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Biofilms- A Never Ending Battle!!

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Abstract:
Microbial communities formed in root canals of teeth constitute the heart of the infected root canal ecosystem, and yet their establishment and development remains challenging to measure and predict. Biofilms are dynamic systems with attributes of both primordial multicellular organisms and represent a protected mode of growth that allows cells to survive. Although there is an initial understanding on the mechanisms of biofilm formation in root canals and its associated resistance to clinical antimicrobial regimens, this topic is still under investigation. A greater understanding of biofilm processes should lead to novel, effective control strategies for endodontic biofilm control and a resulting improvement in patient management.

Keywords:

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I. Introduction

Biofilms are recognized as one of the earliest ecosystems on earth. They are composed of aggregates of microbial cells enclosed in a self-produced matrix adherent to a surface. (1) According to ingle, Biofilm can be defined as a sessile multicellular microbial community characterized by cells that are firmly attached to a hard or soft surface and enmeshed in a self-produced matrix of extracellular polymeric substance (EPS), usually polysaccharide. (2)Biofilm mode of growth is advantageous for microorganisms, as they form three-dimensional structured communities with fluid channels for transport of substrate, waste products, and signal molecules.(3)

HISTORY

- Anthony van Leeuwenhoek is known as the first biofilm experimenter. He noticed a vast accumulation of microscopic bodies in his own tooth scrapings, which he termed as "animalcules". In a report to the British Royal Society, he quoted, "The number of these animalcules in the scurf of a man's teeth is so many that I believe they exceed the number of men in a kingdom."(4)
- Few years later, Heukelekian and Heller observed that: "Surfaces enable bacteria to develop in substrates either as bacterial slime or colonial growth attached to surfaces". (4)
- In 1943, Claude ZoBell described many of the fundamental characteristics of attached microbial communities in seawater. (4)
- Such communities were described and named "Biofilms" in 1978 by Paul Harremoes a Danish physicist.(4)
- In 1987, Nair - described and presented the ultrastructural visualization of intracanal microbial flora.(5)

BASIC CRITERIA FOR A BIOFILM

- Caldwell et al. in 1997 [6] highlighted four characteristics of biofilm as follows:
  - *Autopoiesis* – Must possess the ability to self-organize
  - *Homeostasis* – Should resist environmental perturbations
  - *Synergy* – Must be more effective in association than in isolation
  - *Communality* – Should respond to environmental changes as a unit rather than as single individuals.

STRUCTURE OF BIOFILM

A biofilm community comprises of:
1. Bacterial micro colonies
2. Extracellular polysaccharide layer
3. Fluid channels
4. Primitive communication system

- Basic structural unit of a biofilm is the micro colonies or cell clusters formed by surface- adherent bacterial cells.
- Glycocalyx matrix made of EPS surrounds micro colonies,anchor bacteria to substrate.
- EPS are hydrated biopolymers (usually polysaccharides, but also proteins, nucleic acids, and lipids) secreted by biofilm cells [(3).]
- The EPS matrix serves the following important functions in the microbial community:

(i) It mediates biofilm adhesion to surfaces, very often acting as a "biological glue";
(ii) It provides mechanical stability to the biofilm;
(iii) It allows for extracellular enzymes to accumulate and exert important activities, which include nutrient acquisition and co-operative degradation of complex macromolecules;
(iv) It keeps biofilm cells in close proximity, thus allowing for interactions including quorum sensing, genetic exchanges, and pathogenic synergism;
(v) In periods of nutrient deprivation, it can serve as a nutrient source, although some components of the matrix may be only slowly or partially degradable;
(vi) It retains water and maintains a highly hydrated microenvironment surrounding the biofilm populations;
(vii) It plays a protective role against host defense cells and molecules as well as antimicrobial agents [(7)]

- The water channels are regarded as a primitive circulatory system in a biofilm.

(i) Establish connection between micro colonies
(ii) Facilitates efficient exchange of materials between bacterial cells and bulk fluid
(iii) Coordinate functions in a biofilm community.

- As biofilm get matured, its structure and composition are modified according to the environmental conditions.

DEVELOPMENT/ LIFECYCLE OF BIOFILM

- The three major components involved in biofilm formation are bacterial cells, a solid surface, and a fluid medium. [(8)]
- The development can be described in 4 stages:

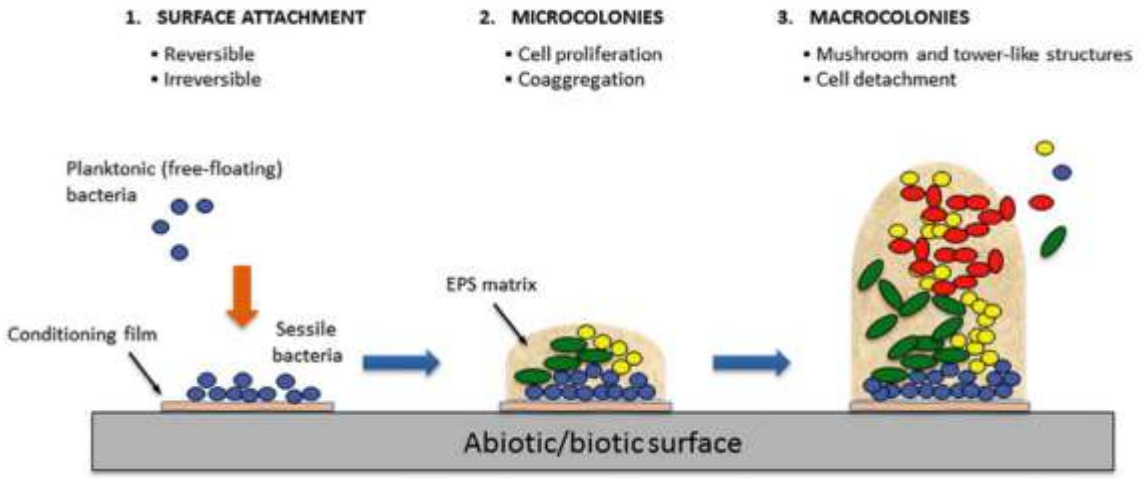
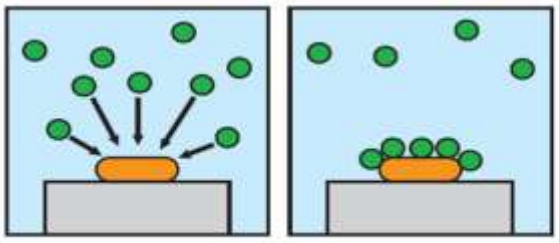


*Figure*2: *Schematic representation of the distinct steps in microbial biofilm development*.[(4)]

- ***Stage 1: Formation of conditioning layer***: involves the adsorption of macromolecules from tissue fluids such as saliva onto a biomaterial surface, leading to the formation of a conditioning layer. The conditioning layer will selectively promote the adhesion of microbial cells to the surface. It may also serve as a source of nutrition for adherent bacteria.[(8)]
- ***Stage 2: Adhesion of microbial cells to this layer***: Can be described in 3 phases:

- PHASE 1: Transport of microbe to substrate surface: this reversible interaction is determined by physicochemical properties such as surface energy and charge density. The bacteria adhere to a substrate by bacterial surface structures such as fimbriae, pili, flagella, and EPS (g1ycocalyx). Bridges are formed between the bacteria and the conditioning film by these bacterial structures.(3)
- PHASE 2: Initial non-specific microbial–substrate adherence phase: molecular-level nonspecific interactions between the bacterial surface structures and the substrate. The bridges formed between bacteria and substrate are a combination of electrostatic attraction and covalent/hydrogen bonding. Initially the bonds between bacteria and substrate may not be strong. However, with time these bonds gain in strength, making the bacterial attachment irreversible.(3)
- PHASE 3: Specific microbial–substrate adherence phase: a more specific bacterial adhesion to the substrate is established via polysaccharide adhesin or ligand formation. Adhesin or ligand molecules on the bacterial cell surface will bind to receptors on the substrate.(3)
- It is critical to realize that these phases occur as a function of time. The reversible and irreversible steps in phase 1 occur in a few seconds to minutes, while phase 2 and 3 interaction take a few hours to days to occur, depending upon the bacteria and the environment conditions. (8)
- ***Stage 3: Multiplication and metabolism of attached microorganisms***: In this stage, the mono-layer of microbes (primary colonizers) attracts the secondary colonizers, forming micro-colonies, and the collection of micro-colonies gives rise to the final structure of the biofilm. (8)
- Two types of microbial interactions occur at the cellular level during the formation of biofilm. One is the process of recognition between a suspended cell and a cell already attached to substratum. This type of interaction is termed co-adhesion.(9)
- In the second type of interaction, genetically distinct cells in suspension recognize each other and clump together. This type of interaction is called co-aggregation. This association is highly specific and occurs between co-aggregating partners only. (9)



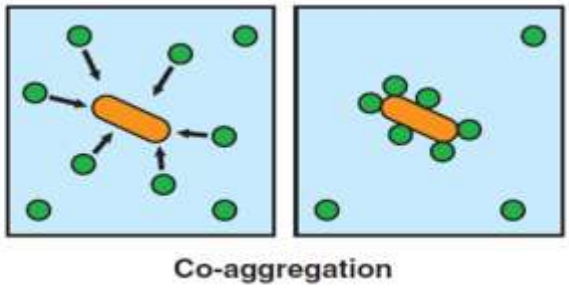


Figure 3: Co-adhesion(10)Figure 4: Co-aggregation(10)

- Interestingly, most oral bacteria recognize each other as co-aggregating partners. Fusobacterium nucleatum, a Gram-negative filamentous anaerobe, can co-aggregate with all oral bacteria tested, and can act as a bridging bacterium that binds together even non-aggregating bacteria. The association of long-filamentous bacteria and surface-adsorbed spherical-shaped cocci produce the characteristic corncob structure of oral biofilms.(3)
- ***Stage 4: Detachment of microorganisms from biofilm***: During detachment, particulate constituents are transferred from the biofilm to the fluid bathing the biofilm.Detachment plays an important role in shaping the morphological characteristics and structure of mature biofilm.Also considered as an active dispersive mechanism- Seeding Dispersal. (2)
- Brading et al. (2) have emphasized the importance of physical forces in detachment, stating that the three main processes for detachment are:
  - Erosion or shearing (continuous removal of small portions of the biofilm)
  - Sloughing (rapid and massive removal), and
  - Abrasion (detachment due to collision of particles from the bulk fluid with the biofilm)

CHARACTERISTICS OF A BIOFILM

- A mature biofilm will be a metabolically active community of microorganisms where individuals share duties and benefits. This signifies the relevance of a polymicrobial biofilm over a mono-species biofilm. The physiological characteristics of the resident microorganisms in a biofilm also offer an inherent resistance to antimicrobial agents. (8)
- ***Protection of biofilm bacteria from environmental threats:***(12)

- EPS covers biofilm communities and creates a microniche favourable for the long-term survival and functioning of the bacterial communities.
- EPS protects the biofilm bacteria from a variety of environmental stresses, such as UV radiation, pH shifts, osmotic shock, and desiccation
- It allows for extracellular enzymes to accumulate and exert important activities, which include nutrient acquisition and co-operative degradation of complex macromolecules
- In periods of nutrient deprivation, it can serve as a nutrient source, it retains water and maintains a highly hydrated microenvironment surrounding the biofilm population.
- It plays a protective role against host defense cells and molecules as well as antimicrobial agents

- ***Nutrient trapping and establishment of metabolic cooperativity in a biofilm:***[(12)]
- Biofilms growing in a nutrient-deprived ecosystem has the ability to concentrate trace elements and nutrients by physical trapping or by electrostatic interaction.
- Highly permeable and interconnected water channels in the biofilm provide an excellent means for material exchange.
- The water channel connects the outer fluid medium with the interior of the biofilm, ensuring nutrient availability to microbial communities deep inside the biofilm structure.
- Bacterial micro colonies in a biofilm structure are exposed to distinct environmental signals. For example, cells located near the center of a microcolony are more likely to experience low oxygen tensions compared to cells located near the surface.
- Due to the juxtapositioning of different microorganisms, cross feeding and metabolic cooperativity between different species of microorganisms are seen in a biofilm
- ***Organized internal compartmentalization in biofilm:***
- Mature biofilm structure displays gradients in the distribution of nutrients, ph., oxygen metabolic products, and signalling molecules within the biofilm. [(12)]
- This would create different microniches that can accommodate diverse bacterial species within a biofilm.
- According to Stewart & Franklin[(11)]three different physiological states are anticipated related to the oxygen and nutrient gradients found in a monospecies biofilm. Cells located in the upper biofilm layers consume all available oxygen and grow aerobically, while an anaerobic micro-niche developed underneath the aerobic layer. Oxygen- and nutrientdepleted regions are found at the bottom layers of the biofilm structure and under these circumstances, most of the sessile cells are metabolically inactive or dead. Consequently, the individual bacterial cell response to the local microenvironment leads to phenotypic heterogeneity. [(4)]

- ***Bacterial cells residing in a biofilm communicate, exchange genetic materials, and acquire new traits:***
- Communications between bacterial cells residing in a biofilm is attained through signalling molecules, by a process called quorum sensing.[(4)]
- Exchange of genetic materials between bacterial species residing in a biofilm will result in the evolution of microbial communities with different traits.
- The horizontal gene transfer is of importance in human diseases caused by bacterial biofilm as it can result in the generation of antibiotic-resistant bacterial population.
- When bacteria are growing within a biofilm, they secrete signalling molecules (auto inducers) that increase in concentration as a function of bacterial cell density. In a process called quorum sensing, bacteria communicate with one another by using auto inducers to regulate their gene expression in response to fluctuations in the cell population density. Differential gene expression results in heterogeneity within the biofilm. Two types of quorum-sensing systems are recognized in bacteria: intra-species communication and inter-species communication. Gram-negative bacteria usually use acyl homoserine lactone (AHL) as signal molecules, while Gram-positive bacteria utilize small peptides.[(4)]
- During inter-species communication, bacteria use autoinducer-2 (AI-2), a furanosyl borate diester. proposed as the universal signal for inter-species communication. [(4)]
- The signals are thought to allow cross talk between species, causing them to increase their production of EPS and the factors that increase their virulence.[(3)]
- ***Resistance of microbes in the biofilm to antimicrobials:***[(4)]
- The nature of biofilm structure and physiological characteristics of resident microorganisms offer an inherent resistance to antimicrobial agents, such as antibiotics, disinfectants, or germicides.
- ***Mechanism responsible for resistance:***
- Resistance associated with extracellular polymeric matrix:[(4)]

- EPS matrix can act as an impermeable barrier to limit antimicrobial penetration. Upon antibiotic treatment, cells at the top of the liquid– biofilm interface die due to their closer exposure, while bacteria embedded deep inside the biofilm are able to survive.
- The biofilm matrix can also be considered a chemically active barrier. Anionic EPS matrix can bind and sequester toxic cationic heavy metals, cationic antimicrobial peptides, and positively-charged antibiotics.
- Altered microenvironment and stress responses:[4]
- Microbial cells, especially those in the deeper layers of the biofilms where nutrients and oxygen are limited, are associated with a lower growth rate. Conventional antibiotics used to treat infections are mostly effective at killing rapidly growing cells. The decreased metabolic activity of cells found within the deeper biofilm layers may thus contribute to antibiotic tolerance and the persistence of biofilm infections.
- One mechanism that has recently been explored is the role of drug efflux pumps to explain the recurrence of biofilm infections. There has been evidence that many membrane-bound drug efflux pumps found in both Gram-negative and Grampositive bacteria are induced by exposure to sub-lethal concentrations of various antibiotics. [13] The origin of these transporters was to remove metabolites and by-products within bacterial cells and, over time, they evolved to efflux out other harmful molecules such as antimicrobial agents.
- Bacteria in biofilms and planktonic cultures can also express stress-responsive genes and switch to more tolerant phenotypes upon environmental stressors (e.g. starvation, heat or cold shock, cell density, pH, osmolarity).[4]
- Persister cells:
- Within a given population of bacteria, a small subpopulation known as non-growing persisters exists. It has been suggested that these specialized cells enter into a state of dormancy, which allows them to survive stress conditions and prevents death because antibiotics target cell growth. [4]
- An initial treatment with antibiotic kills planktonic cells and the majority of biofilm cells, leaving persisters intact. The host immune system targets and kills planktonic persisters, but the biofilm persisters are protected from host defenses by the biofilm matrix. After the antibiotic treatment is stopped, persister cells repopulate the biofilm and the infection relapses.[12]

ENDODONTIC BIOFILM

- Endodontic bacterial biofilms are classified as:[3]

➢ *Intracanal biofilms*: microbial biofilms formed on the root canal dentin of the infected tooth. Identification of biofilm was reported by Nair in 1987 under transmission electron microscopy. [5] Major bulk of the organisms existed as loose collections of filaments, spirochetes, cocci, and rods.

➢ *Extraradicular biofilms*: formed on the root surface adjacent to the root apex of endodontically infected teeth. F. nucleatum, Po. gingivalis, and Tannerella forsythensis were found to be commonly associated with extraradicular biofilm.

➢ *Periapical biofilms*: isolated biofilms in the periapical region of endodontically infected teeth which can be seen even in the absence of root canal infections.

➢ *Biomaterial-centered infections*: found when bacteria adhere to an artificial biomaterial surface and form biofilm structures. It is a major complication associated with prosthesis and also in implant-supported prosthesis.

METHODS TO STUDY BACTERIA IN BIOFILMS

- A variety of microscopic in situ methods have been developed to identify subpopulations and assess the physiological status of bacterial cells in biofilms. [12]
- ***SEM and LSM:***
- Electron microscopy (EM) in the transmission and scanning mode provides resolution and magnification to offer a more detailed insight into the ultrastructure of the biofilm as well as its environment. One of the main drawbacks of this technique, however, is that it requires the sample to be dehydrated prior to its analysis.[12]
- The LSM technique, usually called confocal laser scanning microscopy (CLSM), is nowadays the most important and indispensable tool for three-dimensional in situ imaging of microbial communities. By means of this imaging procedure, it is possible to analyze the structure, composition, microhabitats, activity, and processes using a variety of specific colour probes. One of the main disadvantages of LSM, however, is that the information captured from detailed ultrastructure of the biofilm is difficult. [12]
- Very recently, this problem of LSM has been overcome with the advent of super-resolution microscopy (SRM). SRM encompasses a suite of cutting-edge microscopy methods able to surpass the resolution limits of common light microscopy. It is foreseen that the application of SRM in combination with rRNA FISH would allow the tracking of ribosome-associated changes in activity levels and subcellular localization at the single-cell level.[8]

- ***rRNA Fluorescence In Situ Hybridization (FISH):***
- The combination of FISH with CLSM is one of the most powerful tools in modern microbiology as it allows visualization of specific subpopulation of cells while maintaining unaltered the 3D structure of the biofilm. [12]
- ***Markers of Cell Viability:***
- In root canal infections, culture techniques have been the standard method used to assess bacterial viability. However, the bacteria in low active states may be undetectable by regular culture techniques. The LIVE/DEAD kit tests the integrity of the cell membrane by applying two nucleic acid stains, SYTO-9 and propidium iodide (PI), which can simultaneously detect dead/injured (fluorescent red by stain with PI) and intact cells (fluorescent green by staining with SYTO-9). [12]
- ***Atomic force microscopy (AFM):***
- To study the forces of interaction between bacterial cells and between bacterial cell and substrate.[8]
- ***Laser-based optical tweezers:*** [8]
- Non-invasive and non-contact tools that can probe the interaction between microscopic objects such as bacteria and collagen.
- They give more information about the forces of interaction between bacteria and substrate quantitatively.
- ***FTIR (Fourier transform infrared) spectroscopy:***[8]
- Can be used for the qualitative and quantitative analyses of the chemical constituents on a biofilm structure
- ***NMR (solid-state nuclear magnetic resonance) spectroscopy:*** [8]
- Is useful to obtain metabolic information in planktonic cells, adherent bacterial cells, and in situ biofilm bacteria

ANTIBIOFILM STRATEGIES

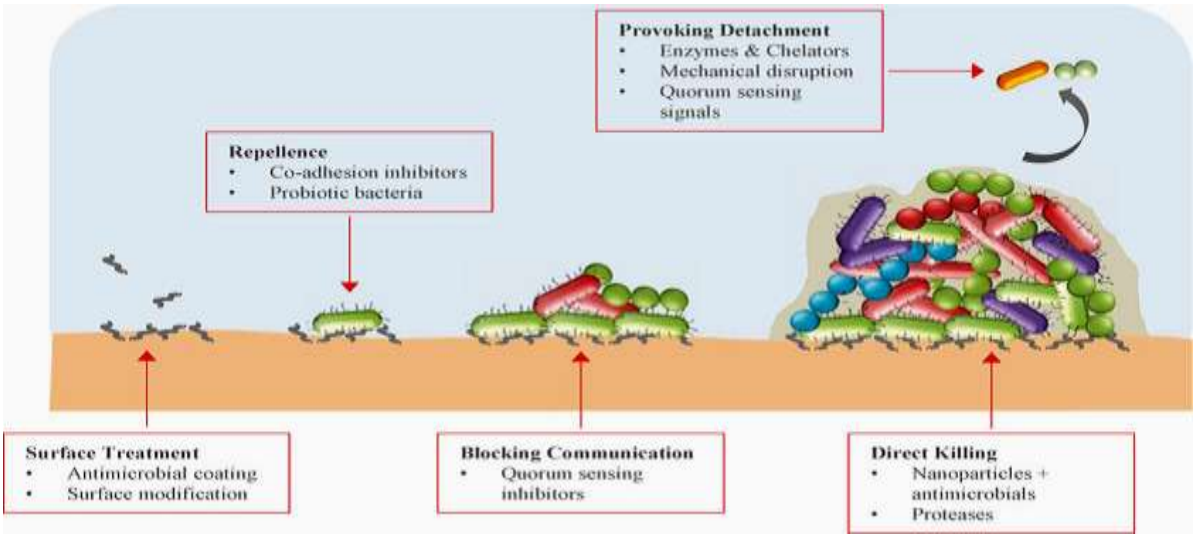


Figure 5: Schematic outline of the general approaches for biofilm eradication currently used and under research[12]

- ***Surface modification***
- A reasonable approach to prevent or reduce secondary biofilm formation in root canals is to replace the conditioning film with repelling substances that will alter the chemical composition of the substrates. [12]
- Surface preconditioning with biocides has the potential to prevent bacterial adhesion; biocides can increase the cell wall charge of bacteria and therefore reduce their ability to attach and form biofilms. [12]
- Surface coating with a solution of benzalkonium chloride was found to exhibit an overall 70-fold reduction in the biofilm accumulation. [12]
- However, one of the main problems with this method to prevent biofilm formation is that the coating at some point in time may get exhausted; thus, its antibiofilm effect may stop.

- ***Mechanical strategies:***
- Mechanical instrumentation is the core method for bacterial reduction in the infected root canal. With the launch of nickel–titanium (NiTi) rotary systems, perhaps too much credit was given to these systems as being the sole solution to challenges in root canal treatment.[14]

- Dalton et al. [15] compared the ability of stainlesssteel K-type files and NiTi rotary instruments to remove bacteria from infected root canals and no significant differencewas detected between canals instrumented with hand files and rotary instruments.
- Carver et al. [16] evaluated the in vivo antibacterial efficacy of a hand/ rotary technique in mesial root canals of necrotic mandibular molars. Root canal cleaning and shaping with hand and rotary instrumentation and irrigation with 6.0% sodium hypochlorite showed a significant reduction in the log colony-forming unit (CFU) counts.
- Self-adjusting file (SAF) was designed to address the shortcomings of traditional rotary files byadjusting itself to the cross-section of the canal [17]. The SAF system uses a hollow vibrating instrument, which allows for continuous irrigation with NaOCl or EDTA throughout the instrumentation process. Irrigants are exchanged and taken to the apical root canal as a result of the vibration and in-and-out motion of the SAF. The compressible NiTi tube can adapt itself to the oval-shaped canal while its abrasive blades are pressed against the walls to promote effective cleaning. [12]
- Bao et al.,[18] in 2017 found the following 3- step irrigant protocol with XP- endo finisher suitable for removal of biofilm from the main canal.

- Use XP-endo finisher for 20 secs with 3% NaOCl
- Followed by 10 sec irrigation with 0.5ml of 3% NaOCl
- Repeat the sequence for 3 times.

- In summary, instrumentation plays an important role in helping to remove biofilm from those areas where the instrument can gain direct contact with the root canal wall. In addition, shaping of the main canal facilitates effective irrigation by creating the necessary space for needle penetration and sufficient irrigantflow. Regardless, challenges remain in many areas due to anatomy and the resistance of biofilms.

- ***Effect of various irrigating solutions on biofilms:***

- **Sodium hypochlorite:**
- Dunavant et al. [19] compared the efficacy of 1% or 6% NaOCl with that of 2% CHX, Smear Clear, and MTAD againstE. feacalis biofilms and theresults showed that both concentrations of NaOCl provided statistically significantly better biofilm killing than any of the other agents tested.
- The poor in vivo performance compared to the in vitro effect may be caused by problems in penetration to the most peripheral parts of the root canal system such as fins, anastomoses, apical canals, lateral canals, and dentin canals. Also, the presence of inactivating substances such as exudate from the periapical area, pulp tissue, dentin collagen, and microbial biomass counteracts the effectiveness of NaOCl.

- *Methods to enhance the action of NaOCl on biofilms:*[20]

- Adding various detergents such as cetrimide or benzalkonium chloride to lower the surface tension and enhance penetration of the solution into dentinal tubules.

- Adding calcium hydroxide, which enhances its antimicrobial actions and reduces the levels of bacterial endotoxins, and physical activation of the solution, by using ultrasonic instruments or pulsed middle infrared lasers (such as Er: YAG or Er, Cr: YSGG lasers).

- "Continuous chelation" where both the chelating agent and the NaOCl are present at the same time (i.e., mixed together). This is not possible using EDTA since it inactivates NaOCl. Non-nitrogen-containing bisphosphonates, such as clodronate, are suggested for use in continuous chelation, since they do not react with NaOCl, and give stable mixtures with excellent smear layer removal capabilities as well as powerful antimicrobial actions.

- **Chlorhexidine digluconate and CHX-Plus**
- 2% CHX killed the biofilm bacteria but was not able to disrupt the biofilm structure. Although CHX may kill the bacteria, the biofilm and other organic debris are not removed by it. [14]
- To improve the antibacterial activity of CHX, surface-active agents has been added. CHX-Plus showed higher levels of bactericidal activity at all exposure times compared to 2% CHX, which may indicate that the surfactant component in CHX-Plus facilitated penetration of the disinfectant into the biofilm.[14]

- **Ethylenediaminetetraacetic acid (EDTA):**

- Alternating the use of NaOCl and EDTA during root canal treatment appears to be a promising approach to remove the organic and inorganic debris, in addition to disrupting microbial biofilms.[21]
- Another demineralizing agent, maleic acid has been shown to be effective against E. fecalis at a concentration of 0.88% for 30 seconds. [21]
- A 2.25% peracetic acid solution was recommended as a final irrigant after the use of sodium hypochlorite during instrumentation. Peracetic acid has been shown to be more effective than chlorhexidine against root canal mono-species E. fecalis biofilm. [21]

- ***Mechanical agitation by sonic and ultrasonic appliances:***
  - The EndoActivator uses sonic energy to agitate the irrigants in the root canal system. The EndoActivator System has been reported to be able to clean debris from lateral canals, remove the smear layer, and dislodge clumps of simulated biofilm within the curved canals of molar teeth.
  - Studies suggested that the combined use of ultrasonic or sonic vibration and chlorhexidine produced a better antimicrobial effect against biofilms than chlorhexidine alone.
  - Ultrasonic agitation can cause shear stress and dis-agglomeration of bacterial biofilm, thus re-suspending the bacteria in planktonic form making them more susceptible to antimicrobial agents. Cavitation causes temporary weakening of the cell membrane increasing the bacterial cell permeability to antimicrobial irrigants.

- ***Microbubble Emulsion***
  - Halford et al., were the first to employ a microbubble emulsion to enhance the effect of sonic and ultrasonic agitation of sodium hypochlorite. [22]
  - Essentially, the technique employs unstable gas-filled microbubbles that expand when exposed to ultrasonic waves. The dynamics thereby induced in the fluid would help in detaching surface adherent bacteria or biofilm destruction. In addition, it may also generate reactive oxygen species to exhibit an antibacterial effect. [22]

- ***Photo-activated disinfection:***
  - Photo-activated disinfection (PAD) involves the use of a photo-active dye (photosensitizer) that is activated by exposure to light of a specific wavelength in the presence of oxygen. The transfer of energy from the activated photosensitizer to available oxygen results in the formation of toxic oxygen species, such as singlet oxygen and free radicals. These very reactive chemical species can damage proteins, lipids, nucleic acids, and other cellular components. PAD achieved a reduction in bacterial viability of up to 80%.

- ***Laser-activated irrigation:***
  - When laser irradiation pulses, the cavitation effect produces a shockwave that can move the irrigating solution within the canal. One brand of Erbium: YAG (Er: YAG) laser propose its use in combination with a special tip to achieve the so-called Photon-induced photoacoustic streaming (PIPS) of irrigant in the canal.This device has been researched for removing debris and smear layer from the root canal system and the results seem positive.
  - Neelakantan et al., demonstrated that both diode and Er: YAG lasers were more effective than ultrasonic activation or syringe irrigation method for removing E. fecalis biofilms.[21]

- ***Ozone against biofilm:***
  - Ozone (O3) is an energized, unstable gaseous form of oxygen that readily dissociates back into oxygen (O2), liberating a reactive form of oxygen, the singlet oxygen (O1). The singlet oxygen is capable of oxidizing cells. [23]
  - Ozone had an antibacterial effect on planktonic E. feacalis cells and those suspended in fluid; little effect on cells embedded in a biofilm structure. [23]
  - Ozone gas concentration currently used in Endodontics is 4 g/m3. This concentration has been shown to be slightly less cytotoxic than NaOCl (2.5%). Aqueous ozone (up to 20 mg/mL) showed essentially no toxicity to oral cells in vitro. [23]

- ***Local intracanal medicaments:***

- Evidence suggests that the association of calcium hydroxide with CMCP has a broader antibacterial spectrum, a higher radius of antibacterial action, and kills bacteria faster than mixtures of calcium hydroxide with inert vehicles. (14)
- Addition of chitosan nanoparticles to calcium hydroxide appears to enhance the bacterial killing in a multi-species model over a 7 and 14-day period. (21)
- It has been shown that triple antibiotic paste(TAP) is significantly better than calcium hydroxide and chlorhexidine in disrupting biofilms of E. fecalis. It has been suggested that 1 mg/mL DAP is needed to demonstrate any significant antibiofilm activity. (21)
- In addition, incorporation of polymer nanofibers with TAP has been shown to enhance the antibacterial activity.(21)
- There is also growing interest in non-antibiotic antimicrobial agents which can penetrate biofilms for possible inclusion in endodontic medicaments, including plant-derived phenolics, and nanoparticles.
- Both silver nanoparticles and biomimetic iron oxide nanoparticles have been shown to impair biofilm formation and to prevent dentinal tubule infection by E. faecalis. (21)

- ***Antibacterial nanoparticles:***

- Nanoparticles are microscopic particles with one or more dimensions in the range of 1–100 nm. The electrostatic interaction between positively charged nanoparticles and negatively charged bacterial cells, and the accumulation of a large number of nanoparticles on the bacterial cell membrane, have been associated with broad spectrum of antimicrobial activity and a far lower propensity to induce microbial resistance than antibiotics.(23)
- Chitosan (CS-np), Zinc oxide (ZnO-np), Copper Oxide (CuO-np), and Silver (Ag-np) nanoparticles possess a broad spectrum of antimicrobial activity
- root canal surface treated with cationic antibacterial nanoparticulates such as ZnO-NP, CS/ZnO-NP, or CS-layer-ZnO-NP significantly inhibited bacterial adherence to dentin, which, in turn, would prevent bacterial recolonization and biofilm formation.(24)
- Rose bengal-functionalized CS-np: effective against monospecies and multispecies biofilms. (23)
- Root dentin treated with chlorhexidine and then with nanoparticulates shows the maximum reduction (97%) in bacterial adherence. (24)
- Mesoporous bioactive calcium silicate nanoparticles and bioactive glass powder loaded with AgNp demonstrated significant reduction in adhesion of E. feacalis biofilms and this was further exemplified by ultrasonic activation.(23)

- ***Herbalalternatives:***

- Some recent trends in anti-biofilm research are directed toward the application of natural extracts from plants to treat biofilm-mediated infection.(23)

EXTRACT	PLANT	ACTION
Anacardic acid	Extract of cashew nut shells	Effective against step mutans and staph aureus biofilms
Morinda citrifolia	From coffee family- Noni	Similar to NaOCl when used in conjunction with EDTA.
Curcumin	Turmeric (Curcuma Longa)	Antimicrobial, anti-inflammatory and antioxidant. Phototoxic effects against Gram+ and Gram- bacteria.
Triphala	From three medicinal fruits- Terminalia Bellerica, Terminalia Chebula, Emblica officinalis	100% killing of E.feacalis in 6 min
Green tea polyphenols	Young shoots of tea plant- Camellia Sinensis	Antibacterial activity against E.feacalis biofilms

- The major advantages of herbal alternatives: easy availability, cost effectiveness, increased shelf life, low toxicity and lack of microbial resistance.

- ***Miscellaneous Interventions:***

- ***Enzymatic irrigation*** was introduced by Niazi and coworkers, who evaluated the effectiveness of 1% trypsin and 1% proteinase K, with or without ultrasonic activation, on a multi-species biofilm. Trypsin with ultrasonic activation was able to effectively kill both aerobic and anaerobic bacteria and has the capability of disrupting the biofilm. (21)
- The two carbohydrate-containing moieties of staphylococcal biofilms, a linear poly-b-(1- 6)-N-Acetyl-D-glucosamine (PNAG) and teichoic acid, have been targeted using enzymes such as dispersin B and proteinase K (168–170). These studies have shown that rinsing an implant surface with enzymes can prevent the formation of staphylococcal biofilms.(23)

- ***Agents that interfere with the cell wall***, such as D-amino acids, specifically D-leucine has been demonstrated to bring about efficient dispersal of E. feacalis biofilms. It has been suggested that the dispersal of biofilms by sub-toxic concentrations of this agent reduces the success of resistant organisms.[21]

- ***Interference with bacterial communication systems:*** [25]

- The bio-film formation can be disrupted by disturbing the quorum sensing mechanism - inhibition of quorum sensing is commonly referred to as "quorum quenching."
- Quorum sensing can be blocked by stopping the signal molecule production, destroying the signal molecule, and by preventing the signal molecule from binding to its receptor.
- *Blockage of autoinducer synthesis:*
- AHL production can be blocked by developing structural analogs of S-adenosyl methionine and acyl carrier protein. (E.g. Molecules like - L/D-S-adenosyl homocysteine, *S*-adenosylcysteine)
- Macrolide antibiotics like erythromycin are capable of repressing AHL synthesis when applied at lower concentrations.
- *Inactivation of AI*
- Enzymes such as - acylase, lactonase, oxireductases can selectively inactivate AHL in Gram-negative bacteria and due to this AHL accumulation in the extracellular environment does not occur and QS regulated genes are not expressed.
- *Inhibition of AHL signal reception*
- Can be inhibited by preventing the AHL molecule from binding to its receptor. It can be competitive inhibition by molecules that bind to the receptor in preference to the AHL molecule.
- Quorum sensing blockage by molecules produced by various plants, algae, and other organisms.
- Eg: Horseradish, Garlic, Turmeric-curcumin, Citrus flavonoids, Nutmeg, Sweet basil, Clove extract

- ***Probiotics:*** [4]

- A promising alternative strategy to treat chronic biofilm infections is bacteriotherapy or the use of selected harmless bacteria to displace pathogenic organisms.
- The introduced probiotic strains can be either wild-type commensals or avirulent (genetically modified) bacterial strains.
- Recent studies showed that different lactococci probiotics (L. rhamnosus, L. reuteri, L. plantarum) were able to inhibit the formation of biofilm by different clinical isolates of S. mutans, and even to significantly reduce their viability.

- ***Persister eradication:***[4]

- Due to the multiple genetic and redundant mechanisms that appear to be involved in persister formation, ascertaining a drug for target selection may be difficult
- However, antimicrobials that target the bacterial membrane organization may be promising. Such membrane-acting antimicrobials would be lipophilic to directly bind and permeabilize the bacterial membrane bilayer to disrupt the physical integrity and numerous cellular functions.
- Two recently approved membrane-acting antibiotics, daptomycin and telavancin, have been in clinical use for treating S. aureus infections. Both of these have shown activity against S. aureus biofilms through the permeabilization of the bacterial membrane.

- ***Specifically targeted antimicrobial peptide (STAMP) technology***: [4]

- Broad-spectrum antibiotics may disrupt the patient's normal bacterial flora. Antimicrobial treatment specifically targeting each undesirable pathogen represents an attractive strategy.
- In 2006, a research group from the University of California, Los Angeles, reported the design of narrow spectrum molecules known as specifically targeted antimicrobial peptides (STAMPs).
- STAMPs have been shown to effectively eliminate the cariogenic biofilm organism S. mutans from a multi-species biofilm without affecting closely related non-cariogenic organisms. These STAMPs were constructed with peptides derived from the quorum sensing CSP signal molecule for selective S. mutans binding.
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II. Conclusion

Microbial biofilms in the root canal are highly resistant to disinfecting agents used in endodontic treatment. The complex and unpredictable nature of root canal anatomy and the multi-species biofilms amplify

the difficulty in eradication of the microbial biomasses from there. The objective of this review was to discuss the microbiological aspects of root canal biofilms, clinical antibiofilm strategies and research methods to study biofilms. It is likely that an optimal approach for biofilm removal will require the combination of several of the technologies discussed in this review, including improved methods for mechanical removal of biofilms using instrumentation combined with irrigation fluids, as well as enhanced chemical treatments and improved biocides that can inactivate microorganisms. The use of antimicrobial nanoparticles is an attractive avenue for further work, particularly as these could readily be added to existing irrigation fluids.

References

[1]. De Paz LE, Sedgley CM, Kishen A. The Root Canal Bio lm.
[2]. Rotstein I, Ingle JI, editors. Ingle's endodontics. PMPH USA; 2019 Jun 1.
[3]. Jhajharia K, Parolia A, Shetty KV, Mehta LK. Biofilm in endodontics: a review. Journal of International Society of Preventive & Community Dentistry. 2015 Jan;5(1):1.
[4]. Dufour D, Leung V, Lévesque CM. Bacterial biofilm: structure, function, and antimicrobial resistance. Endodontic Topics. 2010 Mar;22(1):2-16.
[5]. Ramachandran Nair PN. Light and electron microscopic studies of root canal flora and periapical lesions. J Endod 1987;13:29-39
[6]. Caldwell DE, Atuku E, Wilkie DC, Wivcharuk KP, Karthikeyan S, Korber DR, Schmid DF, Wolfaardt GM. Germ theory vs. community theory in understanding and controlling the proliferation of biofilms. Advances in dental research. 1997 Apr;11(1):4-13.
[7]. Siqueira JF, Rôças IN, Ricucci D. Biofilms in endodontic infection. Endodontic Topics. 2010 Mar;22(1):33-49.
[8]. Kishen A, Haapasalo M. Biofilm models and methods of biofilm assessment. Endodontic Topics. 2010 Mar;22(1):58-78.
[9]. Love RM. Biofilm–substrate interaction: from initial adhesion to complex interactions and biofilm maturity. Endodontic Topics. 2010 Mar;22(1):50-7.
[10]. Busscher HJ, Van der Mei HC. Physico-chemical interactions in initial microbial adhesion and relevance for biofilm formation. Advances in dental research. 1997 Apr;11(1):24-32.
[11]. Stewart PS, Franklin MJ. Physiological heterogeneity in biofilms. Nat Rev Microbiol 2008: 6: 199–210.
[12]. Basrani B, editor. Endodontic irrigation: chemical disinfection of the root canal system. Springer; 2015 Jul 17.
[13]. Lynch AS, Robertson GT. Bacterial and fungal biofilm infections. Annu Rev Med 2008: 59: 415–428.
[14]. Haapasalo M, Shen YA. Current therapeutic options for endodontic biofilms. Endodontic Topics. 2010 Mar;22(1):79-98.
[15]. Dalton BC, Ørstavik D, Phillips C, Pettiette M, Trope M. Bacterial reduction with nickel–titanium rotary instrumentation. J Endod 1998: 24: 763–767
[16]. Carver K, Nusstein J, Reader A, Beck M. In vivo antibacterial efficacy of ultrasound after hand and rotary instrumentation in human mandibular molars. J Endod 2007: 33: 1038–1043.
[17]. Metzger Z, Teperovich E, Zary R, Cohen R, Hof R. The self-adjusting file (SAF). Part 1: respecting the root canal anatomy—a new concept of endodontic files and its implementation. J Endod 2010: 36: 679–690.
[18]. Bao P, Shen Y, Lin J, Haapasalo M. In vitro efficacy of XP-endo Finisher with 2 different protocols on biofilm removal from apical root canals. Journal of endodontics. 2017 Feb 1;43(2):321-5.
[19]. Dunavant TR, Regan JD, Glickman GN, Solomon ES, Honeyman AL. Comparative evaluation of endodontic irrigants against Enterococcus faecalis biofilms. J Endod 2006: 32: 527–531.
[20]. Walsh LJ. Novel approaches to detect and treat biofilms within the root canals of teeth: a review. Antibiotics. 2020 Mar;9(3):129.
[21]. Neelakantan P, Romero M, Vera J, Daood U, Khan AU, Yan A, Cheung GS. Biofilms in endodontics—current status and future directions. International journal of molecular sciences. 2017 Aug;18(8):1748.
[22]. Halford, A.; Ohl, C.D.; Azarpazhooh, A.; Basrani, B.; Friedman, S.; Kishen, A. Synergistic effect of microbubble emulsion and sonic or ultrasonic agitation on endodontic biofilm in vitro. J. Endod. 2012, 38, 1530–1534.
[23]. Kishen A. Advanced therapeutic options for endodontic biofilms. Endodontic Topics. 2010 Mar;22(1):99-123.
[24]. *Kishen A, Shi Z, Shrestha A, Neoh KG. An investigation on the antibacterial and antibiofilm efficacy of cationic nanoparticulates for root canal disinfection. J Endod 2008: 34: 1515–1520*
[25]. *Yada S, Kamalesh B, Sonwane S, Guptha I, Swetha RK. Quorum sensing inhibition, relevance to periodontics. Journal of international oral health: JIOH. 2015 Jan;7(1):67*