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Endodontic Biofilms - A Review

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Abstract: ***Biofilm like structures are the main form in which bacteria infecting the root canal system are organized. Bacterial biofilms can be found in virtually all areas of the root canal system, including the main canal, apical and lateral ramifications, isthmuses, and recesses. Biofilms are very frequent in the apical part of root canals of teeth with primary or posttreatment apical periodontitis. It consists of microbial cells of different species and is organized in multiple cell layers, embedded within a matrix. The composition and structure of this endodontic biofilm are highly variable. Biofilm infection may be very difficult to reach and eradicate and may require special strategies for successful management. This review article briefly describes different types of endodontic biofilms, its characteristics and eradication.***

Keywords: Biofilm, Quorum sensing, Persister cells, Intracanal biofilm, Casernae

1. Introduction

Biofilms are defined as a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth and gene transcription [1]. Biofilm is the manner of bacterial growth which survives unfavourable environmental and nutritional conditions; the root canal environment will favour biofilm formation [2]. The importance of biofilm was for the first time enlighten by Bill Costerton, recognized as the "Father of Biofilm" [3]. The occurrence of biofilm structures in infected root canal was demonstrated by PNR Nair (1987) [4]. The term biofilm was coined for the first time by Poul Harremoes, a Danish physicist, in a paper dealing with the diffusion kinetics of fluids into slimy biofilters that he called 'biofilms'.

2. Criteria for Biofilm

According to Caldwell, microbial biofilm is considered as a community and the microorganisms living in the community must possess the ability to self organize (Autopoiesis), should resist environmental perturbations (Homeostasis), must be more effective in association than in isolation (Synergy), should respond to environmental changes as a unit rather than as single individuals (Communality) [5] [6].

3. Ultrastructure of Biofilm

Biofilms are heterogenous arrangement of microbial cells on a surface. In bacterial biofilms, individual cells grow and aggregate to form microcolonies (populations) that are embedded and non randomly distributed in the EPS matrix/Glycocalyx and separated by water channels [1]. Open water channels contain sessile cells [9]. These water channels provide circulating nutrients and helps in exchanging metabolic products (Fig.2) [8]. The basic building block or structural unit of the biofilm is the microcolony. Fully hydrated biofilms are composed of cells (±15% by volume) and of matrix material (±85%by volume), and the cells are located in matrix enclosed "towers" and "mushrooms" (Fig.1) [1]. Dental biofilms can exhibit up to 300 or more cell layers of thickness. EPS are hydrated biopolymers (usually polysaccharides, but also proteins, nucleic acids & lipids) [7]. Bill coined the phrase ' casernae' to convey the concept of EPS having a functional structural role [26]. The important functions include: [4]

1) Act as a biological glue
2) Mechanical stability to biofilm
3) Keep biofilm cells in close proximity
4) Act as a nutrient source
5) Retains water and maintains highly hydrated microenvironment
6) Protective role against host defense cells and molecules
7) Exchange of genetic information

Figure 1: Cells located in matrix enclosed 'towers' and 'mushrooms' [1]

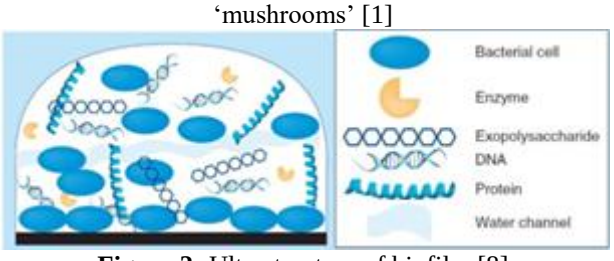

Figure 2: Ultrastructure of biofilm [8]

4. Development of Biofilm

The development of biofilm is a dynamic process involving successive steps (Fig.4). The pattern of oral biofilm formation is spatiotemporal pattern [4]. It is a five stage process which includes:[10] (Fig.3)

1) Initial attachment of cells to the surface
2) Production of EPS – more firmly adhered irreversible attachment
3) Early development of biofilm architecture
4) Maturation of biofilm architecture
5) Dispersion of single cells from biofilm

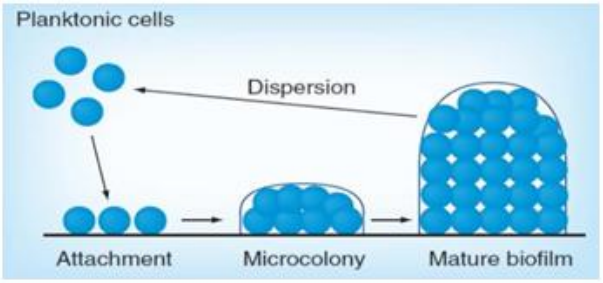

Figure 3: Development of biofilm [8]

First stage: Attachment of bacterial cells to selected abiotic or biotic surface.Usually adhere to a conditioning film which is composed of organic molecules (nutrients, salivary proteins, large macromolecules).Weak and reversible contact between the cell and conditioning film, which results from brownian motion, gravitation, diffusion or electrostatic interactions. [11]

Second stage: Once attachment to a surface has been affected by reversible attachment, the bacteria must maintain contact with the substratum and grow in order to develop a mature biofilm. There is a change from reversible to irreversible (Zobel 1943). Transition from weak interaction of the cell with substratum to a permanent bonding mediated by extracellular polymer [10]. The main components of EPS includes:

1) Water (major component)
2) Exoplysaccharides
3) Extracellular proteins
4) Extracellular DNA (eDNA)

Exoplysaccharides provides structural stability; act as a scaffold for proteins which mediate intercellular attachment and adhesion of biofilm to a surface and protection of biofilm from host defense

Extracellular protein has structural and enzymatic functions. It maintains biofilm architecture by linking bacteria and exoplysaccharides

Extracellular DNA (eDNA) has role in initial attachment of biofilm, adhesion, aggregation, cohesion and exchange of genetic materials. The primary source of eDNA is autolyzed cells controlled by altruistic suicide and fractricidal release of DNA in e.faecalis [4].

Phase 1 - transport of microbe to the substrate surface
Phase 2 - initial non - specific microbial substrate adherence phase Phase 3- specific microbial – substrate adherence phase [5]

Third stage: Bacterial growth and biofilm expansion occur. Microcolony is formed by the monolayer of microbes which attracts secondary colonizers, and give rise to final structure of biofilm. Two types of microbial interaction occur at the cellular level during formation of biofilm:

- Co- adhesion
- Co- aggregation

Co – aggregation is a process by which genetically distinct bacteria become attached to one another via specific molecules Co – adhesion is a process by which bacterial cells in suspension specifically adhere to cells in biofilm [8] [13].

Fourth stage (Biofilm maturation): – Maturation results in generation of complex architecture, channels, pores and redistribution of bacteria away from substratum. Macrocolonies surrounded by water channels helps to distribute nutrients and signaling molecules [10].

Fifth stage (Detachment): – When nutrients become limited to survive or to spread and colonize to other niches, some biofilm cells can detach individually or in clumps. Dispersing biofilm cells revert to the planktonic mode of growth; thus, the biofilm developmental life cycle comes full circle [10].

Mechanism of biofilm dispersal:

- **Active dispersal**
- **Passive dispersal**
  Active dispersal is initiated by bacteria themselves
  Passive dispersal is mediated by external forces like fluid shear and abrasion Three distinct mode of bofilm dispersal:
  - **Erosion** - continuous release of single cells or small clusters of cells at low levels over the course of biofilm formation
  - **Sloughing** – sudden detachment of large portions of biofilm, during the later stages of biofilm formation
  - **Seeding dispersal / Central hollowing** – rapid release of large number of single cells or small cluster of cells from hollow cavities inside biofilm [14]

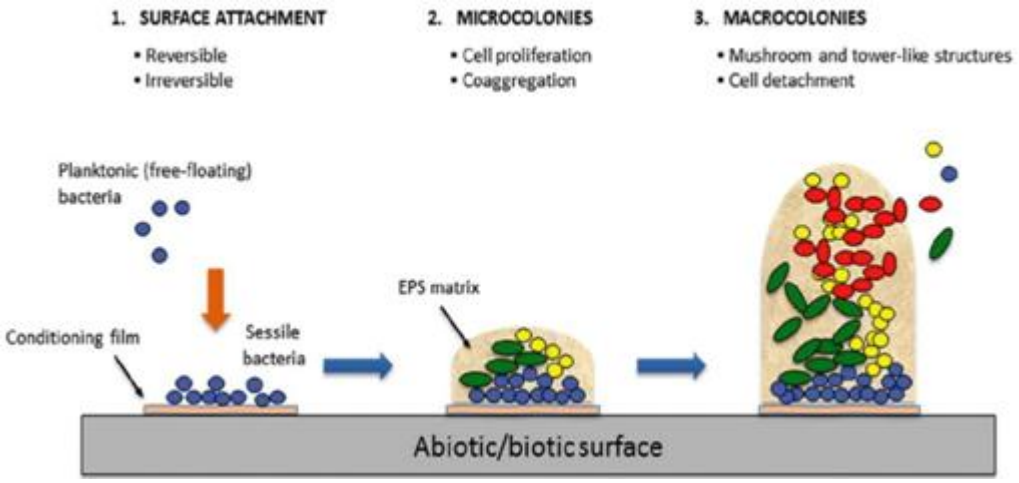

Figure 4: Schematic representation of the distinct steps in microbial biofilm development [11]

5. Characteristics of Biofilm

Antimicrobial resistance [12]
Biofilm formed by oral bacteria is more resistant to chlorhexidine, metronidazole than planktonic cells. Resistance depends upon substrate, microenvironment, age of the biofilm. There are two types of resistance. (Fig.5)

1) Physical resistance
2) Acquired resistance
   a) Differentiation of cells with low metabolic activity
   b) Differentiation of cells – actively respond to stress
   c) Differentiation of cells with high persistent phenotype

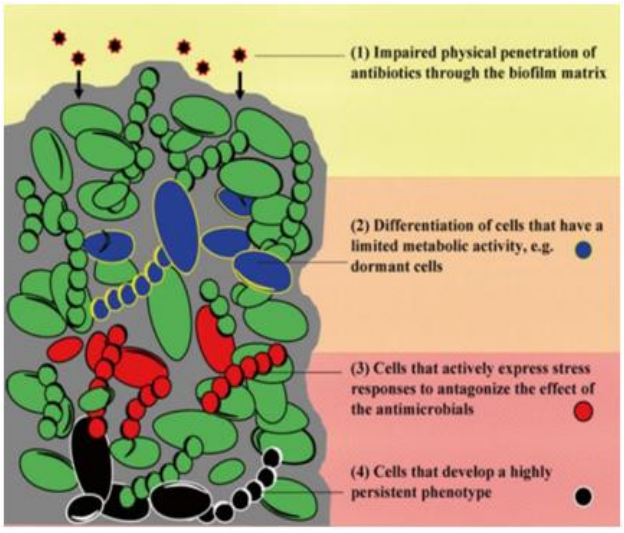

Figure 5: Mechanism of resistance by biofilm bacteria

Cell – Cell Communication and Quorum Sensing (Fig.6)
Cell – cell communication is used as an aid in coordination of bacterial population behaviour for biofilm formation and community development. Bacteria use small diffusible molecules or peptides as signal for cell – cell communication. Autoinducer 2 is a non- specific signalling molecule for inter bacterial communication. Quorum sensing is a cell – cell communication process that regulates gene expression at high density. When a high density population reaches a certain threshold ("quorum"), the concentration of normally low levels of certain signal molecules become high enough to act as autoinducers that trigger a synchronized response throughout the biofilm population. Two types of quorum sensing: Intra – species communication and Inter – species communication. [4] [11] [15]

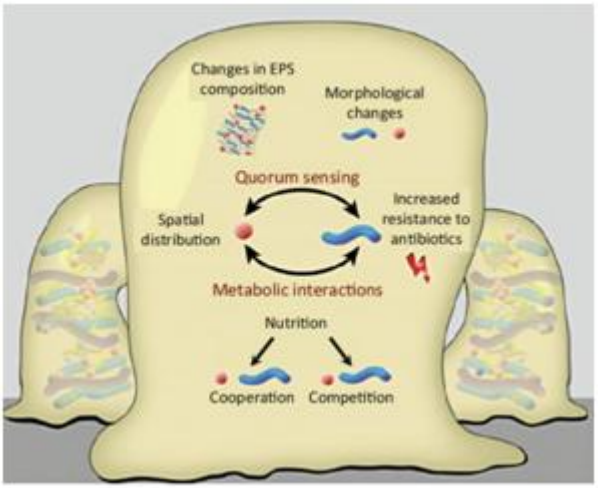

Figure 6: Individual and social processes occurring within biofilm communities

Horizontal Gene Transfer
HGT (Horizontal gene transfer) allows movement of genetic information both within and between species. Three basic modes:

1) Transformation
2) Transduction
3) Conjugation

Most efficient HGT process in bacteria is conjugation. Biofilms are uniquely suited for genetic exchange. They sustain high bacterial density and DNA can be trapped within extracellular matrix. Open channels and pores enable more frequent cell collisions, thus rapid spread of plasmid borne genes by conjugate gene transfer [4].

Stress Responses
Microbial cells in deeper layer of biofilm have limited amount of nutrients and oxygen. Thus reduced metabolic activity results in enhanced tolerance toward antibiotics and persistence of biofilm infection. Different types of stresses

like nutrient stress, oxidative / nitrosative stress, envelope stress, heat stress, ribosomal stress. Stress response includes down regulation of error correcting enzymes, upregulation and activation of error – prone DNA polymerases. Regulators that control these responses include alternative sigma factor RpoS, RpoH, gene repressor LexA, Toxin antitoxin system [4] [16].

Persister cells

Persister cells are small subpopulation of susceptible and genetically homogenous population of bacteria that survive antibiotic exposure [17]. These specialized cells enter into a state of dormancy which survive stress conditions and prevents death. They are phenotypic variants of wild type that form stochastically in a clonal population of genetically identical cells. These are formed through a combination of continuously occurring random events (stochastic) or in response to an environmental stimulus (deterministic) [4].

Upregulated efflux pumps

Efflux system can actively pump toxic substances and antibiotics out of cells. Multidrug resistant efflux pump also contribute to antibiotic resistance in biofilm. Addition of efflux pump inhibitors (verapamil) to endodontic antimicrobial medicaments (calcium hydroxide etc) will enhance antibiofilm activity. [4] [18]

Heterogeneity and oxygen gradients

According to Stewart & Franklin, Cells located in upper biofilm layer consume all available oxygen and grow aerobically, while an anaerobic micro – niche developed underneath the aerobic layer. Oxygen – and nutrient-depleted 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] [19].

6. Endodontic Biofilm

Endodontic biofilm formation mechanism

For biofilm formation in root canal, pulp would have to become necrotic and liquefied before bacterial invasion. Caries is a disease caused by biofilms. As the caries lesion advances toward the pulp, so does the biofilm that causes it. Eventually when the last dentin layer is destroyed in advanced caries lesions, the pulp becomes exposed primarily to the caries biofilm and also to planktonic bacteria floating in saliva. Thus the pulp portion beneath the carious lesion becomes severely inflamed, necrotic, and eventually the front line of infection advances to involve first the tissue in the pulp chamber and then moves inward in the pulp in an apical direction. Biofilm is present at the front line of infection. These events of aggression, inflammation, necrosis, and infection occur by compartments of pulp tissue and gradually move in an apical direction. Finally the apical canal will become necrotic and infected. In the advanced front of a root canal infection, the biofilm enters into contact with the host tissue, which is inflamed. Biofilm not only adhered to canal walls, but also covering the surface of the inflamed tissue in the forefront of the infection [7].

Biofilms and the Community – As – Pathogen Concept

Most endogenous infection is caused by mixed biofilm communities. The concept of the community as pathogen is based on the principle that 'team work is what eventually counts'. By this, the community behaviour and the outcome of host/ bacterial community interaction will ultimately depend upon the community membership and the myriad of associations within the community. The pathogenesis of apical periodontitis is resultant of the concerted action of bacteria in a multispecies community. Bacterial virulence factors involved in the pathogenesis of apical periodontitis consist of a summation of structural cellular components, antigens and secreted substance that accumulate in the biofilm. The concentration and virulence of this bacterial "soup" will depend upon the population density, species composition, and bacterial interactions in the community. Once the biofilm forms in the apical canal, this" soup" of antigens and virulence factors becomes in constant and direct contact with peri radicular tissue to cause damage and stimulate or modulate the host immune response [20] [4].

Biofilm and Apical Periodontitis

Nair (1987) described biofilm as "bacterial condensation on the surface of the dentin wall, forming thin- or thick- layered bacterial plaques." In addition to main root canal, bacterial biofilms also seen in anatomical variations of the root canal system, including apical ramifications, lateral canals, and isthmuses. In 2003, Parsek and Singh proposed 4 criteria to determine whether a given infectious disease can be classified as a disease caused by biofilm communities.

1) Infecting bacteria are adhered to or associated with a surface
2) Bacteria forming clusters or micro colonies encased in an extracellular matrix
3) Infection is generally confined to particular site, and although dissemination may occur, it is a secondary event
4) Infection is difficult or impossible to eradicate with antibiotics Later, a 5th criterion was suggested by Hall – Stoodley and Stoodley (2009)
5) Ineffective host clearance. This may be evidenced by the location of microbial colonies in areas usually surrounded by host defense cells. Accumulation of PMNs and macrophages near bacterial aggregates/ co aggregates
6) 6th criteria was added by Ricucci and Siqueira (2010)
7) Elimination or significant disruption of the biofilm structure and ecology leads to remission of the disease process. [21] [22] [23]

Types of Endodontic Biofilm

1) Intracanal biofilm
2) Extra radicular biofilm
3) Periapical biofilm
4) Biomaterial centered biofilm

Intracanal Biofilm

Intracanal biofilms are microbial biofilms formed on the root canal dentin of an infected teeth. Biofilms, most often composed of several morphotypes, grow in multilayers or as aggregates on the dentin walls of root canal or as dense

aggregates in the necrotic tissue. Spatial organization for root canal biofilms were seen as a palisade structure of filaments, chains of cocci perpendicular to canal wall, corn cob like structure of cocci attached to biofilm. The extracellular matrix material of bacterial origin was also found.

Extraradicular Biofilm

Extraradicular biofilms formed on the root surface adjacent to the root apex of endodontically infected teeth are root surface biofilms. Such biofilms have been composed of various microbial forms, even including yeasts. They have been demonstrated mainly in cases with acute symptoms with and without sinus tracts (fistulas), or in cases not responding to endodontic treatment. Sometimes, the extraradicular biofilm becomes calcified and gets associated with periapical inflammation and delayed periapical healing in spite of adequate orthograde root canal treatment.

Periapical Biofilm

They are isolated biofilms found in the periapical region of endodontically infected teeth. Periapical biofilms may or may not be depend on the root canal. These microorganisms have the ability to overcome host defense mechanisms, thrive in the inflamed periapical tissue and subsequently induce a periapical infection.

Biomaterial- Centered Infection

Biomaterial centered infection is caused when bacteria adhere to an artificial biomaterial surface and form biofilm structures. Presence of biomaterial in close proximity to host immune system can increase the susceptibility to biofilm. It can be form on root canal obturating materials These biofilms can be intraradicular or extraradicular depending on whether obturating material within canal or has extruded beyond the root apex [2] [24].

7. Biofilm Detection

a) Atomic force microscopy- detects forces of interaction among bacterial cells and between bacterial cells and substrates
b) Scanning electron microscopy – detects structure of microbial ecosystem (Fig.7)
c) Confocal scanning laser microscopy (CSLM) – 3D reconstruction of microbial biofilm, reveals highly heterogeneous structure of biofilm (Fig.8)
d) Fluorescent probes along with CSLM – discriminate between classes, genera, species, viability of individual organism in microbial ecosystem
e) Fluorescence in situ hybridization (FISH) – simultaneous detection of phylogenetically different bacteria
f) FISH with oligonucleotide probe – specifically identifies targeted bacteria [4] [12]

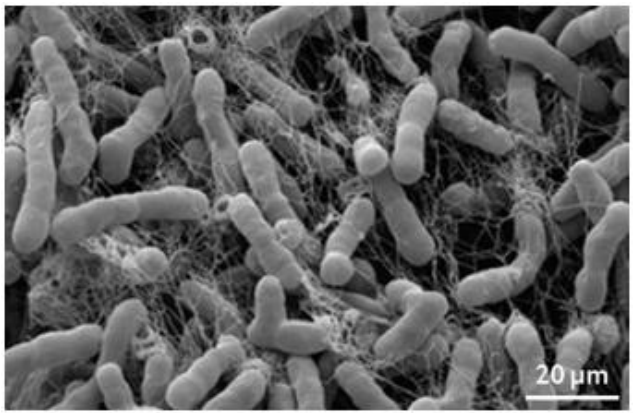

Figure 7: SEM clearly showing the bacteria connected by nanotubules which were hypothesized to have conductive properties [26]

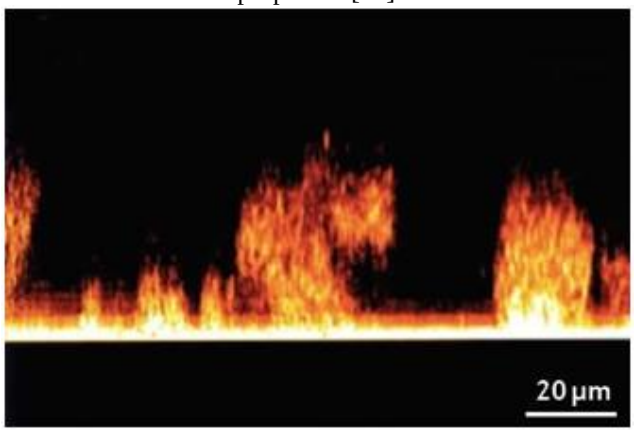

Figure 8: The confocal image of a laboratory – grown biofilm shows cell clusters (that is bacterial cells held together and to the glass surface in an EPS matrix) separated by water channels [26]

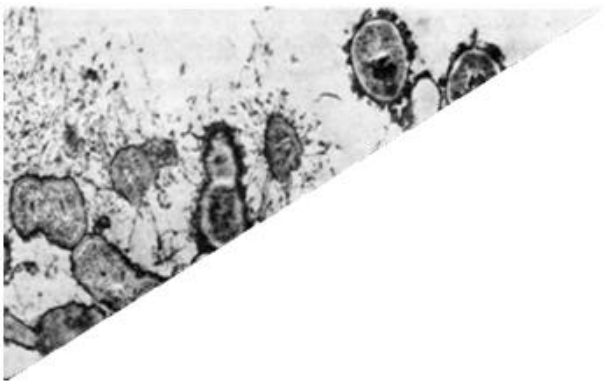

Figure 9: The TEM (transmission electron microscopy) of a biofilm community shows that cells are held together by extracellular bacterial glycocalyx (EPS) [26]

8. Eradication of Biofilm

Along the years, different therapeutic strategies have been developed to prevent biofilm formation and to eliminate established biofilm – related infections. Most of the strategies are summarized in Fig.10. Instrumentation has an important role in removing biofilm, where the instruments can gain direct contact with root canal wall. Sodium hypochlorite 1%, 6% dissolve biofilm in addition to direct killing of microbes. 2% Chlorhexidine also kill biofilm bacteria but not disrupt the biofilm structure. Other

strategies includes antibacterial nanoparticles like chitosan, zinc oxide nanoparticles, antimicrobial photodynamic therapy, herbal products like phenolics, terpenoids, alkaloids, lectins and polypeptide, combination of polyisoprene or polycaprolactone and nanomeric bioactive glass 45S5, surface coating with solution of benzalkonium chloride. [4] [12] [25]

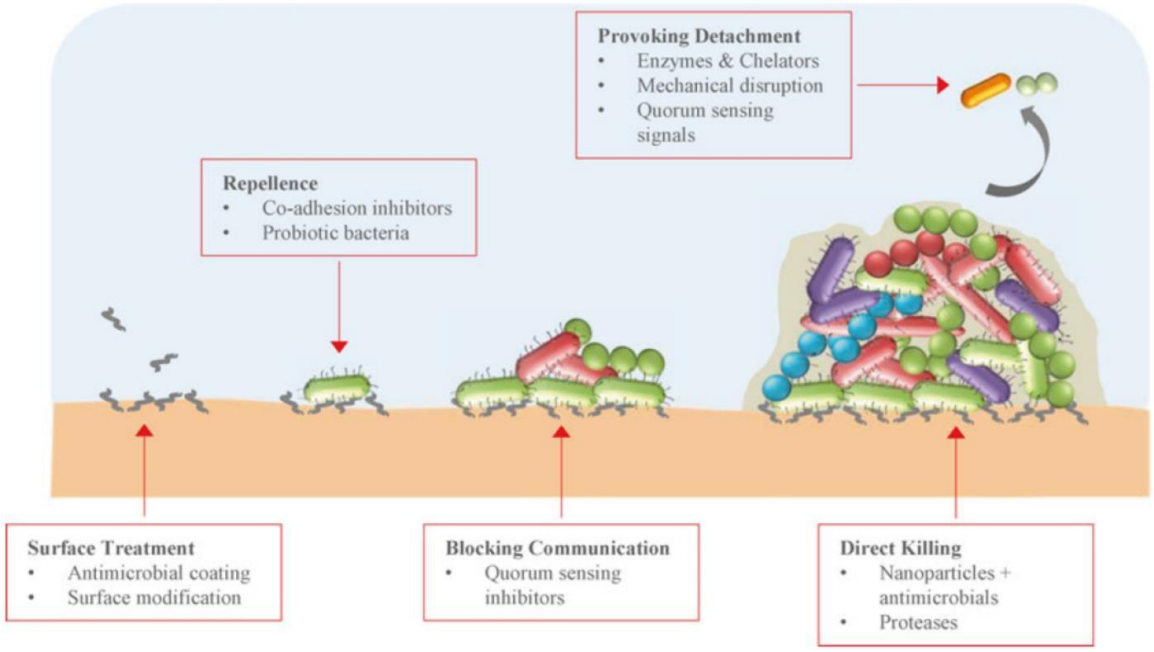

Figure 10: Antibiofilm strategies

9. Conclusion

Endodontic infections are caused by multispecies biofilms and that the interaction between different organisms can contribute to apical periodontitis progress and clinical outcome. Elimination of bacterial biofilm from root canal system and exterior root surface is necessary to maximize a favourable outcome .Root canal environment is a challenging locale to accomplish the goal. Different protocols ranging from antimicrobial root canal irrigation to advanced methods are used. Further research in basic microbiological process such as molecular basis and biological effect of these host- bacterial connections may lead to an improvement of treatment regimens and also may identify new strategies for disease control.

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