Bacterial adhesion, Dental plaque, Disinfection, Orthodontic appliances

INTRODUCTION

Oral biofilm, also known as dental plaque is an extensive network of bacteria encased in an extracellular substrate generated by microorganisms that mostly cling to gingival epithelial cells or tooth surfaces. It serves as acellular protein-based coating that covers the enamel surface and serves as a site for bacterial attachment, which is regarded as a prerequisite for the bacterial colonisation of the tooth and other oral surfaces [1].

Most common complication of orthodontic appliances are enamel demineralisation and gingivitis [2]. Orthodontic appliances alter and increase the microbiota because they offer areas for bacteria to congregate and make cleaning difficult [3]. Maintaining oral hygiene during orthodontic treatment is critical as orthodontic appliances promote food accumulation and plaque retention. This increases the risk of enamel demineralisation and gingival inflammation [4]. White Spot Lesions (WSL) are consequences of enamel demineralisation caused by acid producing caries-associated bacteria. WSL appear as chalky white opaque enamel. It is an unfavourable complication of orthodontic treatment [5]. The risk of caries is linked to oral microenvironment, and the later plays a pivotal role in the genesis and progression of caries [6].

Orthodontic fixed appliances tend to alter the oral microbiota, with increase in the levels of *Streptococcus mutans* and *Lactobacillus* species, noted especially after bonding [7]. These appliances favour biofilm formation [8,9]. A study by Yan D et al., observed higher levels of *P. gingivalis*, *P. intermedia/Prevotella nigrescens*, *T. forsythia*, and *Fusobacterium* spp. in orthodontic patients three months after orthodontic treatment [9,10]. Similarly, Pan S et al., observed a increase in several periodontopathic bacteria three months after the start of orthodontic therapy [11].

Invisible orthodontic treatment, being preferred by many patients involves the use of clear aligners [12]. These are transparent polymer materials which can be thermoformed or directly printed and are available as a series of trays. These trays are to be changed every two weeks for the predicted tooth movement to take place. Compared with the traditional orthodontic appliances, clear aligners cover all teeth surfaces, which hinder the antibacterial, remineralising potential, and cleansing ability of saliva [13]. Drawbacks of these clear aligners include discolouration, appliance loss, biofilm build-up, the emission of potentially cytotoxic or oestrogenic substances, and the necessity for patient participation [14].

Understanding the biofilm formation and microbial adhesion on tooth surfaces is essential for maintaining good oral health and preventing enamel decalcification. And it is also essential to encourage patients to keep their aligners clean to prevent the development of WSL. Hence, the present review aimed to assess the existing literature evidence on the initial microbial adhesion and biofilm formation on clear aligner materials.

A thorough search was run in PubMed, Scopus and Web of science along with the use of google scholar search engine. Articles published in English and dated from 2010-2024 were included.

The following search strategy was used: (Microbial adhesion OR microbial colonisation) AND (orthodontic treatment OR orthodontic therapy OR clear aligners OR clear aligner therapy).

Eligibility criteria: Studies which have quantified biofilm formation and microbial adhesion in patients treated using clear aligner therapy have been included. Studies that include methods of cleaning or disinfecting aligners have been included in the current review.

Plaque and oral microbiota: There are over 700 distinct types of microorganisms, including both anaerobic and aerobic bacteria [15]. the The gram-positive and gram-negative organisms in the oral cavity are represented in [Table/Fig-1] [16]. Supra gingival plaque is considered to be a causative agent for enamel demineralisation, with the presence of the above bacterial species [17].

DISCUSSION

Bacterial Adhesion

Bacterial adhesion depends on various factors such as duration of exposure, bacterial characteristics, nutrients, and bacterial density [18]. Initially bacteria attach to a surface by vanderwaals forces and electrostatic forces caused by ionic groups [19].

Streptococcus (*S.*) *sanguis* appears to have a preference for colonising on tooth surfaces, whereas *S.salivarius* has a marked affinity for epithelial surfaces and shows little affinity to adhere to teeth. It has been observed that plaque bacteria *S. sanguis*, *S.mitis*, and *Actinomyces* species, but not *S. mutans* or *S. salivarius* aggregate in presence of human saliva. Also, *S. sanguis* and *Actinomyces*

Gram positive organisms	Gram negative organisms
Streptococcus	E. coli
Lactobacillus	Klebsiella
Staphylococcus	Enterobacter
Clostridium	Enterococcus
Bacillus	Citrobacter
Corynebacterium	Vibrio spp.
Actinomyces	Campylobacter
Propionibacterium	Helicobacter pylori
Rothia	Proteus
Eubacterium	Fusobacterium
Gemella	Neisseria
Peptostreptococcus.	Moraxella
Enterococcus faecalis	Kingella
Leuconostocaceae	Actinobacillus
Listeriaceae	Bacteroides
	Porphyromonas
	Prevotella
	Selenomonas
	Cardiobacterium
	Eikenella
	Veillonella
	Leminorella
	Morganella
	Providencia
	Yersinia
	Serratia

[Table/Fig-1]: Microorganisms in the oral cavity.

have been observed to adhere to saliva coated enamel, whereas lactobacilli, *S. mutans* and *S. salivarius* appear to be unaffected or demonstrate little ability to adhere to saliva coated enamel.

Straub H et al., classified bacterial adhesion into three different levels, which includes: (1) specific physical-chemical interactions; (2) Non specific interactions; and (3) surface mechanosensing [Table/Fig-2] [20-22].

Type of interactions	Key features
Specific microscopic interactions	Occur at very short distances between surface components that are stereochemically complimentary; particularly hydrogen, ionic, and may be chemical connections [20]
Non specific interactions	Interactions due to change in surface free energy. These interactions can occur over very long distances, making it impossible for a substratum to discern between different molecules on the cell surface [21]
Mechanosensing	Flagellar appendages of bacteria act as mechanosensors by sensing their rotation inhibition upon surface attachment. Pilus retraction also plays a mechanosensing role [22].

[Table/Fig-2]: Classification of microbial adhesion [20-22].

Methods to test microbial adhesion: The gold standard for quantitative bacterial detection is plate counting, often known as Colony Forming Unit (CFU) counts. However, it takes several days to cultivate bacteria, colony aggregation causes errors in detection and it is impossible to immediately detect bacterial attachment sites; and materials with irregular shapes or varied textures cannot be evaluated as a whole [23]. Alternative methods include Adenosine Triphosphate (ATP) bioluminescence, a non destructive bioluminescence technology, and crystal violet staining. While non destructive bioluminescence technology uses bacteria engineered with the luciferase gene to monitor bioluminescence signals in real-time, ATP bioluminescence assays rely on the oxidation reaction between ATP and fluorescein in living cells to emit fluorescence, indirectly determining the number of adherent bacteria through the proportion between luminous intensity and microbial count [24]. Methods to detect microbial adhesion are shown in [Table/Fig-3] [21,25-30].

Quantitative methods		Qualitative methods	
Colony forming units	Method for quantifying bacteria: The pour-plate and surface-spread methods [25].	Crystal violet staining	It is a type of spectrophotometry; It is based on the quantitative relation between optical density and colony counts which is obtained from standard curves prepared for each bacterial species [25] It is to evaluate the early stages of bacterial biofilm formation [26].
16S rRNA gene sequencing	Accurate identification of microorganisms [27].	Fluorescent microscopy	Immunofluorescent method and observed under a microscope and are visualised using dyes.
Polymerase Chain Reaction (PCR)	Used for quantifying adhesion to intestinal epithelial cells.	Confocal scanning microscopy	Allows real-time visualisation of fully hydrated, living specimens [28] enable quantification of the forces involved in the adhesion of microbes on a single cell basis [21,29,30].
ATP bioluminescence	To assess bacterial adhesion with hydrophobic polystyrene tubes as the attachment surface.	Scanning Electron Microscopy (SEM)	For morphological assessment of bacteria adhered on a surface, the morphology of the material surface, and the relationships between them.

[Table/Fig-3]: Methods to detect microbial adhesion [21,25-30].

Changes in Oral Microbiome with Fixed Orthodontic Appliances

When a patient undergoes orthodontic treatment with fixed appliances, there is alteration in the microbiota, microbes initially adhere to the brackets and tissue surfaces of removable appliances, leading to biofilm formation [31]. There are numerous studies on interaction of oral bacteria with orthodontic brackets, but specific bracket type that favours biofilm development is unknown [3,32,33]. Ceramic brackets have been implicated to have more plaque retention than metal brackets but it remains controversial [33,34]. In a study by Abutayyem H et al., seven different bracket systems were assessed for microbial adhesion. *E. coli* had the highest concentration in ceramic brackets [35]. Highest concentration of *S. mutans* was observed in self-ligating brackets, while *E. faecalis* was most common in SS wires [35].

Microbial Adhesion on Different Aligner Materials

In recent years, there has been an increased demand for aesthetic, metal-free orthodontic devices called aligners due to their less visibility and aesthetic appeal. These are recommended to be worn for 20-22 hours daily. Invisalign aligner, made from a polyurethane-based material, was introduced in the late 1990s is one of the most popular aligner systems used worldwide [36]. A systematic review by Ajwa N et al., reported that the overall changes of oral microbiota are higher in patients with fixed appliances than removable appliances [37]. Even though clear aligner therapy is associated with better oral hygiene levels in the short term there are concerns of plaque formation and WSL occurrence [38]. The included studies on microbial adhesion on clear aligners and cleaning methods of clear aligners [Table/Fig-4] [39-45].

Tektas S et al., reported no significant differences in the biofilm formation [39]. Rouzi M et al., and Sfondrini MF et al., observed microbial shifts, with a notable increase in *Streptococcus* spp [41-43]. While Yazdi M et al., observed that C.albicans formed the most

Authors	Intervention	Method of assessment	Organisms tested	Outcomes assessed	Outcome
Tektas S et al., 2020 [39]	CA-medium, copolyester, Duran, Erkodur Comparison - enamel surfaces and metal brackets	Colony forming units of aerobic and anaerobic bacteria	Both aerobic and anaerobic organisms	Bacterial adhesion at 2 hours and biofilm formation after 72 hours	No differences in initial microbial attachment and biofilm formation of aerobic and anaerobic species
Bozkurt A et al., 2024 [40]	Invisalign, Clarity, Clear Correct , Smartee, Orthero and Graphy.	Microwell titre assay Crystal violet assay	*Streptococcus mutans* (ATCC 25175) and Lactobacillus acidophilus (ATCC 4356)	Time-dependent biofilm formation and microbial adhesion	More biofilm formed on Clear Correct at 168 hours. *Streptococcus mutans* formed more biofilm on Invisalign at 240 hours Lactobacillus acidophilus bacteria formed significantly more biofilm on Invisalign at 240 hours
Rouzi M et al., 2022 [41]	15 Patients treated with invialign aligners Aligner tray plaque and subgingival plaque was tested	16S rRNA Gene Amplicon Sequencing Sequence analysis	Plaque collected and tested	Plaque index (PI), salivary flow, PD, BI, sequence analysis, microbial composition and diversity	Abundance of *Streptococcus* increased, as well as the richness and microbiota decreased substantially as the duration of treatment time.
Baybekov O et al., 2023 [42]	A microbiological examination of 10 persons using aligners obtained from adolescents aged 13 to 19 years PETG Aligner	1. CFUs 2. Crystal violet staining - OD values for biofilm formation 3. Confocal laser scanning microscopy	Used aligners were subjected to microbial isolation and testing		13 Gram-positive and 13 Gram-negative bacteria, and 2 yeast-like fungi. There are currently no means of ensuring the direct and complete destruction of biofilms.
Sfondrini MF et al., 2021 [43]	Intervention- clear aligner Control - no treatment	Real Time PCR-based test	Aggregatibacter actinomycetemcomitans, Tannerella forsythensys, Porphyromonas gingivalis, Troponema denticola, Prevotella intermedia and Fusobacterium nucleatum.	Probing Pocket Depth (PPD), Bleeding on Probing (BOP) and Plaque Index (PI), and microbiological tests.	Porphyromonas gingivalis and Prevotella Intermedia increased in both groups Conversely, Tannerella forsythia and Fusobacterium nucleatum increased in the clear aligner group. During the first two months of treatment, CAT has no discernible effect on periodontal and microbiological markers as compared to patients who are not receiving treatment.
Yazdi M et al., 2024 [14]	3D printed CAD/CAM material (Detax) and four thermoformed retainers Erkodent; EasyVac; DB and Clear Tech	Microtiter plate assay	*Streptococcus mutans Streptococcus sanguinis*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, and *Lactobacillus casei*	Biofilm formation on 5 thermoformed and 3D printed CAD/CAM orthodontic retainers in 3 intervals	*Candida albicans* biofilm was more intense
Mummolo S et al., 2019 [44]	30 subjects (aged 21.5±1.5 years) were treated with removable clear aligners (CA), while for other 30 cases (aged 23.3±1.6 years) a fixed multibrackets appliance (MB)	*CRT® bacteria* CFU	*S. mutans* and *Lactobacilli*	Investigate salivary concentrations of *S. mutans* and some Lactobacilli, and plaque index (PI) in patients wearing fixed versus removable orthodontic appliances.	Only about 10% of CA patients and 13.3% of RP patients achieved a microbial colonisation which may lead to high risk of caries development, about 40% of MB patients - and 20% after 3 months - showed a high level of vulnerability to developing caries.
Levrini L et al., 2015 [45]	Invisalign® group, fixed orthodontic appliances group and control group.	Total biofilm mass and periodontal pathogens were analysed and detected via real-time PCR.	P. intermedia, A. actinomycetemcomitans, P. gingivalis, Tannerella forsythia.	Total biofilm mass and periodontopathic bacterial species Periodontal indices	The microbiological analyses detected the presence of A. actinomycetemcomitans only in one patient treated with fixed orthodontic appliances.

[Table/Fig-4]: Characteristics of included studies [39-45,65].

biofilm formation with Detax retaining the least biofilm, followed by Erkodent and Clear Tech [14]. Baybekov O et al., observed that the biofilm formation varied based on the type of aligner, with clear correct aligner exhibiting higher count of S.mutans while Invisalign showed more biofilm than Clarity and Orthero . Additionally, Graphy demonstrated increased biofilm formation when *S. mutans* and L. acidophilus were combined. In a study by Baybekov O et al., results revealed that 28 different microbial species were isolated from the aligners, with a predominance of Bifidobacterium spp., Candida albicans, E. coli, and various *Streptococcus* and *Peptostreptococcus* species [42]. These species were found in significant concentrations (≥5 lg CFU/1 mL), indicating their dominance in the oral microflora. A systematic review by España-Pamplona P et al., analysed the impact of orthodontic appliances, including clear aligners, on oral microbiota and periodontal health. It found that clear aligners tend to promote a healthier oral microbiota, with improved periodontal indicators such as lower plaque and bleeding indices compared to fixed appliances [46].

In a review by Rouzi M et al., it was observed that clear aligners show reduced incidence of WSLs compared to fixed appliances. This could be attributed to their simplified oral hygiene routines, leading to lower plaque accumulation [41]. No significant changes observed in bleeding on probing or probing depth in aligner patients. Improper cleaning of aligners can lead to the growth of harmful bacteria, underlining the importance of regular cleaning, the authors recommend a multistep cleaning approach that combines mechanical and chemical methods for optimal hygiene [47].

Properties of Aligners Influencing Microbial Adhesion

Microbial adhesion to clear aligners is influenced by surface properties, material composition, and intraoral conditions. Over time, aligner surfaces may undergo changes such as increased roughness and hydrolytic degradation, potentially enhancing bacterial colonisation. Surface properties such as roughness and wettability are linked to microbial adhesion [48,49]. Understanding these factors is crucial for maintaining aligner efficacy and minimising microbial accumulation through proper hygiene and regular dental check-ups. Surface properties such as roughness and wettability are linked to microbial adhesion [48,49]. Methods to reduce microbial adhesion on clear aligners [Table/Fig-5].

The changes associated with the intraoral aging of aligners, have been evaluated with respect to mechanical properties and surface roughness [50]. Hydrolytic degradation has been described as the main mechanism of the disintegration of polymers exposed

Mechanical methods	Chemical methods
• Brushing with toothpaste • Brushing with toothpaste and chlorhexidine • Sonic or ultrasonic bath	• Aligner cleaning crystals (e.g., Invisalign Cleaning crystals, retainer brite • Tablets • Brushing with toothpaste+effervescent tablet • Rinsing with water • Cupral ®, a copper-calcium hydroxide solution

[Table/Fig-5]: Methods to reduce microbial adhesion on clear aligners.

to aqueous media [51]. Ihssen BA et al., demonstrated that water absorption can reduce the material's stiffness and may therefore promote a decrease in the orthodontic forces in PETG aligners [52].

Consequences of Microbial Adhesion - WSL Incidence

WSLs are clinically defined as opaque, white areas caused by the subsurface loss of enamel minerals [53]. WSL incidence was observed to be less in patients treated with CAT than traditional braces,which partially due to shorter treatment duration, or better pretreatment oral hygiene [54]. Approximately, 1.2% of the aligner patients developed WSLs, compared to 26% of the traditionally treated patients [55,56]. In a study by Liu Q et al., 35.5% of patients developed WSL during CAT. The incidence was higher in maxillary dentition with the lesions appearing symmetrical side to side. The order of incidence of WSL is as follows- maxillary lateral incisor, canine, central incisors, first premolars, second premolars and the first molars [57].

The variation in WSL incidence among studies could be attributed to differences in number of teeth examined, the methods and standardisation of the examinations, the location of the study sample, initial age, treatment duration, type orthodontic therapy, supplemental fluoride application and orthodontic materials [56]. In a meta-analysis by Llera-Romero, it was reported that, at the end of treatment (9-24 months), aligners patients have ten times less risk of presenting new WSLs than conventional fixed appliance patients [58].

In a study by Song Z et al., it was observed that the relative abundances of 14 taxa were significantly higher in the WSL group [59]. After clear aligner therapy, there were changes observed in the oral microenvironment, including inflammatory cytokine levels such as CXCLs and ILs, with CXCL8 expression significantly associated with the occurrence of WSLs [60].

Consequence Of Microbial Adhesion - Periodontal Problems

Periodontal health is defined by the absence of detectable signs of inflammation that may affect periodontal physiology [61]. Orthodontic appliances can promote plaque retention and a change in the composition of the oral microbiota, with possible effects on periodontal health and risk of caries. Some authors reported a different microbiota composition in plaque or saliva in aligner patients compared to patients wearing FAs [62,63]. This may be attributed to the fact that aligners are removable, allowing the maintenance of good oral hygiene.

During the assessment of periodontal parameters, aligners demonstrated significantly lower scores of plaque indices [64]. In a meta-analysis, it was observed that clear aligners were better for periodontal health than fixed appliances and might be recommended for patients at high-risk of developing gingivitis [45,65]. In another systematic review by Abay F et al., it was reported that the percentage of patients who developed new WSLs after clear aligner therapy varied between 1.2% and 41.18%, with a heterogeneity in the included articles.

Methods to Reduce Microbial Adhesion on Clear Aligners

There is possibility for adhesion and growth of bacterial biofilm in clear aligners though they are removable type of orthodontic appliances. There are several methods to clean clear aligners. In a study by Levrini L et al., it was observed that brushing with toothpaste alone is more efficient than rinsing with water, while better results are obtained when brushing with toothpaste is combined with an effervescent tablet [66]. Ultrasonic bath was observed to be efficient in removing bacterial biofilms from aligners. However, the SEM pictures obtained showed that the surface of the aligner was damaged by the ultrasonic vibrations, showing signs of ultrasonic cavitation [67].

In a trial by Shpack N et al., it was observed that the use of vibrating bath along with Cleaning Crystal solution was observed to be more efficient than using toothpaste and chlorhexidine to brush, but it did not exhibit bacterial inhibition zone [68]. The cleaning crystals used in the vibrating bath is composed of Sodium Sulfate, Sodium Carbonate, Sodium Tripolyphosphate , Sodium Dichloroisocyanurate and Sodium lauryl sulfate, which act as a pH neutraliser (e.g., Sodium Sulfate), disinfectant (e.g., Sodium Dichloroisocyanurate) and detergent (e.g., Sodium lauryl sulfate).

In a study by Levrini L et al., patients were instructed to follow different aligner cleaning procedures for six weeks rinsing with water, soaking with cleaning tablets and brushing, and brushing with a soft toothbrush and toothpaste [66]. Scanning Electron Microscopy (SEM) analysis of the external and internal surfaces of aligners revealed that those cleaned using a combination of cleaning tablets and brushing were significantly cleaner compared to those rinsed with only water. Despite this, bacterial contamination, predominantly composed of spheroidal microorganisms, was observed on all aligners, with occasional mineral crystal deposits. The internal surfaces exhibited higher contamination levels, likely due to their concave shape, which made thorough cleaning more difficult.

Meto A et al., investigated the disinfectant properties of Cupral®, on aligners contaminated with biofilm and found it effective in eliminating bacteria [69]. Additionally, studies by Zhang M et al., and Xie Y et al., demonstrated that applying a gold nanoparticle coating to the aligner surface reduced initial bacterial adhesion, thereby preventing biofilm formation [70,71]. In-vitro findings were particularly promising, showing significant suppression of Porphyromonas gingivalis, a periodontopathogenic bacterium, and *Streptococcus* mutans, a key contributor to dental caries.

CONCLUSION(S)

Streptococcus mutans and lactobacillus species have been observed to be the most common organisms linked to biofilm formation. The incidence of WSL has been linked to biofilm formation, leading to enamel demineralisation. Use of Cleaning Crystals for cleaning aligners has been reported to be more effective in eliminating bacterial contamination.

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