Elaboration of an artificial polybacterial biofilm as a model for endodontic disinfection procedures

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DISSERTATION

To obtain PhD degree

Elaboration of an artificial polybacterial biofilm as a model for endodontic disinfection procedures

By

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To my little princess

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Abbreviations

ATCC	American Type Culture Collection
CHX	Chlorhexidine
CLSM	Confocal Laser Scanning Microscopy
E.	*Enterococcus*
ECM	Extra cellular matrix
EDTA	Ethylenediaminetetraacetic acid
F.	*Fusobacterium*
FISH	Fluorescence In Situ Hybridization
NaOCl	Sodium hypochlorite
P.	*Porphyromonas*
PDT	Photodynamic Therapy
PS	Photosensitizer
PUI	Passive Ultrasonic Irrigation
rRNA	Ribosomal Ribonucleic Acid
S.	*Streptococcus*
SEM	Scanning Electron Microscope
TEM	Transmission Electron Microscopy
WL	Working length

1

Introduction

Periapical periodontitis is an inflammatory response of the tissues surrounding the apex of the tooth root against the bacterial invasion of the tooth pulp. The prevalence of periapical infections increases with age. One of two individuals of 50 years of age and more experiences this problem and this ratio augments to nearly 62% above the age of 60 (1).According to the 2005-06 Survey of Dental Services rendered by the American Dental Association, out of 23 million endodontic treatments, 15 million were root canal treatments The failure rate was 13% which is about 1,900,000 teeth (1, 2). Microbial infection plays an important role in the development of dental pulp necrosis and the possible formation of periapical infection and future tooth loss. Elimination of microorganisms from the root canal space is the main goal of root canal treatment of the infected teeth. It is apparent that mechanical debridement in combination with chemical irrigation removes the bulk of the infecting microorganisms; however, due to tri-dimensional structure of root canal system (3, 4), residual bacteria could still be detected just before filling the root canal. The previous studies have shown that treatment might achieve a success rate up to 94% in a microorganism free root canal environment prior to root filling, treatment may achieve a success rate up to

94%. This success rate drops to 68% in the presence of bacteria inside the root canal at the moment of obturation (5). In biofilms like other microenvironment, the adaptive characteristics of each microorganism evolve subsequent to the biofilm formation. This ecological point of view on root canal infection is the base for the concept of collective pathogenicity of biofilm components rather than infection caused by an individual species. Polymicrobial unit goes through different physiological and genetic alteration following series of root canal environmental changes such as pH modifications, reduction of nutriment, arrival of different bacterial species and etc.(6, 7).

1.1 What is a biofilm?

Biofilms can be defined as a sessile population of microorganisms attached to a surface and embedded in a self-generated extracellular matrix of polysaccharides and proteins. The matrix typically takes 85% of the volume of a biofilm (8) and acts as the first environment for integrated microorganisms (9). The majority of microorganisms in nature are found in biofilms. The ability to attach to and be retained on a surface is a fundamental survival strategy for most prokaryotic organisms. Gene expression can be markedly altered when cells form a biofilm, resulting in a radically different phenotype following the attachment to a surface. Intercellular communication systems are used by some bacteria within the biofilm to synchronize gene expression (10). Quorum sensing is one of these regulatory mechanisms. Quorum sensing is a bacterial intercellular communication system for controlling bacterial functions, such as virulence and biofilm formation (11). Autoinducers are diffusible chemical molecules, which regulate the signaling process. Presence of a minimal stimulatory concentration of autoinducer can trigger alteration of the gene expression (12).

This intercellular communication could not occur without the ECM, which immobilizes microorganisms and keeps them close to each other (9). In addition, due to its capability to accumulate nutrients as a source of energy, ECM acts as a water retainer to protect bacteria inside the biofilm from desiccation. ECM could serve also as a source of energy for the biofilm through its biochemical compounds (13).

Microorganisms in biofilm communities have enhanced abilities against antimicrobial agents (14). Extracellular matrix acts as a diffusion barrier and inhibits binding of certain antibiotics to biofilm forming bacteria (15). Thus, bacteria organized in biofilm might escape

to host immune system (16) and are protected from ecological competition of other microorganisms and increase their pathogenicity (10).

Acute diseases caused by planktonic pathogens have been mainly eliminated, since causes of these illnesses have been identified and neutralized with vaccines and antibiotics. The new microbial pathogens are common and abundant in nature; they live in protected communities where they resist antibiotics and host defenses through many barriers, and they can raise small or large acute attacks on the host that may eventually accomplish when his or her defense system is down (17). The immune system may overleap bacteria incorporated within biofilm due to hidden antigens; this may lead to repression of phagocytic cells' ligand expression. The biofilm matrix can act as a shield against physical damage (18).

1.2 Oral Biofilm and Plaque

Biofilm formed on tooth surfaces is called dental plaque. The oral cavity consists of different tissues: hard and soft surfaces of these tissues act as a substrate for biofilm formation. However, the high superficial shedding rate of soft tissues (with the exception of dorsum of the tongue) disturbs significant plaque buildup (19).

In adults, microbial populations of a mature dental plaque consists of about 500 different species (20) enclosed in a matrix of bacterial and salivary origin. The very first bacteria attach to the tooth surface by means of the acquired pellicle; this pellicle contains salivary molecules such as proline rich proteins, histatins and statherins which have intention to bind to tooth surface (21). The acquired pellicle forms shortly after cleaning of teeth and bacterial colonization is detectable in minutes (22).

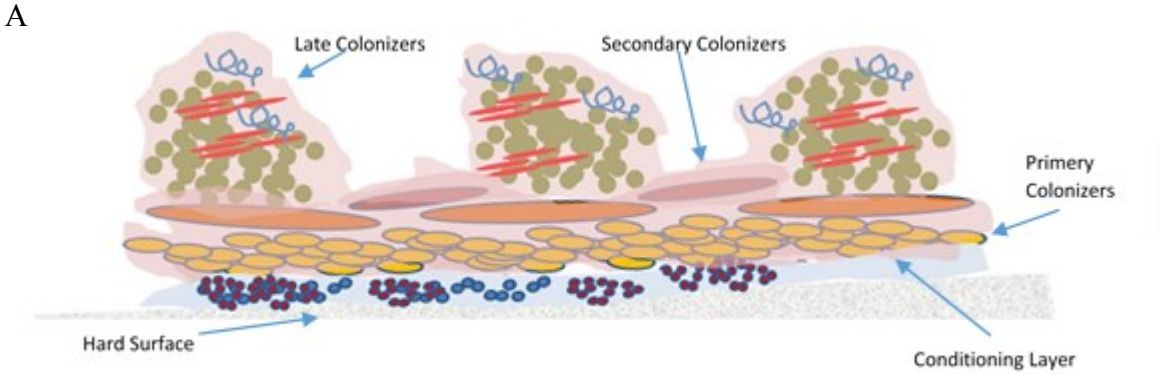
Cocci (mainly *Streptococci*) are identified as pioneer colonizers. Colonization comprises two steps: first phase, bacterial adhesion to the pellicle by means of adhesins on cell surface and special binding-site on acquired pellicle and second phase, bacterial multiplication by cell division (doubling) and binding to other bacteria through the process of co-adhesion (23).

The characteristics of the bacterial community begin to alter. For instance, gram-negative bacteria of the genus *Fusobacterium* act as a bridge between primary colonizers and later colonizers. That is, primary colonizing *Streptococci* cannot aggregate with late colonizers directly, but can do so via their ability to co-aggregate with *Fusobacterium* species. *Propionibacteria, Prevotellae, Veillonellae*, and *Selenomonas flueggei* are among the late colonizing organisms (Fig. 1A) (24).

Aerobic organisms such as *Neisseria* and *Nocardia* are in inverse proportion with the progression of plaque development. However, the number of anaerobic organisms like *Fusobacterium* and *Veillonella* increase as plaque grows. Nevertheless, growth of anaerobic organisms is dependent upon prior growth of aerobic and facultative anaerobic organisms leading to an increase in plaque thickness granting suitable condition for anaerobic growth (25).

The final phase of biofilm formation is the process of detachment. The role of cell detachment during the course of biofilm formation is not known. It is reasonably possible that the detachment of adherent cells helps bacteria to spread to a new site (Fig. 1B) (26).

A



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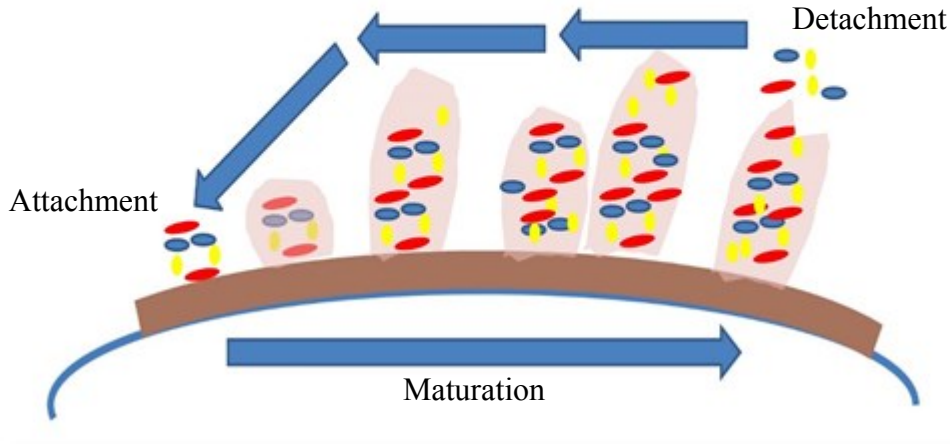


Figure 1. A: Layer by layer assembly of Dental Biofilm consisting of conditioning layer, primary, secondary and late colonizers. **B:** Biofilm formation is a cycle consisting of three stages: Adhesion, Colonization and Detachment.

1.3 Root Canal Anatomy, Histology and Physiology

The pulp tissue is present inside the pulp cavity of the teeth, which is not exposed to the micro flora and subsequently remains usually sterile. The pulp cavity is divided in pulp chamber and radicular pulp. Blood, lymph vessels and nervous fibers enter in and exit from the pulp cavity via apical foramina. They ramify in the pulp tissue bringing blood and innervation (Fig. 2A).

Pulp and dentin act as a unit (Pulp-Dentin complex; Fig. 2B-2C). Pulp is responsible for dentin formation. Enamel and dentin protect the pulpal tissue from physical and microbial attacks. Dental pulp tissue is formed of four different layers. The outer most layer of a healthy pulp consists of odontoblast cells. These cells are responsible for secretion of dentinal matrix. Their bodies remain in the pulp chamber and odontoblast processes go through predentin and dentin into dentinal tubules. The second layer called the cell-poor or cell-free layer of Weil, is a space of about 40 μm subjacent to the odontoblast layer. Arterioles, venules and nerves cross this layer. We find in the third layer called the cell-rich zone, high density of fibroblasts, undifferentiated mesenchymal cells and cells present in the last layer.

Lastly, the main bulk of pulp is called pulp proper, which consists of loose connective tissue. Main blood and nervous supply of pulpal tissue are present in this part.

The most specific cells of the pulp are odontoblasts, which are responsible for dentinogenesis during tooth life. However, fibroblasts are most numerous cells present in the pulp, these cells control the collagen turnover of pulp tissue via their collagen generation and digestion functions. Macrophages, dendritic cells and lymphocytes are main cells of the immune system present in pulpal tissue.

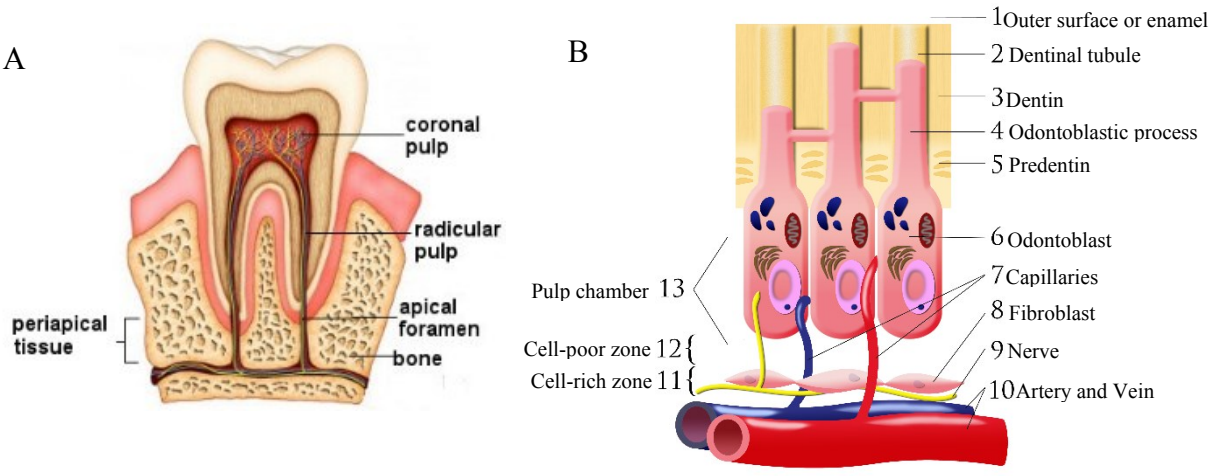


Figure 2. A. Schema of Dental pulp. (http://www.infodentis.com/tooth-anatomy/tooth-structure.html), **B**. Illustration of odontoblast layer and subodontoblastic region of the pulp (Furst I, Wikipedia), **C**. Histological view of Pulp-Dentin complex (Courtesy of Rocca JP, UNSA)

Dentin is mainly composed of hydroxyapatite crystals covered by collagen type I. Other collagen types (III, V, and VI) and non-collagenous proteins and proteoglycans are present as minor elements (27). Dentinal tubules are the result of continuous deposition and mineralization of the dentinal matrix around the cytoplasmic extension of odontoblasts so-called Tomes Fibers (Fig.2B). The anatomy of the Root canal system is complex: in general it consists of a main canal, lateral and accessory canals and microscopic dentinal tubules. Dentinal tubules extend from the pulp (with an S shape form) to the dento-enamel junction in the crown of the tooth and outer cement in the root (28). The number of dentinal tubules is higher at the pulpal side (about 55000/mm^2) than at the extremities (about 15000/mm^2) (Fig. 3). The deposition rate at the distal end of dentinal tubules is more than the surface near the pulp which makes dentinal tubules narrower near the dentino-enamel junction (0.9 µm). The openings are about 2.5µm which is big enough to allow bacterial penetration into the dentinal tubules (27).

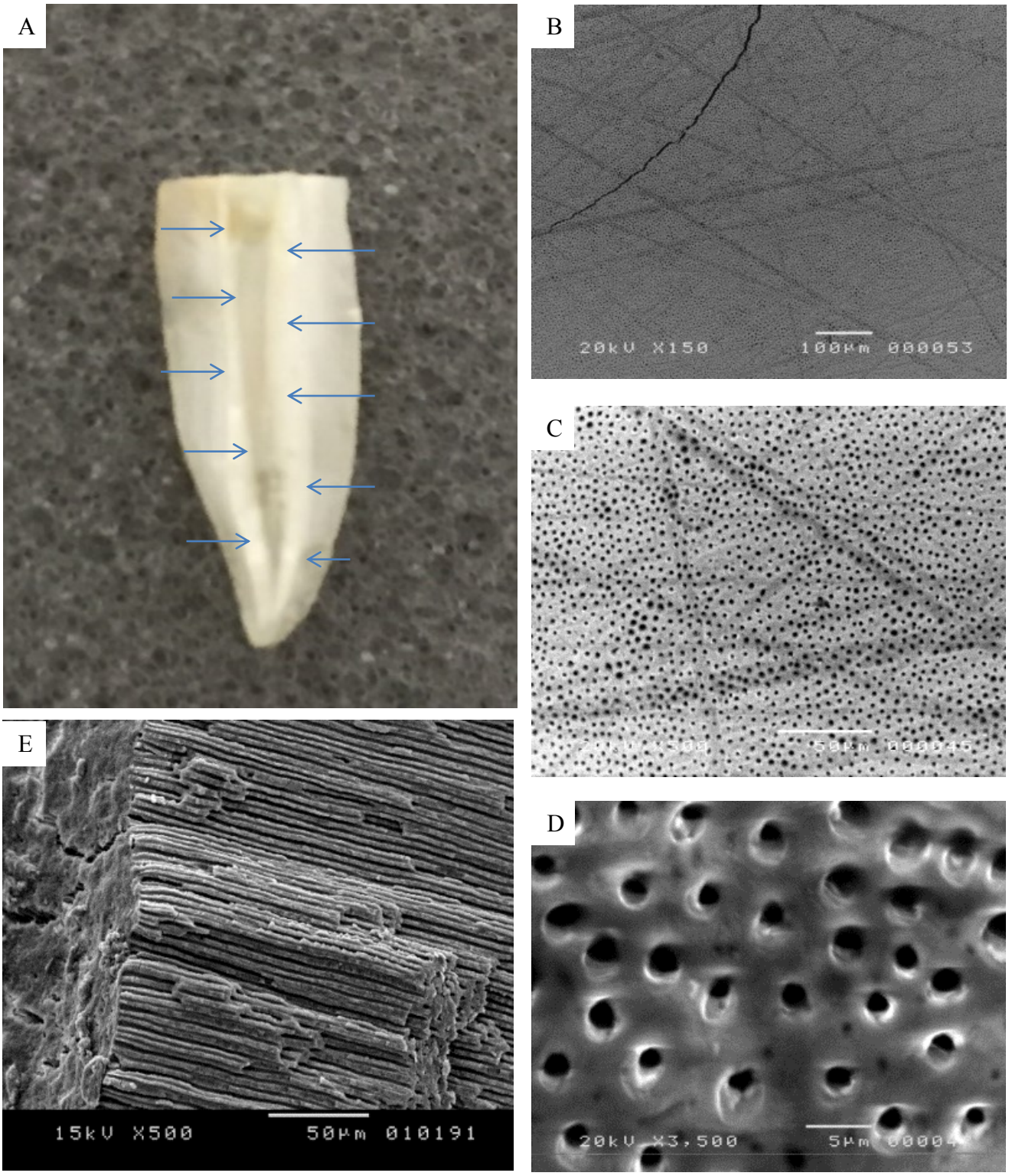


Figure 3. Anatomy of Root Canal, **A:** Root Canal space. **B, C and D:** Different magnification of Dentinal surface with dentinal tubules openings. **E:** Lateral view of Dentinal tubules.

1.4 Root Canal Infection

Microbial biofilm is the main cause of apical periodontitis. Success of root canal treatment of infected teeth is linked to removal of the biofilm from the root canal space (29, 30). Miller *et al.* first reported the ability of cocci and rods to invade dentinal tubules (31) and Kakehashi *et al.* demonstrated the pathogenic role of microorganisms during pulpal and periapical disease of germ-free rats when pulp is surgically exposed to outer zone (32).

The contamination of this sterile space mainly occur after demineralization of enamel, cementum and dentin resulted by caries which allows the penetration of bacteria into the dental pulp (Fig. 4). However, pulp tissue contamination may occur following dental trauma (33), congenital deformities of teeth (34), defective dental restoration (35) and possibly anachoresis (36, 37). Dentinal tubules are pathways for microorganisms to invade the root canal space (Fig. 5), though the degree of bacterial invasion varies according to the patency of dentinal tubules in different regions of root canal walls (38). However, the dentinal invasion is not significant in case of viable pulp. Odontoblastic processes and their associated structures within dentinal tubules restrict the movement of bacteria toward the pulp. In case of colonization, the immune system of a vital pulp tissue can eliminate the microorganisms and their injurious product (39). If there is no treatment, persistent bacterial attack of the pulp and a continuous inflammatory process will lead to pulpal necrosis and later to periapical disease. Untreated endodontic infection may lead to tooth loss.

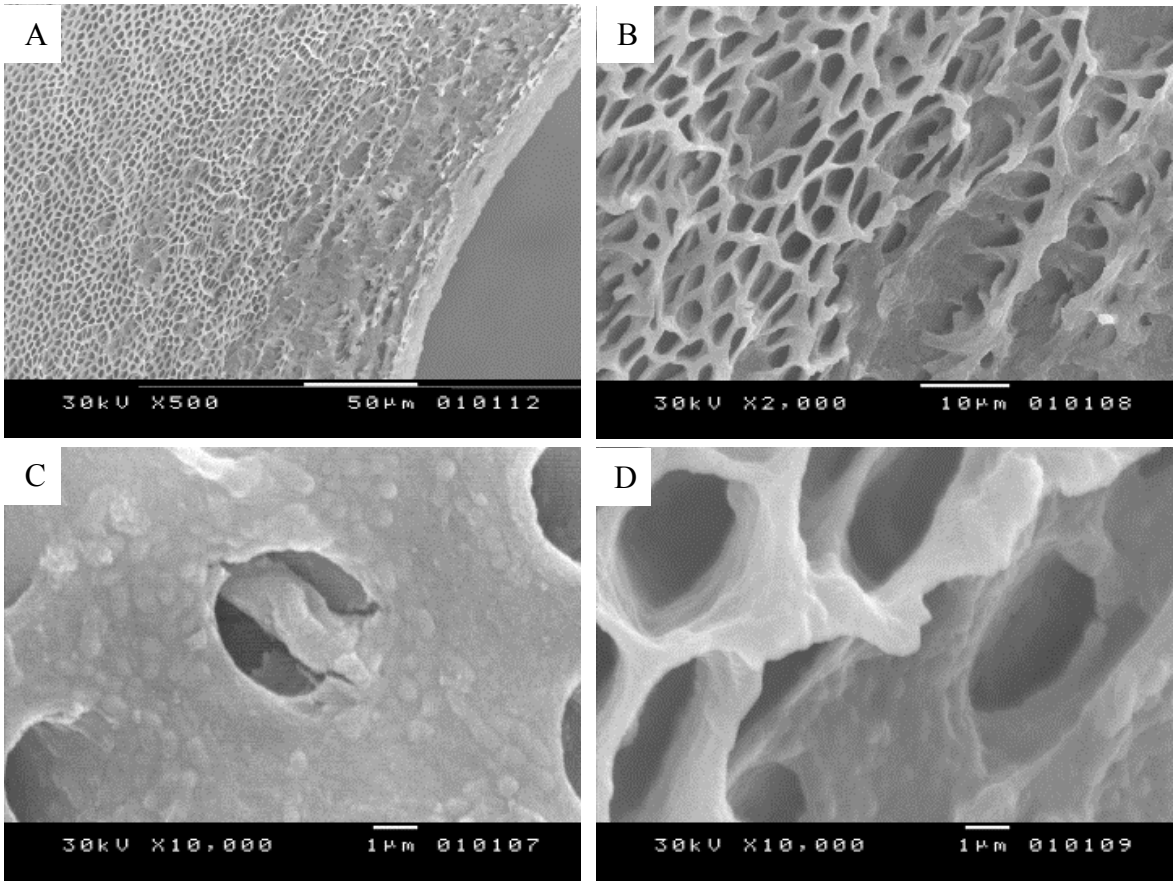


Figure 4. Anatomy of Root Canal. **A and B:** Different magnification of an artificially infected dentin. **C and D:** Higher magnification of same samples, modifications in size and form of openings of dentinal tubules and bacteria attached to dentinal surface could be noticed.

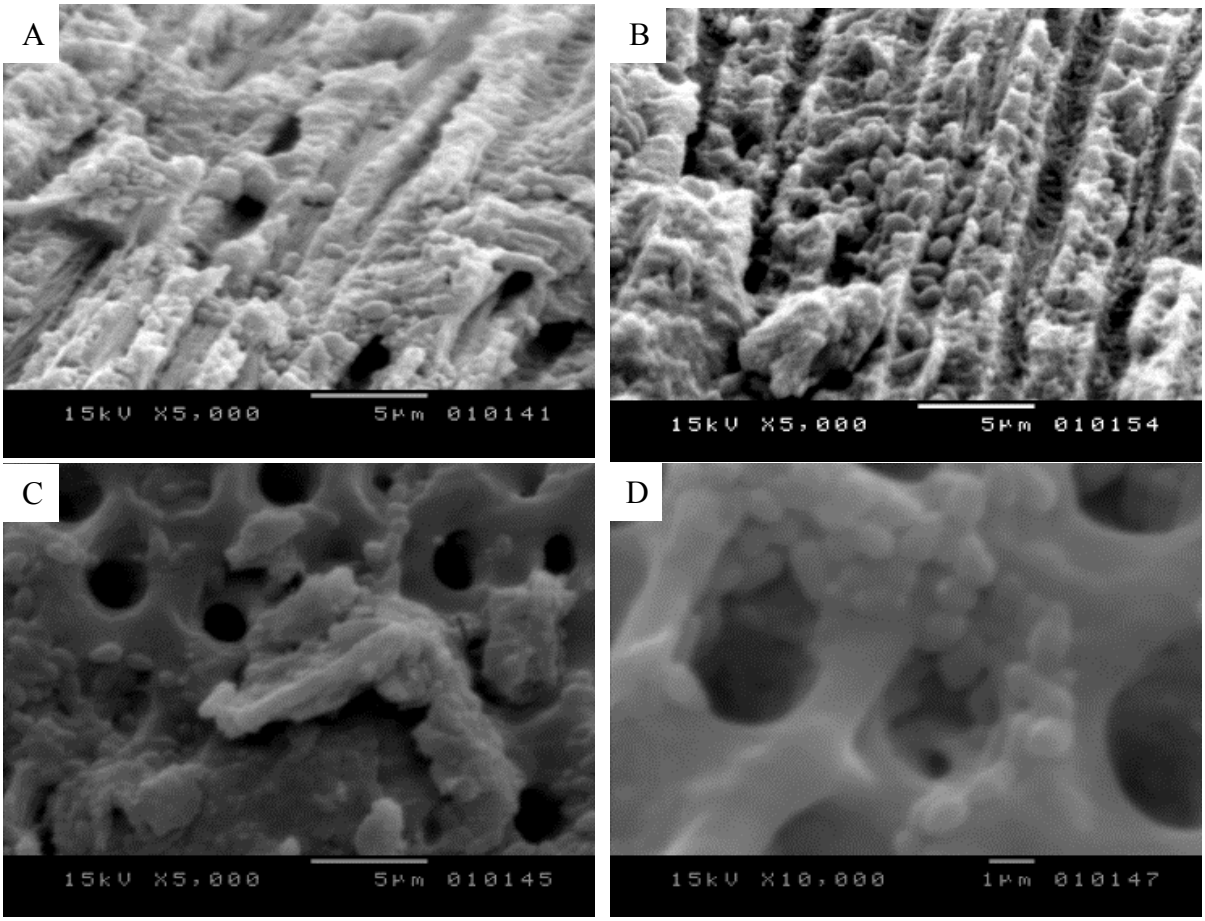


Figure 5. SEM of bacterial invasion of dentinal tubules, **A and B:** Bacteria could be seen individually or in packs inside dentinal tubules, **C and D:** The presence of bacteria at dentinal tubule level could be outlined.

1.5 Bacterial Composition of the Endodontic Biofilm

In the oral cavity, normal microbiota is present following a permanent colonization in a commensal relationship with the host. In this conditions oral normal flora participates in many beneficial relationships such as the possibility to protect the host from exogenous infections by excluding other microorganisms (40). These harmless microorganisms become pathogen in case of gaining access to a normally sterile zone like pulp. When enamel integrity is impaired, bacteria can colonize dentinal tubules and through them contaminate the dental pulp. This type of infection is called opportunistic infection, which is the main type of endodontic contamination. The development of endodontic infection includes different phases. It starts with microbial invasion of pulpal cavity. Afterward, microorganisms colonize the root canal space by multiplication and finally they begin their pathogenic activity associated with host response (11). Obligate anaerobic microorganisms are main colonizer of the pulp space during root canal infection (38). These bacteria do not need the oxygen for their growth. Different anaerobic bacteria have different sensitivity to oxygen (41). Facultative anaerobic and strict aerobic bacteria are present in the endodontic infectious community (Table. 1).

The bacterial composition of the biofilm of the endodontic system covers a distance usually from the pulp chamber roof to the apex of the tooth. Thus, there are different distribution gradients of bacteria inside the root canal space. The growth of anaerobic is favored to other respiratory types of bacteria by consumption of oxygen and production carbon dioxide and hydrogen (42). In endodontic infection, primary colonization of dentin is performed by *Streptococci*, which remove oxygen from the environment favoring the

condition for growing anaerobic species. Bacteria in root canal infections can penetrate into dentinal tubules and travel inside as far as 500 μm from the main canal (3)

This feature along with distribution of nutrients make the root canal system a selective habitat. Availability of oxygen inside the pulp space results in an aerobic region in the coronal part with an anaerobic zone at the apical third with a gradient between the two poles. Likewise, there is a gradient of different bacteria (e.g. starving non-resistant and resistant bacteria) from the coronal part to the apex in correspondence to the nutrition supply (e.g. including host diet via micro-leakage near crown and breakdown of pulp tissue and apical tissue fluid at apical zone) (19). Furthermore, as in every natural microenvironment, the adaptive capabilities of individual organisms are exponentially increased when growing in biofilm communities, which are communities of unicellular microorganisms living together and attached to a substantial surface. The base of this ecological approach of root canal infection is founded on the following concept: the most dangerous pathogen is not an individual species, but is a polymicrobial entity or an infectant group, which undergoes different physiological and genetic alternations initiated by changes in root canal environment. However, monospecies infections do occur (e.g. by *Enterococcus* species), particularly in the apical and periapical region (6, 7).

	Aerobic spp.	Facultative spp.	Anaerobic spp.
Gram-positive cocci		*Enterococcus faecalis* *Enterococcus faecium* *Staphylococcus warneri* *Staphylococcus lentus* *Streptococcus anginosus* *Streptococcus constellatus* *Streptococcus intermedius* *Streptococcus gordonii* *Streptococcus mitis* *Streptococcus mutans* *Streptococcus oralis* *Streptococcus salivarius* *Streptococcus sanguis*	*Peptostreptococcus micros* *Peptostreptococcus prevotii* *Peptostreptococcus magnus* *Peptostreptococcus asaccharolyticus*
Gram-positive rods		*Corynebacterium xerosis* *Lactobacillus acidophilus* *Lactobacillus catenaforme* *Lactobacillus fermentum* *Lactobacillus salivarius*	*Actinomyces naeslundii* *Actinomyces israelii* *Actinomyces meyeri* *Actinomyces odontolyticus* *Actinomyces viscosus* *Atopobium minutum* *Cryptobacterium curtum* *Eubacterium brachy* *Eubacterium lentum* *Eubacterium nodatum* *Mogibacterium timidum* *Propionibacterium acnes* *Propionibacterium granulosum* *Propionibacterium propionicus* *Pseudoramibacter alactolyticus* *Slakia exigua*
Gram-negative cocci		*Neisseria spp.*	*Veillonella parvula*
Gram-negative rods	*Pseudomonas aeruginosa*	*Campylobacter curvus* *Campylobacter rectus* *Campylobacter sputorum* *Capnocytophaga ochracea*	*Dialister pneumosinites* *Eikenella corrodens* *Fusobacterium nucleatum* *Fusobacterium necrophorum* *Porphyromonas gingivalis* *Porphyromonas endodontalis* *Prevotella oralis* *Prevotella oris* *Prevotella buccae* *P. intermedia* *Prevotella denticola* *Prevotella dentalis* *Prevotella melaninogenica* *Prevotella loescheii* *Selenomonas sputigena*

Table 1. Diversity of species isolated for root canal infections

Medical Biofilms: Detection, Prevention and Control. Edited by Jana Jass, Susanne Surman and James Walker, 2003 John Wiley & Sons, Ltd. ISBN: 0-471-98867-7

1.6 Methods for characterization of the Oral Biofilm

For a long time, conventional phenotypic identification of microorganisms has provided information on bacterial characterization (43); this technique basically requires cultivation of microorganisms, Gram staining and colonial morphology which all are inconclusive (44). Nowadays, we know that more than 99% of microorganisms are not cultivable under laboratory conditions (45). Advancing in biological science allows us to detect those micro-organisms through genotypic identification, different microscopic techniques or a combination of both. Bacteria can now be identified directly inside the clinical specimen without need to culture (43).

1.6.1 Bacterial Culture

Although the *in vitro* replication of the root canal condition for endodontic biofilm bacteria is difficult, many specially designed culture media have been produced which allow different bacteria to be selectively harvested from root canal space. However, recovering all bacteria seems impossible because of difficulties in providing suitable growth condition for different species.

1.6.2 Polymerase Chain Reaction (PCR)

PCR is a technique frequently used in molecular biology to amplify a small DNA fragment in order to produce enough genetic material for various analysis such as identifying of pathogenic microorganisms. As mentioned by Siqueira *et al.* (43, 46, 47), PCR technology facilitates the detection of bacterial species that are difficult or even impossible to culture. PCR could be utilized to amplify genes useful for taxonomy of bacteria, for instance 16S rRNA (48). In Endodontics, PCR has developed the information about bacterial

communities present in root canal infections. This technique helped to discover unknown organisms present in pulpal necrosis (49). Amplification of 16S rRNA provides and improves the knowledge about the pathogenic microorganisms incorporated in both primary and persistent endodontic infection (50). PCR technology is more rapid, sensitive and accurate compared to the traditional identification methods. Different protocols have been developed since the first application of conventional PCR. Nested PCR, reverse transcriptase PCR, Q-PCR, real time PCR and broad-range PCR are some examples of new approach of PCR.

1.6.3 Imaging techniques

Light microscopy is used for differential identification of gram-positive and gram-negative species as well as morphological differentiation of bacteria present in the biofilm (Cocci, Bacilli, filaments etc.). Moreover, in terms of inflammatory process light microscopy could be of help in studies of pulpal tissue. (51). The scanning electron microscope (SEM) is an interesting tool to study biofilms; its ability to image the surface topography with very high resolution and magnification helps to visualize the state of bacteria in attached microbial communities (52). However, light microscopy may not be helpful to discover bacteria in endodontic surgical specimens and concerning electron microscopy might not offer a clear identification of individual bacteria because of thick extracellular matrix. In addition, Electron microscopy provides a limited observation field of the sampled area which is another disadvantage of this technique (53). Drying artifact is another disadvantage of SEM. To visualize biological samples under SEM, they should be dehydrated and metal coated prior to observation. These procedures result in removal of extracellular matrix and distortion of biofilm (54). To overcome this problem Low-Vacuum Scanning Electron Microscopy

(LVSEM) could be of help. This technique does not rely on metal coating and preserves the cytopathological information and provides topographic images without any loss of specimen for future reexamination (55-57). For the same purpose, the Environmental Scanning Electron Microscopes could be used. This system uses a second detector system and can provide *in situ* perspective of wet samples without metal coating (58, 59).

Another imaging technique is the Transmission Electron Microscope (TEM). TEM helps to analyze the internal as well as peripheral structures of bacterial cells. It also becomes possible to distinguish the outer Lipopolysaccharides (LPS) from peptidoglycans.

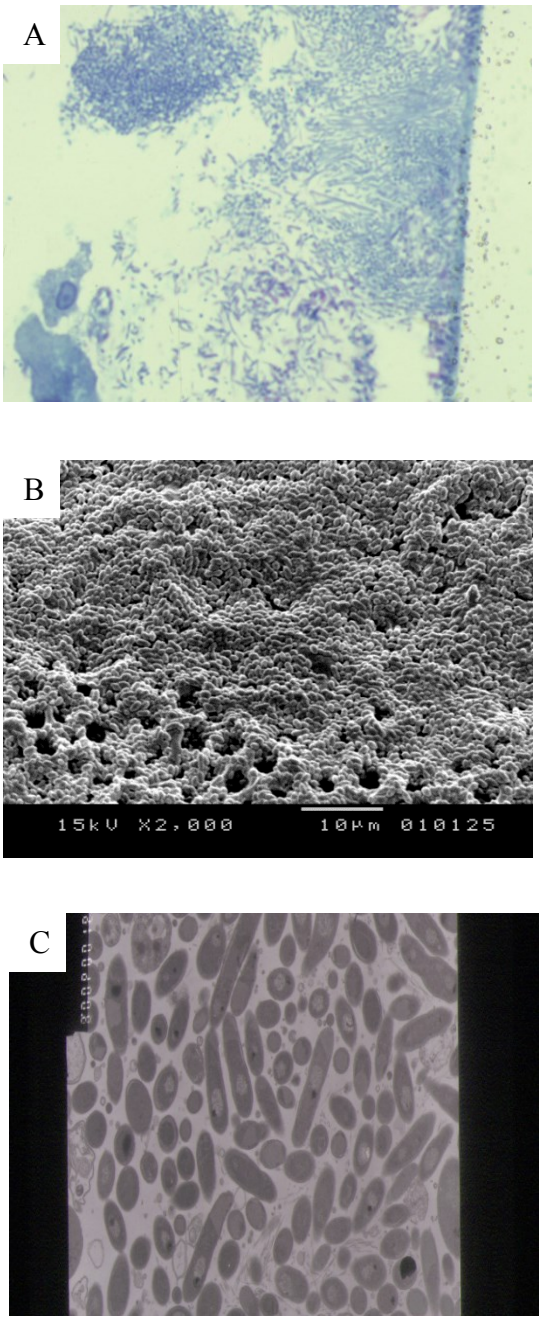


Figure 6. A: Cross section view of a dental plaque (courtesy of JP ROCCA), presence of different form of microorganism could be demonstrated, **B:** A SEM view of covered dentinal tubules by an artificial biofilm, **C:** A TEM of dental plaque, the morphology of components could be noted (courtesy of JP ROCCA).

1.6.4 Fluorescence *in situ* Hybridization and Confocal Microscopy

To overcome limitations of light and electron microscopy, fluorescence *in situ* hybridization (FISH) in combination with confocal laser scanning microscopy could be of help. This technique allows the visualization of matrix associated bacteria in dense biofilms.

FISH technique simultaneously presents the accuracy of molecular genetics and illustrates visual information of microscopy. Microorganisms can be spotted and studied in their habitat or morbid tissue. *In Situ* Hybridization was first introduced by Pardue *et al.* (60) and John *et al.* (61). They introduced radioactive RNA into target cells to form hybrid with nuclear DNA. Afterward, the established hybrids were visualized through autoradiography.

Direct fluorescent labeling of oligonucleotide is usually used during *In Situ* Hybridization. Fluorescent marker molecules (Fluorochromes) attach to the 5' end of oligonucleotide probes. Fluorochromes have different excitation and emission ranges. This could help simultaneous identification of two or more microorganisms. Main molecular target in microbiology is 16s rRNA thanks to its genetic stability, its domain structure with conserved and variable regions, and its high copy number (Fig. 7) (62). 16S rRNA sequences for most cultured and many uncultured microbial species have been collected in databases available for public (63, 64). It should be noted that probes designed for the majority of 16s rRNA are stocked in different online programs like ARB (65) and probeBase (66).

A typical FISH protocol comprises four steps: the stabilization and permeabilisation of the sample; hybridization; removing unbounded probes through washing steps and detection of labelled cells by microscopy (67).

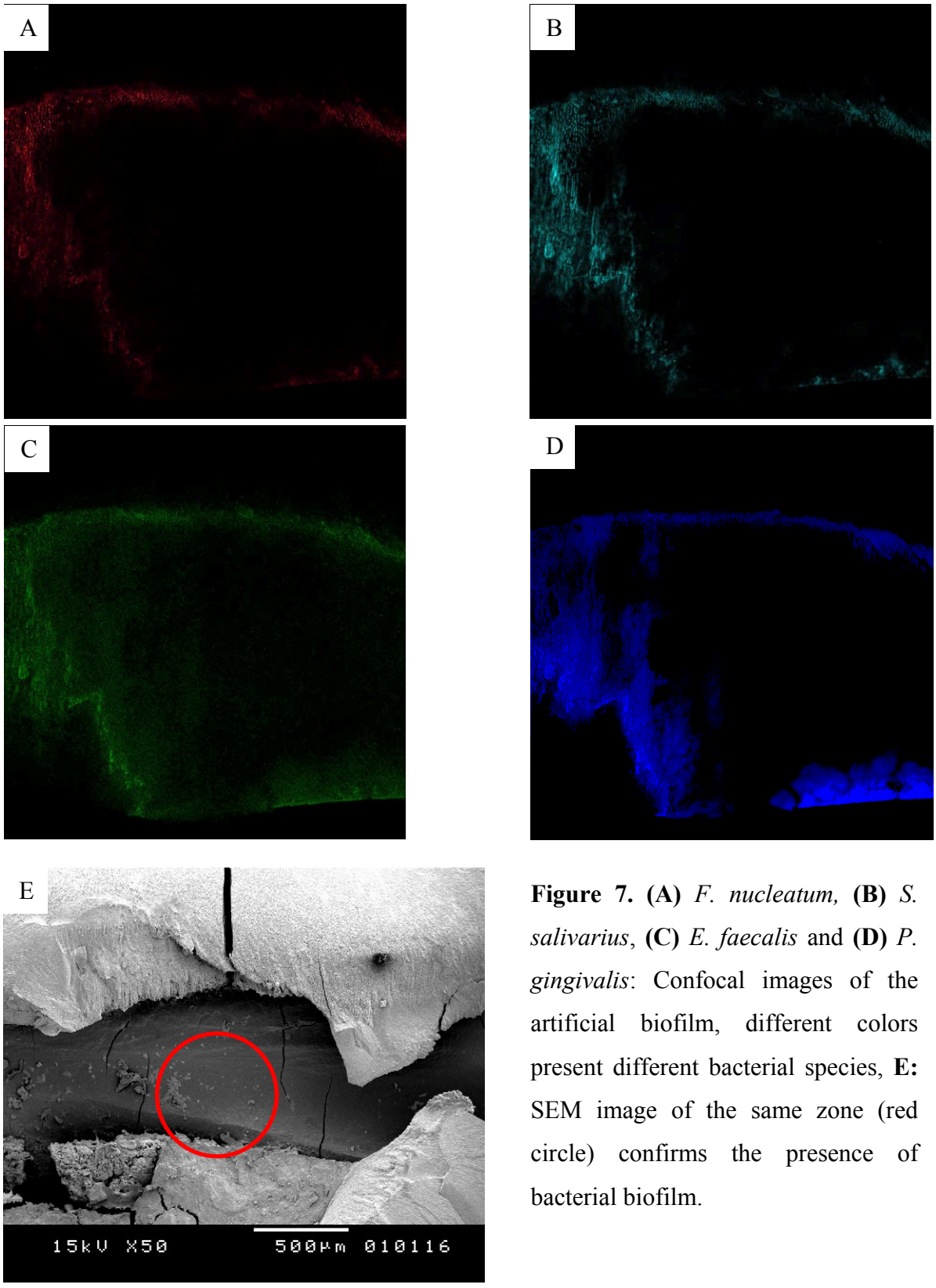


Figure 7. (A) *F. nucleatum,* **(B)** *S. salivarius*, **(C)** *E. faecalis* and **(D)** *P. gingivalis*: Confocal images of the artificial biofilm, different colors present different bacterial species, **E:** SEM image of the same zone (red circle) confirms the presence of bacterial biofilm.

1.7 Artificial Biofilms

To design an effective protocol to decontaminate the root canal space, it is crucial to develop a microbial biofilm model which closely resembles an *in vivo* infected root canal for *in vitro* and *ex vivo* studies.

Every endodontic instrument, chemical product or treatment protocol should be optimized in the laboratory before any clinical use. Preparation of the bacterial biofilm is an important challenge for endodontic treatment. The effect of chemo-mechanical or any other approach on biofilm removal determines the efficiency of product or protocol. A reproducible artificial biofilm resembling the wild type biofilm in main structural features seems crucial.

The design of a study model is affected by several important factors, especially for an endodontic microbial biofilm. Diversity of microbial species composing the biofilm, age and nutritional availability are some of these factors.

The microscopic analysis highlighted a distinct variation in the ultrastructure of the biofilms formed under different experimental conditions. It has been shown that the penetration of bacteria into dentinal tubules is in direct relation with nutrient availability. The Energy Dispersive X-ray microanalysis showed a significant increase in the levels of Calcium (Ca^{2+}) of the biofilm formed under anaerobic, nutrient-deprived condition. Meanwhile the depth of bacterial penetration was significantly greater in nutrient-rich condition (68). It has also demonstrated that the age and the nutritional conditions of biofilm may interfere with effects of antimicrobial agents (69).

In the literature, Monobacterial biofilms have been used as microbial study model for several endodontic disinfection studies. Biofilms used in these essays are easy to construct because of their simplicity (70, 71). However, rapid killing of planktonic bacteria by various disinfecting agents does not reflect the actual outcome of the same agent on bacteria in *in vitro* and *in vivo* biofilms. It has been demonstrated that bacteria incorporated in biofilm can be 100–1000 times more resistant to antibacterial agents than their planktonic counterparts (72, 73). Because of this great difference, a growing number of studies are now focusing on the destruction of bacterial biofilm instead of planktonic bacteria by disinfecting agents. The biofilm associated with apical periodontitis or chronic pulpitis are polybacterial. The major bulk of the organisms present in the biofilm as a collection of cocci, rods, filaments and spirochetes (74).

In case of apical periodontitis associated with failed endodontic treatment, microorganisms have a limited access to nutrients. However, many microbes defy environments by producing a starvation response (69). Microorganisms are likely to switch from multiplication toward energy gaining in purpose of survival (75, 76).

1.8 Biofilm and Success of Root Canal Treatment

Clinical management of microbial infection by eliminating them from the root canal space is the main goal of endodontic treatment (1, 77). Microbial infection plays an important role in the development of pulp necrosis and the possible formation of periapical lesions (77). Endodontic treatment has an obviously high degree of success (78). Nevertheless, this treatment can go wrong. Most failures occur when treatment procedures, specially the technical quality, have not reached an acceptable level of controlling and elimination of infection (5, 79, 80). It is apparent that the mechanical debridement combined with chemical irrigation removes the bulk of the infecting microorganisms, but because the infection of root canal system is three dimensional, the residual bacteria are still detectable in an important area of the tooth just before filling the root canal (4, 81, 82). Certain operative problems such as insufficient instrumentation, a missed canal or its improper restoration might lead to post-treatment endodontic failure (4). Another obstacle to obtain a completely bacteria free root canal system is the variation in internal root anatomy, which makes chemo-mechanical debridement ineffective even in their highest technical standards to achieve a total bacterial eradication from root canal system (83). This space incorporates small lateral canals additionally to the main canal, which do not allow direct access during the biomechanical preparation because of their location and/or their small diameters (84).

The success rate of endodontic treatments is higher when the canal is bacteria free and filled (85). This was reported to reach 68 – 85% when rigorous radiographic standards were used. The success rates were approximately 66%, 75%, 77%, and 85% for interventions carried out by general dental practitioners, undergraduate students, graduate students, and

specialists, respectively (86). The antimicrobial susceptibility or resistance of the polymorphous microflora, which includes anaerobic, facultative anaerobic and aerobic bacteria, may determine the outcome (84).

Hence, the need for an efficient root canal disinfection method drives the attention of the researchers toward implication of other more effective technologies in Endodontics

1.9 Endodontic Disinfection Protocols

1.9.1 Ultrasonic Irrigation

As an essential part of debridement of the root canal space, irrigation makes it possible to achieve a cleaner root canal space more than that obtained only with mechanical instrumentation (87, 88). An ideal irrigant should compensate the deficiencies occurring during mechanical debridement and should also have the following characteristics: wide antibacterial spectrum and efficiency against anaerobic and facultative microorganisms organized in biofilm, dissolve necrotic pulpal remnants, inactivate the endotoxins, prevent smear layer formation during instrumentation and capable to remove it once it has formed. These products should also be systematically nontoxic to vital tissues and harmless to surrounding tissues when they are in contact (89).

There is no one unique irrigant, which can possess all these criteria; therefor dual irrigant protocols like NaOCl and EDTA are mainly used in actual protocols. This bi-product technique is to overcome the probable defect of the irrigants (90, 91).

The use of ultrasonic devices during irrigation has been proposed to confront the problems observed during cleaning and disinfection of the root canal system (92). The use

of ultrasonic activation of irrigants needs a minimal effort and results in significant reduction of bacterial survival (93, 94).

Two different types of ultrasonic activation have been present. The first type is simultaneous ultrasonic instrumentation and irrigation of the canal. The second type is passive ultrasonic irrigation once instrumentation of the canal has been accomplished (88). Acoustic streaming is the phenomenon that occurred when the ultrasonic file is activated in the canal filled with irrigant. This enhances the ability of irrigants to dissolve remnants and/or smear layer (93), even if new smear layer may partially reformed due to possible contact between the ultrasonic file and root canal walls (95). Cavitation or formation and collapse of bubbles inside irrigation solutions is another evidenced phenomenon which happens during ultrasonic irrigation (96). Macedo *et al.* demonstrated that ultrasonic activated irrigation at clinically relevant ultrasonic power can initiate cavitation inside an irrigation solution. This series of events occurs in both straight and curved root canals, even when the file and root canal walls are in contact (97). The vibration of the endodontic file produced by the ultrasonic device, provides the energy needed to produce the cavitation and acoustic streaming that makes the PUI an efficient technique (98). According to Van der Sluis *et al.* the the final taper of the root canal preparation influences the outcome of PUI (99). A better cleaning in root canals can be achieved when the preparation taper is increased.

1.9.2 Lasers

Since the first laser device was developed by Maiman in 1960 (100), this device has been used in various fields of dentistry (101-108). Among the first endodontical applications of laser, the intracanal sealing of the apical foramina was done by high power CO_2 laser (109). Effects of Nd:YAG laser on apical seal of teeth after apicoectomy and retro fill were

also tested (110). Afterward, the antibacterial concept of laser attracted the researchers to employ this technology for the purpose of disinfection (111-118).

In general, laser light is either reflected, absorbed, scattered or transmitted according to its target tissue; this is called laser-tissue interaction (118). Direct intracanal irradiation of lasers for the purpose of endodontic decontamination has been evoked in the literature. Lasers such as Nd:YAG, Nd:YAP and diodes are called near infra-red lasers (800 nm to 1500 nm). These wavelengths are well absorbed in pigmented tissues (hemoglobin, melanin,…) but they have trifling affinity for hydroxyapatite crystals and water which build up the dentin (118). So, the laser beam will not be absorbed at the superficial layers of dentinal walls. The advantage of this range of lasers is that they may possibly clean root canal space from bacteria even in deeper layer of dentin (119, 120). It should be reminded in case of non-respect of protective measures that the energy of the near infra-red lasers may be focalized in some regions of the endodontic space and a part of it could be transmitted to periodontal tissues, which may lead to photo-thermal damages of these tissues (120-122). Mid infra-red lasers (2500 nm – 3000 nm) are highly absorbed in water and to a lesser degree in hydroxyapatite crystals. Both Er:YAG (2940 nm) and Er,Cr:YSSG (2780 nm) produce photo-ablative as well as photo-thermal effects in their targets (123).

Er:YAG showed to be effective to eliminate the bacterial contamination from root canal spaces. Mehl *et al*. showed that 60 sec of intracanal irradiation was statistically as significant as 2 minutes of contact with NaOCl in terms of bacterial load reduction (124). Recently new laser wavelengths have been examined for their antibacterial properties. KTP is one of these lasers, which has the affinity for pigmented tissues. KTP laser could be efficient to

eradicate endodontic microbial infection when reasonable parameters are applied and tissue protective techniques of irradiation are used (125, 126).

A rise in temperature of the tooth beyond the physiologic tolerable degree of 5.6°C may cause pulpal damage and periapical cell death (127). The study of effects of direct application of high power infra-red lasers with dental tissue showed that these devices may cause extreme overheating and engender zones of charring and carbonization in both soft and hard tissues (128, 129). Melting, cracking and debris formation of the dentinal surfaces could be observed specially after irradiation of root canal system with near infra-red (800 nm – 1500 nm) and far infra-red (9600 nm – 10600 nm) high power lasers (130-133). To these disadvantages should be added the possible complexity and expensive cost of dental lasers (134).

Lasers may be used to activate irrigation solution, which may enhance their fluid dynamics and possibly increase the success rate of decontamination procedure (135).

Laser assisted endodontic decontamination in conjugation with conventional chemical solutions activated by laser should be favored over direct use of laser to remove bacterial biofilm from root canal space. There are 2 ways to activate irrigation solutions with laser: first, through an endodontic optical fiber either with a slow upward movement inside the root canal or by keeping stationary or in motion over a small distance inside the root canal. The second technique is to irradiate the irrigant from the canal orifice (135). It has been demonstrated in the literature that common root canal irrigation solutions have the capacity to absorb different light wavelengths (136). For example chlorhexidine and citric acid could absorb light at 513 nm and 2200 nm respectively. In addition, most irrigants have high absorption rate for wavelengths higher than 2500 nm. These optical properties make all

tested irrigants qualified for laser assisted endodontic irrigation (136). The important point is to match the right irrigant with available wavelengths or vice versa

Laser-activated irrigation using different wavelengths has been reported. Near infra-red laser such as Nd:YAG, diodes have been used to activate irrigation solutions but the effect of these lasers are not significant on non-pigmented bacteria (137). Meire *et al.* demonstrated that Nd:YAG laser had nearly no effect on planktonic *E. faecalis* a non-pigmented bacterium (138). This could be explained by the fact that the Nd:YAG laser beam is not absorbed inside the bacteria and passes through it. Pirnat *et al.* showed that the near infra-red lasers are effective to reduce load of the *P. gingivalis* a pigmented microorganism (139) They suggest that heating of the microenvironment following irradiation with Nd:YAG or diode lasers, contributes to kill the microorganisms. Hmud *et al.* investigated a possible cavitation effect of pulsed irradiation of diode laser inside irrigation solution (140). They demonstrated the formation of bubbles inside irrigation solution, however in another study they measured a rise in temperature about 30°C in the irrigant (141). But it is highly probable that boiling of the irrigant be the origin of this bubble formation (135). Recently, George *et al* showed that a diode laser can produce air bubbles when it delivers its energy via a side firing endodontic fiber called honeycomb fiber (142). This fiber produced more bubbles at its long axis than its tip, this may lead to a better debridement of root canal walls.

The nature of action of erbium doped lasers during laser-activated irrigation is based on cavitation phenomenon (123). Blanken *et al*. demonstrated that a considerable heating of irrigant happens at the first moments of irradiation with an Er,Cr:YSSG laser resulting in vaporization bubble formation (143). This results in fast movement of irrigant inside the root canal.

Erbium doped lasers may enhance the antibacterial effect of NaOCl (144). These mid infra-red lasers can remove effectively organic and inorganic debris from root canal space (123).

There are several factors effecting the formation and quality of bubbles during LAI. According to Olivi and De Moor (135), both number and duration of the pulses and their energy have direct effect on the cavitation inside the irrigants and subsequently the outcome of LAI. Other influencing factors are the size and form of the endodontic fiber as well as its placement inside the root canal space during the irradiation. The latter is important in matter of safety and prevent the extrusion of irrigants to periapical region.

The number of the pulses determine the repetition of cavitation. The number, size and lifetime of the bubbles depend on the pulse duration and the energy of the laser pulse regulates the onset of the cavitation process. The form of the optic fiber tip influences the form and direction of the bubbles and bigger fibers lead to formation of bigger air bubbles.

1.9.3 Photodynamic Therapy

Photodynamic therapy (PDT) is a medical treatment that utilizes light to activate a phototoxic agent called Photosensitizer (PS). The activation of PS in the presence of oxygen results in the formation of toxic oxygen species, such as singlet oxygen and free radicals which are lethal for the microorganisms (134). Synonyms such as photo-activated chemo-therapy (PACT), antimicrobial photodynamic therapy (aPDT) and photo activated disinfection (PAD) are utilized when the PDT is used for the purpose of disinfection (145). The antimicrobial efficiency of PDT especially for drug-resistant pathogens is due to radical oxygen speciecs, which have multiple cellular targets (146). As a consequence, the resistance

for PDT is unlikely. This characterstic makes PDT active against a broad spectrum of bacteria (Gram positive , Gram negative) protozoa and fungi (147).

Many Photosensitizers including chemical photosensitizing agents like Toluidine Blue (148, 149), Methylene Blue (150, 151) and natural Photosensitizers like curcumin (152) have been tested. The outcome of PDT in different protocols with different activating lights has also been tested (153).

As a pioneer, Wilson mentioned bactericidal effects of PDT in oral diseases (154, 155). The number of scientific publication is increasing in recent years (Table. 2; Fig. 8 and 9) (137). A high degree of safety of PDT for the host could be a reason for such significant progress (156). The potential role of PDT in total eradication of root canal infection was outlined in many *in vitro* and *in vivo* researches (157-160). Nevertheless, it should be kept in mind that Photodynamic therapy cannot remove the bacterial infection from the root canal space because of its pure photochemical nature of action (137). But, it has been reported that the antimicrobial activity of PDT is minimized when it interacts with the biofilm (147). Thus, new approaches such as nanotechnology have been introduced to improve the action of PDT against the biofilms (161).

The take-home message of the bibliographic research about the antimicrobial application of PDT in endodontics is that photochemical disinfection in this state could not be a substitution for conventional disinfection methodologies, however, it can improve the outcome of the latter as an adjuvant (137, 147).

[redacted]	[redacted]					
[redacted]	1047	4	55	314	285	403
[redacted]	306	0	4	44	89	174

Table 2. Number of publications related to Endodontics and Laser and disinfection from 01.01.1971 to 31.12.2014

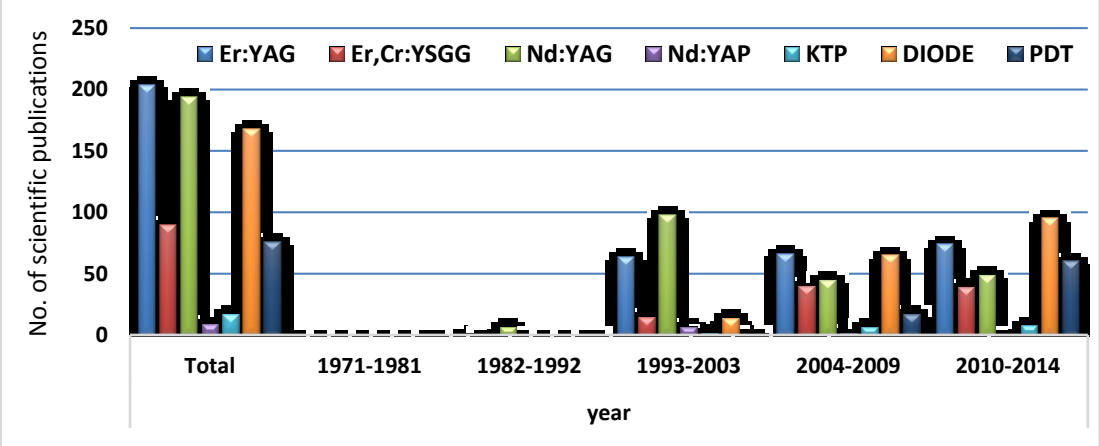


Figure 8. Distribution of scientific publications about endodontics and laser according to specific wavelength and PDT from 01.01.1971 to 31.12.2014.

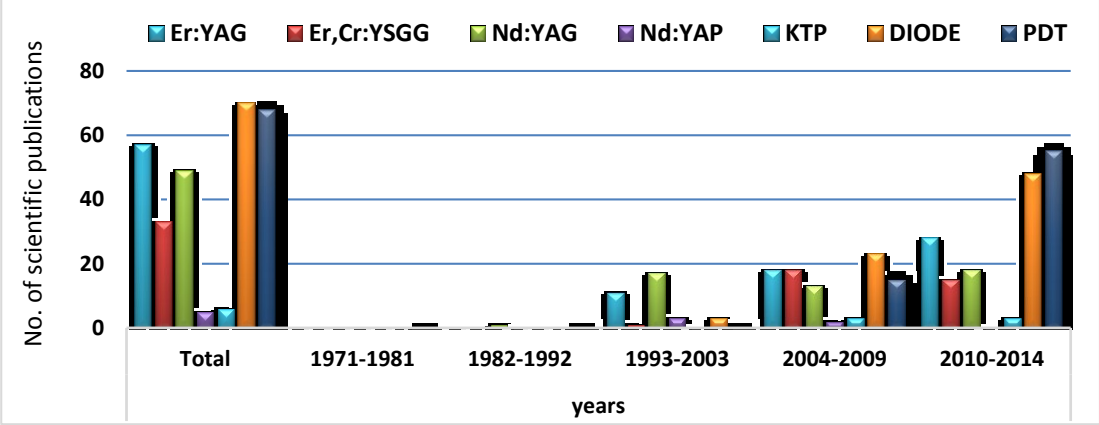


Figure 9. Distribution of scientific publications about Endodontic Disinfection and Laser according to specific wavelength and PDT from 01.01.1971 to 31.12.2014.

2

Aim of Work

The outcome of root canal treatment depends on successful control of endodontic infection. Nowadays, new endodontic disinfection agents, instruments or protocols emerge in a daily rhythm. However, these methodologies should be tested within *in vitro* conditions with a maximum resemblance to those of *in vivo* root canal pathologies. In the current study we aimed to construct, characterize and treat an artificial polybacterial biofilm that could be used as a reproducible model system of endodontic infection. In order to achieve this aim, our study was conducted in 4 distinct phases.

Phase 1: The feasibility to use the root canal as an *in vitro* habitat for different bacteria was tested in a pilot study. For this purpose a biofilm model was used, which was already designed in MICORALIS laboratory (former laboratory of oral health and aging). To designate the gold standard we used PUI. Two different PDT protocols were tested against the artificial biofilm to evaluate the effect of their light sources.

Phase 2: After achieving preliminary results of the pilot study, we used Medline-PubMed research engines to collect data on artificial biofilms used for endodontic *in vitro* studies. This literature review allowed us to design a more complete biofilm model. This artificial biofilm must coexist and be composed of representative microorganisms which can be found in different colonizer groups forming the wild type endodontic biofilm. The age of

the biofilm has direct influence on outcome of decontamination procedure. Therefore, this study was conducted or attempted to outline the growth milestones of the artificial biofilm throughout its maturation process to find out the most proper incubation time to establish a matured artificial biofilm structure.

Phase 3: Afterward, to confirm the structural details of the biofilm, it is indispensable to characterize the artificial biofilm. For this purpose we used bacterial cultures, molecular techniques (FISH technique) and different imaging methods (optical microscopy, scanning electron microscopy and confocal microscopy). This knowledge is essential to confirm the ability of multiple species to coexist and to determine their localization inside biofilm structure and their distribution over the root canal system.

Phase 4: The last objective of this study was to investigate different decontamination protocols on the artificial biofilm and to evaluate their capability to neutralize and eradicate bacterial infection from root canal space. We aimed to assess the effect of PUI as the gold standard as well as to weigh the outcome of rotary instrumentation alone in terms of bacterial charge reduction of an artificially infected root canal space. Moreover, we intended to analyze the effect of laser assisted irrigation with different wavelengths (Er:YAG and diode lasers) and different photon delivery methods (endodontic fiber and sapphire) and PDT on the artificial biofilm.

3

Materials and Methods

3.1 Sample Preparation

3.1.1 Dentinal Disks

-Ten freshly extracted, caries free molars were selected and the outer surface was cleaned from any soft tissue remnant by NaOCl 2.6% (produced in Saint-Roch hospital's pharmacy, Nice, France) and then rinsed with sterile water.

- Teeth then were put in plastic molds ® (Buehler, Lake Bluff, IL USA) and fixed to the base with a putty mixture of Aquasil™ (a vinyl polysiloxane impression material; Dentsply DeTrey GmbH, Konstanz, Germany).

- A mix of EpoxyResin® base and hardener (Buehler, Lake Bluff, IL USA) was prepared in a 5:1 ratio. The resin was mixed thoroughly to obtain a homogenous consistency and to remove any trapped air bubble.

- Prepared resin was carefully poured inside the plastic mold till it completely covered the tooth.

- Resin was left to set for 24 hours at room temperature.

- Then, resin was removed from the plastic mold.

- Using an Isomet®2000 precision saw (Buehler, Lake Bluff, IL USA) resins were cut in 2 mm wafers, for a total of 30 disks (3 disks per each sample; Fig. 10 and 11).

- To eliminate the residual resin layer formed on the disks after cutting, samples were polished by TEXMET® and MASTEREX® polishing disks and Metadi® 0.1, 1 and 3 µm diamond suspension (Buehler, Lake Bluff, IL USA).

- All disks then were rinsed with water.

- For the purpose of removing smear layer and any possible contamination from disk surfaces, NaOCl 2.6% and Salvizol® 8% EDTA solution (ACTEON, Merignac, France) were applied using small adhesive brush for 1 minute for each solution.

- Disks then were rinsed during 5 hours under running water to ensure total removal of solvents.

- Finally, all samples were autoclaved (Systec, Linden, Germany) at 120°C for 20 min.

- Dentinal disks were stored in sterile water at 4°C until use.

Figure 10. The ISOMET2000® precision saw to cut samples.

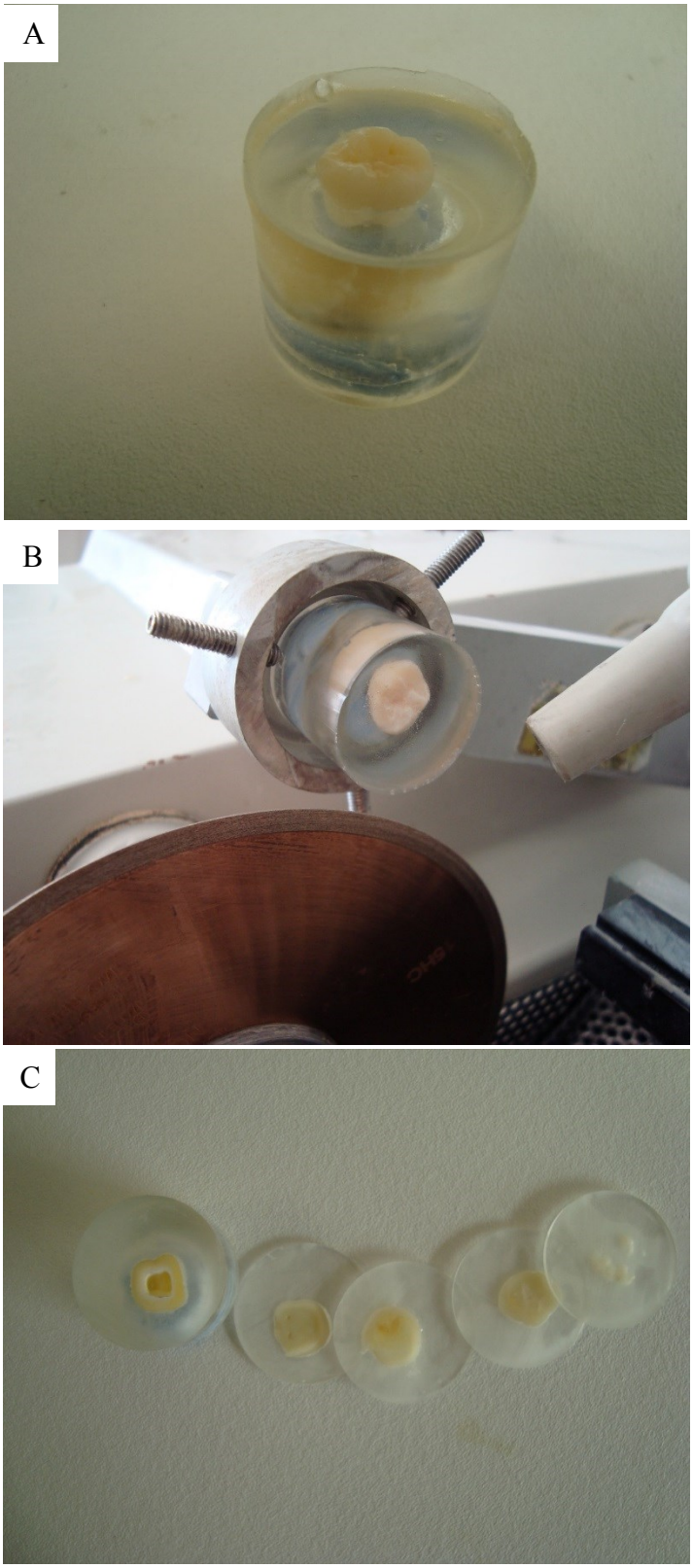


Figure 11. **A:** An extracted molar embedded in epoxy resin, **B:** Cutting of the tooth to obtain dentinal disks, **C:** Dentinal disks of 2 mm thickness obtained from one block.

3.1.2 Root Canal Preparation

- One hundred and eleven single rooted extracted human teeth were selected. The presence of just one canal was confirmed by digital radiography (Fig. 12). Subsequently the outer surface was cleaned from any inorganic deposit or remaining soft tissue using an ultrasonic device (Piezon® Master 400 EMS Electro Medical Systems SA, Nyon, Switzerland) and NaOCl 2.6%. Samples, then, were washed with distilled water and were stored in saline solution at room temperature until starting the experiments. The saline solution was renewed 2 times/week.

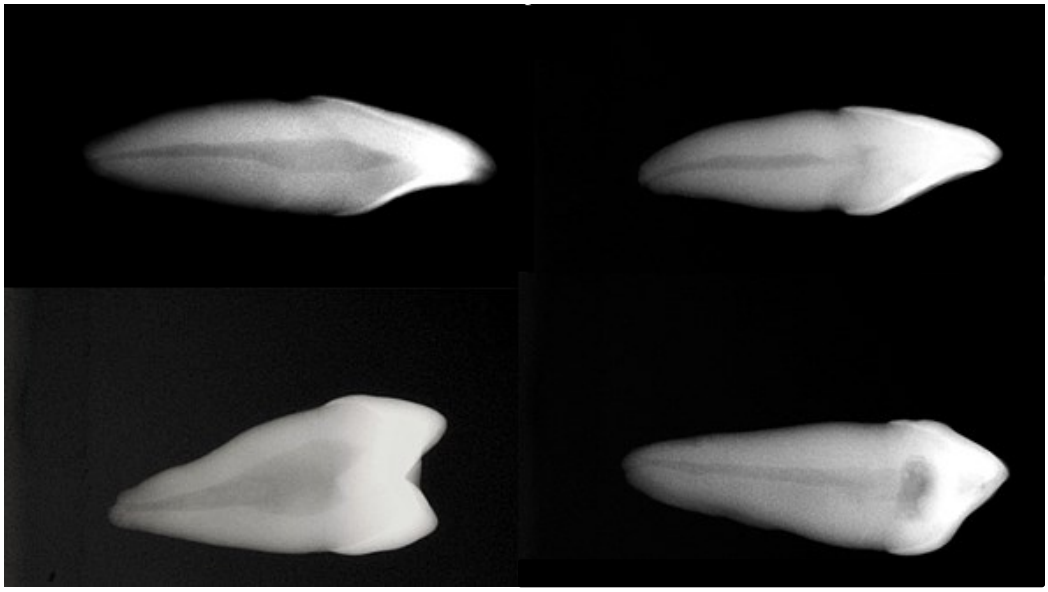


Figure 12. The verification of root canals by means of X-ray.

- The same practitioner performed all preparation steps. The crowns were removed using a diamond disk (Prodont Holliger, Vence, France) and all roots were shortened to approximately 15 mm in height.

- Afterward, all samples were placed in an Ultrasonic bath (Fisher Scientific Inc., Schwerte, Germany) in order to clean them from the dust and dirt of cutting.

- The canals were enlarged manually using K-file (Micro-Mega, Besançon, France) as follows:

1. A #8 K-File was introduced in the canals reaching the working length (root canal length minus 0.5 μm), to remove the canal content and confirm the absence of any obstacles in the canal.
2. The preparation was continued by stepback technique from #10 to #40 K-files (Fig. 13A).
3. The canals were flushed with 1 ml of NaOCl 2.6% using a 26 gauge open ended needle (PentaFerte S.p.A, Campli, Italy; Fig. 13B).
4. A # 10 K-File used to verify the patency of the apical part of root canal.
5. A wash out with 1 ml of NaOCl 2.6% was performed each time the size of the file was changed to a superior one.

- All apices were closed with Photac™ Fil Quick Applicap™ Glass-ionomer cement (3M ESPE AG, Seefeld, Germany) to avoid extrusion of irrigation solution during root canal preparation.

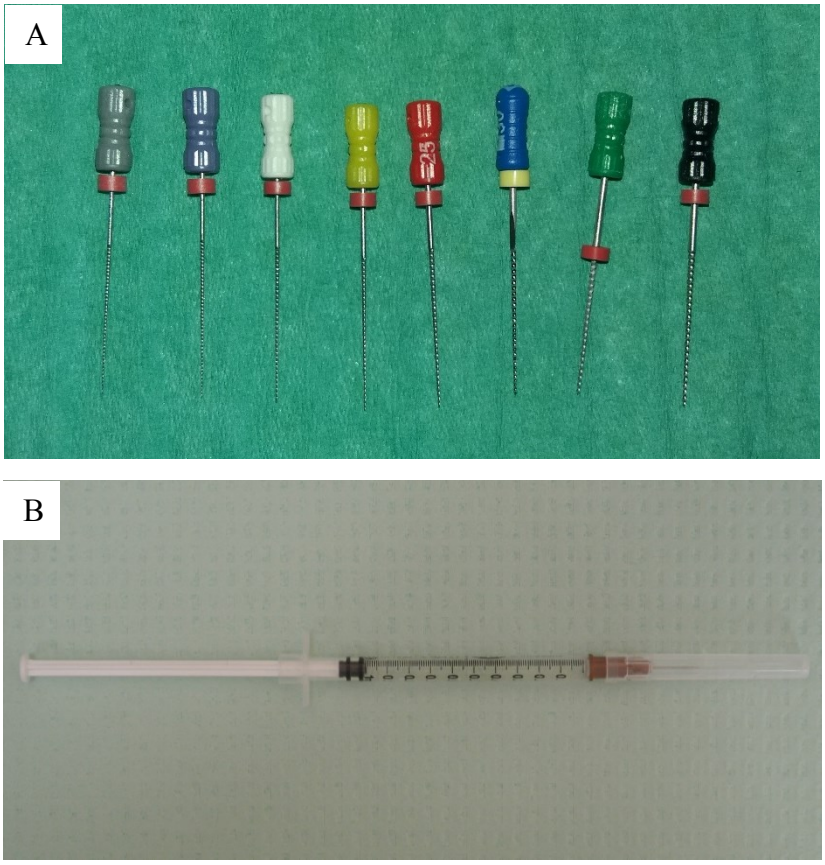


Figure 13. A: File sequence for preparing the root canals, **B:** 2.6% NaOCl was used to irrigate the root canal space after each filing step.

- A final rinsing, aiming to remove smear layer and debris was performed using 1 ml of Salvizol® EDTA 8% in the root canals. The irrigation solution was injected into the root canal space during approximately 10 seconds.

- The EDTA solution was agitated in the canals for one minute by an endodontic ultrasonic file (IRRISAFE® ACTEON, Merignac, France; Fig. 14) mounted on an ultrasonic unit (Piezon® Master 400 EMS Electro Medical Systems SA, Nyon, Switzerland). This unit generates a fixed frequency of 28 KHz. The power was set at 9 o'clock position (162).

Figure 14. IRRISAFE® endodontic ultrasonic file.

- 2 ml of NaOCl 2.6% were injected in the canal and agitated for one minute with the ultrasonic device as mentioned before.

- Rinsing was repeated by injection 2 ml of NaOCl 2.6% into the canals to remove any remnant from the canals without ultrasonic agitation.

- Root canals then were put in a plastic container and kept during 5 hours under running water to ensure total removal of irrigation solutions.

- Finally, all samples were autoclaved (Systec, Linden, Germany) at 120°C for 20 min.

- Root canals then were stored in sterile water at 4°C until use.

The number of samples used in this study is summarized in the Table 3.

- In order to exclude any possible artifact caused by preparation procedures, 3 random samples from the dentinal disks and the root canals were visualized with Scanning Electron Microscope (JSM-5310LW total Vacuum, JEOL LTD, Japan; Fig. 16C) under low vacuum

condition before any experimental procedure. We observed that dentinal surfaces in both groups of samples were free from any organic or inorganic debris.

Study	Number of controls	Number of group	Number of samples per	Number of repetition
Pilot study	3	3	10	1
Design and buildup of biofilm* (Disks)	8	8	4	3
Characterization	2	1	5	2
Treatment protocols	8	6	10	1

Table 3. The number of samples used in different phases of this study. *In this phase, each 3 dentinal disk samples were obtained from 1 tooth.

3.2 Pilot Study

3.2.1 Root Canal Infection

The Biofilm used in this study was composed of four different bacterial species, *Porphyromonas gingivalis* (ATCC 33277), *Streptococcus salivarius* (ATCC 7073), a wild-type strain of *Enterococcus faecalis* and a wild-type strain of *Prevotella intermedia,* which were provided by laboratory of bacteriology of the Hôpital Archet 2 CHU Nice - France.

- *Enterococcus faecalis* and *Streptococcus salivarius* were grown aerobically overnight at 37ºC on Mueller-Hinton agar plates and on 5% sheep blood agar plates (BioMérieux, Marcy l'Etoile, France), respectively. *Porphyromonas gingivalis* and *Prevotella intermedia* were cultivated anaerobically on 5% sheep blood agar plates at 37ºC for 3 and 5 days, respectively.

- For the four strains a standardized suspension containing $1.5x10^8$ cells ml^{-1} in Schaedler broth (Bio-Rad, Marne la Coquette, France) was prepared with following proportions: 5% *Streptococcus salivarius*, 21% *Enterococcus faecalis*, 37% *Prevotella intermedia* and 37% *Porphyromonas gingivalis* (Fig. 15C).

- The biofilm was formed inside the root canal by dispensing 2.2 ml of standardized cell suspension within 24-well cell culture plates (Corning Inc., Union city, CA, USA; Fig. 15D).

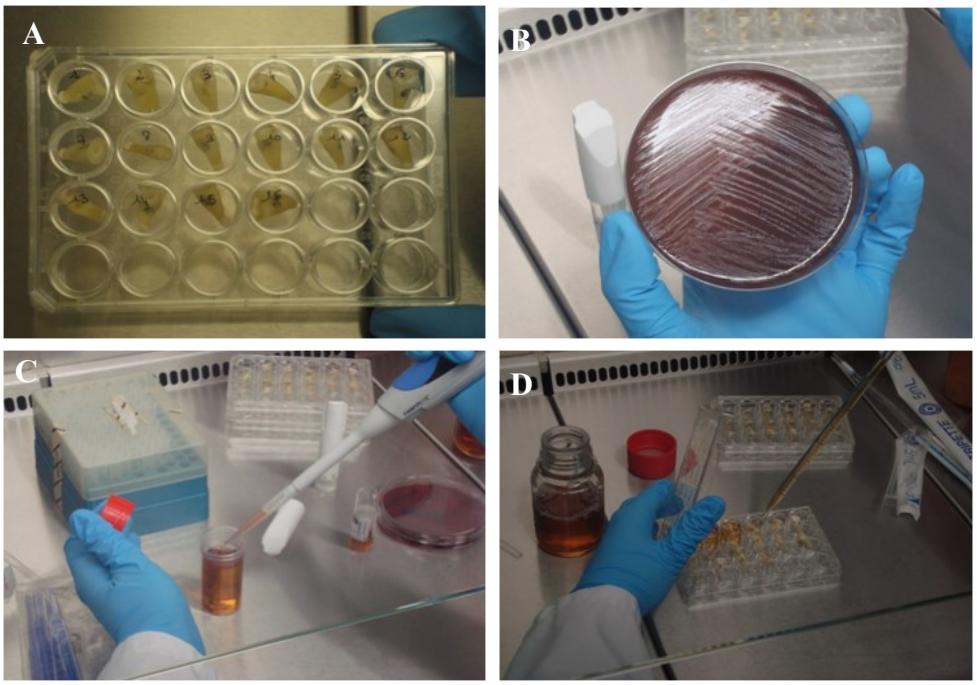


Figure 15. A: Teeth in a 24-well plate, **B:** *Streptococcus salivarius* cultivated on 5% sheep blood agar, **C:** Cell suspension in Schaedler broth. **D:** Inoculation of biofilm in root canals.

- Cell culture plates were incubated anaerobically at 37°C on an orbital shaker (150 r.p.m). After 24 hrs, 0.5 ml of Schaedler broth was added into each well. Seven days after the inoculation, teeth were removed and washed twice for 3 min using 0.1 M phosphate buffer saline.

3.2.2 Biofilm Development Control

One of two control samples was broken in two pieces along its long axis. Samples then were gold coated (Ion sputter, JOEL JFC-11E LTD, Japan; Fig. 16A and B). Then sample was visualized using Scanning Electron Microscope (JSM-5310LW total Vacuum, JEOL LTD, Japan; Fig. 16C), with original magnifications ranging from

75X to 7500X to obtain images of synthetically formed biofilm on the walls of the root canal (Fig. 17).

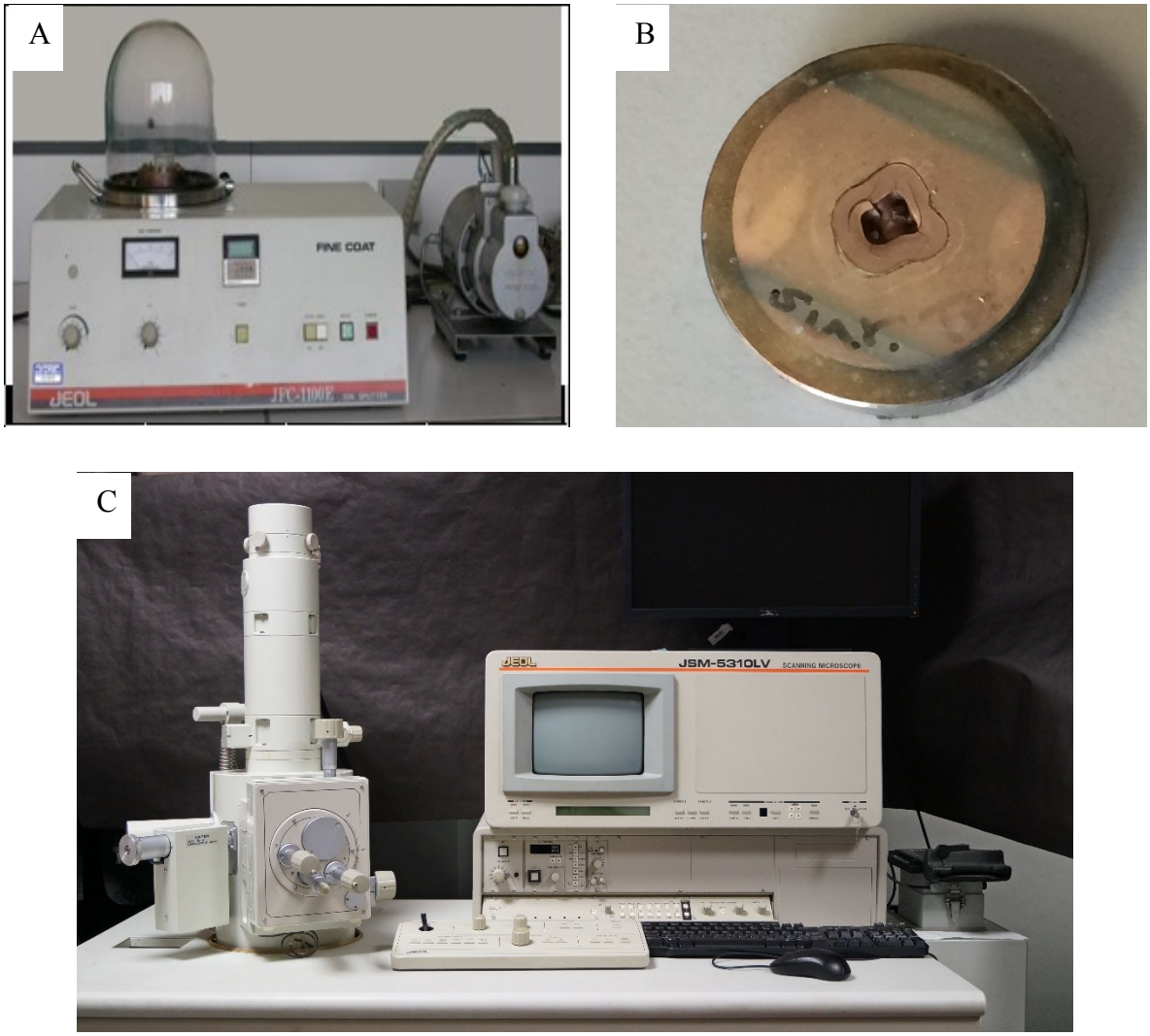


Figure 16. A: Ion sputter® coating machine, **B:** a gold coated dentinal disk, **C:** Joel scanning microscope.

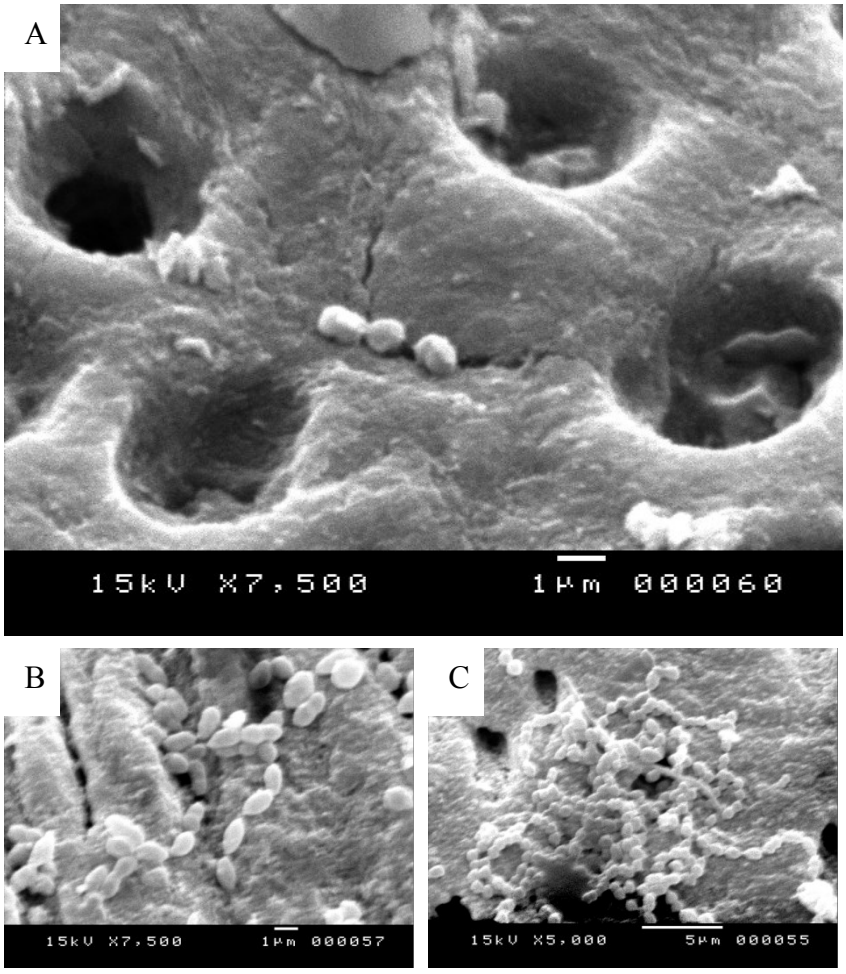


Figure 17. *Enterococcus faecalis* **(A and B)** and *Streptococcus salivarius* **(C)** can be recognized by their chain formation characteristic. Images were captured using Scanning Electron Microscope. Scale bars represent 1 µm and 5 µm.

3.2.3 Grouping

The teeth were randomly divided into four groups, one group of 2 teeth as control group and three groups of 10 teeth.

3.2.3.1 Group 1: Photodynamic Therapy with LED

- **Light source**

The first group of ten teeth was treated following a Photodynamic therapy (PDT) protocol using a Light emitting diode with a wavelength of 635 nm as light source (Aseptim^{TM} Plus, Leutkirch im Allögo, Germany; Fig. 18A). Light was delivered with disposable conical plastic tips (Fig. 18B).

- **Photosensitizer**

The Photosensitizer was a solution of diluted pharmaceutical grade of Toluidine blue (concentration is not revealed by the manufacturer), which was supplied in 0.8 ml syringes. The solution was introduced inside the canal using of a G26 needle (Fig. 18C)

- **Treatment protocol**

For easy performance of the treatment protocol, teeth were mounted in a base made from Aquasil™ (a vinyl polysiloxane impression material; Dentsply DeTrey GmbH, Konstanz, Germany).

The work desk was cleaned with alcohol 70% to avoid contamination. All procedures were carried out under sterile conditions.

The Aseptim™ solution was injected into the canal using a G26 needle to fill up the entire volume of the canal. The excess of the product was collected during injection using a sterile pipette tip and a suction device. The procedure was continued by rubbing the solution inside the canal for one minute, using a size 10 K-file according to the manufacturer's instructions. An especially sterile designed flexible tip was attached to the LED device and was inserted into the canal space till a tug-back sensation was achieved. The photosensitizer

was activated for 120 seconds according to the manufacturer's instructions. Once the procedure completed, the canal was rinsed with 2 ml of sterile water during 10 seconds approximately to remove the photosensitizer from the canal. Afterward, the canal was dried with a sterile paper points. The procedure was repeated for the all specimens.

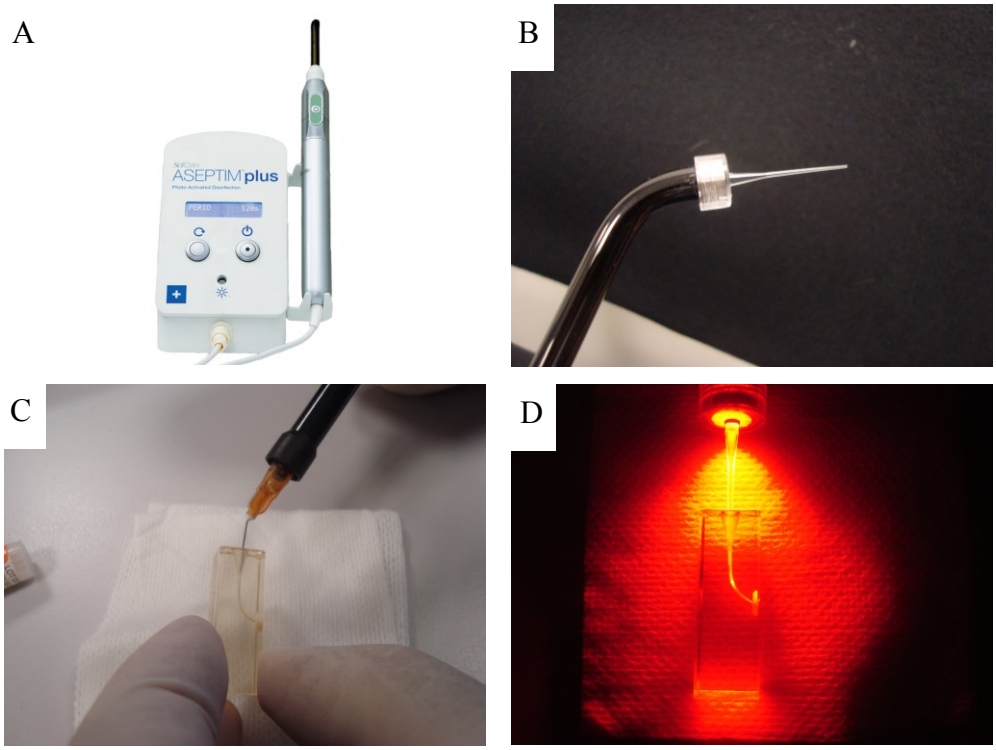


Figure 18. A: Aseptim™ delivers a red light of 635 nm, **B:** Soft plastic transparent tip, through which light travels into the canal, **C:** Photosensitizer solution was supplied by a syringe of 0.8 ml and was injected by a G26 needle, **D:** A demonstration of the process of Photodynamic therapy via LED, red light was diffused in all directions.

3.2.3.2 Group 2: Photodynamic therapy with Diode Laser

- **Light source**

A so-called DeltaCube™ soft laser with a wavelength of 650 nm (Laser 3 S, Pessac, France) and a maximum energy of 60 mW (Fig. 19A) was used. The light was delivered into the canals with a 300 µm optic fiber during 120 seconds. The optic fiber was kept stationary during irradiation. The most efficient irradiation time of the photosensitizer with the diode laser was determined in a pilot study. We exposed the photosensitizer to the red laser light; the color of the photosensitizer changed during the irradiation period from the original blue color to pink, which might change the light absorbance (Fig. 19B and C).

- **Photosensitizer**

A concentrated stock solution prepared from Toluidine blue O powder (Sigma-Aldrich, Schnelldorf, Germany) as follow: 0.075 g of stock Toluidine blue O powder was dissolved in 50 ml sterile water to get a 1.5 mg/ml stock solution. This stock solution was stored in the dark. The working concentration of Toluidine blue O was 15 µg/ml and this was obtained by diluting of 0.1 ml of stock solution in 9.9 ml of sterile water.

- **Treatment protocol**

The teeth were stabilized in a base made of Aquasil™ impression material. The canals were filled with Toluidine blue photosensitizer solution using a 26G needle and the excess of the solution was collected by a suction unit (Fig. 19D, E and F). Before activation, the photosensitizer was agitated for 1 minute using # 10 K-file. Then the optic fiber was inserted into the canal and the Laser device was activated for a period of 120 seconds. Because Laser beam could not diffuse in all directions, the fiber was moved continuously upward and downward to ensure the contact with the photosensitizer along the root canal

wall. After treatment, the canal was rinsed using 2 ml of sterile water during 10 seconds approximately and dried with sterile paper points.

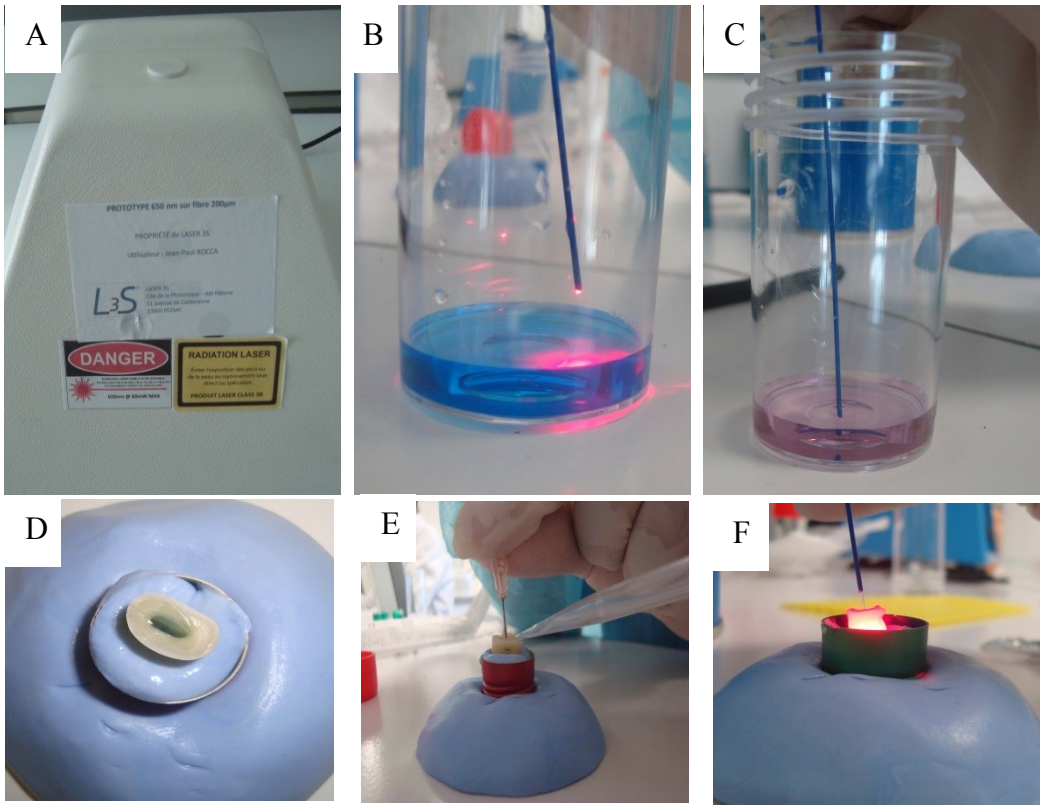


Figure 19. A: DeltaCube™ 650 nm specially designed for this experiment, **(B and C)** An experiment was performed to determine the best duration for activation of the photosensitizer by Laser; the original blue color of the photosensitizer turns in pink after 120 seconds of irradiation **(D and E)** The canal was filled with solution and the excess was collected by means of a suction unit, **F:** Activation of photosensitizer by Laser.

3.2.3.3 Group 3: Passive Ultrasonic Irrigation

- . **Treatment protocol**

The ultrasonic irrigation protocol was performed by injecting 1 ml EDTA 17% (Produit Dentaire S.A, Vevey, Switzerland) in the root canals. The injected EDTA solution was agitated in the canals for one minute by means of an endodontic ultrasonic file (IRRISAFE® ACTEON, Merignac, France) mounted on an ultrasonic unit (Piezon® Master 400 EMS Electro Medical Systems SA, Nyon, Switzerland). Two ml of NaOCl 2.6% was injected in the canal and agitated for one minute with an endodontic ultrasonic file. Between the 2 irrigation steps, the EDTA was rinsed out by injecting 1 ml saline solution into the root canal space.

Another 2 ml of NaOCl 2.6% was injected again into the canals during 10 seconds. Finally the canal was rinsed with 2 ml sterile water and dried with sterile paper points. All injections were done using a 26G needle.

3.2.4 Sampling and Cultures

Once the clinical procedures accomplished, microbiological sampling was done from the canals. A #10 K-file was used to rub the canal walls to collect any possible viable bacteria. The sample was then cultivated in 5% sheep blood agar.

The technique of culturing was inspired by scientific publications of Bonsor *et al*. (163, 164). A design of five parallel lines was created on the agar plate and this was repeated three times more which gave a five growing area to the culture pattern (Fig. 20). It should be reminded that the standardization of this technique was performed according to previously mentioned publications, where score 2 represents $1.5x10^8$ of *S. salivarius* and scores 0 and 5

represent respectively no and heavy bacterial load. During streaking the lines the K-file by which the sample was collected from root canal walls was turned clockwise to ensure that the entire sample was transferred to the agar plate. Bacterial specimens from all teeth were cultivated under aerobic condition, and 3 teeth from each group were randomly selected for anaerobic cultivation. The plates were then incubated at 37ºC. The bacterial growth was observed and recorded after 24, 48 and 72 hours of incubation.

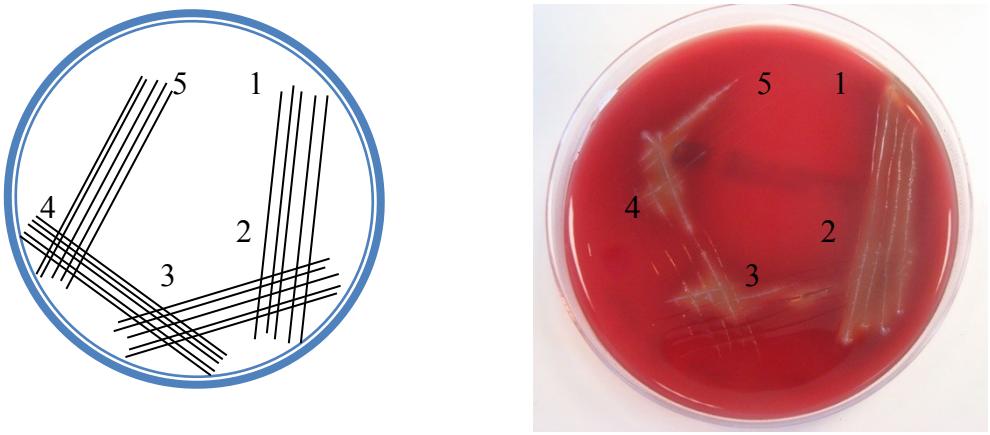


Figure 20. Schematic view of culturing and scoring and an agar plate with 5 score design streaking.

3.2.5 Statistical Evaluation

ANOVA statistical analysis was performed to document any statistically significant difference between groups. All groups were compared by multiple two by two sample tests by PLSD Fisher (Protected Least Significant Difference) test and were confirmed by Student Newman-Keuls test. A probability level of $p \leq 0.05$ was considered to be significant.

3.3 Main Study

3.3.1 Artificial Biofilm Design

Bacterial composition of the biofilm was a combination of *Streptococcus salivarius* ATCC 13419, *Enterococcus faecalis* ATCC 29212, *Fusobacterium nucleatum* ATCC 25586 and *Porphyromonas gingivalis* ATCC 33277. All bacterial species were acquired from Institut Pasteur's Biological Resource Center (Institut Pasteur, Paris, France).

The bacterial composition of the artificial biofilm was chosen based on different scientific publications to guarantee a future possible coexistence (Table. 4; Fig. 21A-E).

Enterococcus faecalis and *Streptococcus salivarius* were grown aerobically overnight and at 37ºC on Mueller-Hinton agar plates and 5% sheep blood agar plates (BioMérieux, Marcy l'Etoile, France), respectively.

Porphyromonas gingivalis and *Fusobacterium nucleatum* were cultivated anaerobically on 5% sheep blood agar plates at 37ºC for 3 and 5 days, respectively.

For the four strains a standardized suspension containing $1.5x10^8$ cells ml^{-1} in Schaedler broth (Bio-Rad, Marnes la Coquette, France) was prepared according the following proportions: 5% *Streptococcus salivarius*, 21% *Enterococcus faecalis*, 37% *Fusobacterium nucleatum* and 37% *Porphyromonas gingivalis.*

Genre and Species	Bacteriological characteristic	Gram	Respiratory mode	Hemolysis type	Catalase	Habitat and pathogenic ability	ATTC	Reference
Streptococcus salivarius	0,5-2µ Cocci ; pairs and chains	+	Aerobic or facultative anaerobic	**γ - α – β**	-	oral *Streptococcus*, presents in supra gingival plaque, gingival sulcus, saliva and over soft tissues isolated from: caries, pulpal necrosis, endodontic reinfection, periapical abscess, chronic gingivitis, peri implantitis and endocarditis	ATCC13419	(165)
Enterococcus faecalis	0,6-2,5µ Ovoid ; pairs and short chains	+	Aerobic or facultative anaerobic	**γ - β**	-	endodontic infections, endodontic treatment failure	ATCC29212	(165, 166)
Porphyromonas gingivalis	1-3µ Coccobacillus	-	Strict Anaerobic	**γ - β**	-	endodontic infection	ATCC33277	(165)
Fusobacterium nucleatum	3-100 µ (3-10 µ in average) Filaments with rounded ends, or in slender shape avec with granules inside; Isolated or in chains	-	Strict Anaerobic	**γ - β**	-	isolated from: pulpal necrosis, endodontic infection and periapical abscess	ATCC25586 ATCC10953	(165) (166, 167)

Table 4. The bacterial composition of the artificial biofilm.

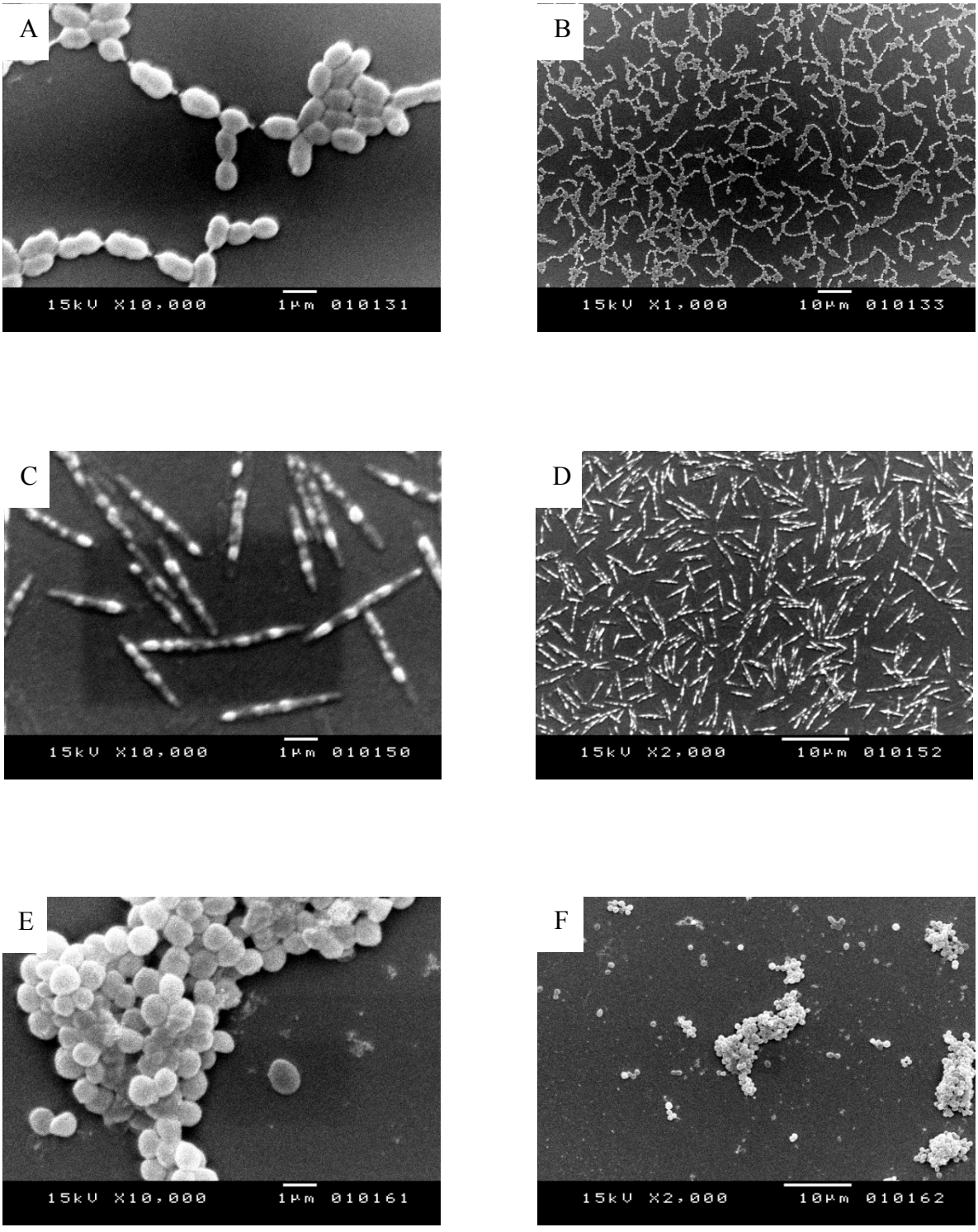


Figure 21. SEM observation of different member of monospecies biofilms before development of the artificial biofilm **A and B:** *Streptococcus salivarius,* **C and D:** *Fusobacterium nucleatum,* **E and F:** *Enterococcus faecalis.*

3.3.2 Infection of Dentinal Disks

For dentinal disks, the biofilm was formed on the disk surface by dispensing 2.5 ml of a standardized cell suspension into 6-well cell culture plates (Corning Inc., Union city, CA, USA). A total of 54 dentinal disk were used.

Cell culture plates were incubated anaerobically at 37°C on an orbital shaker (150 r.p.m). After 24 hrs, 0.5 ml of Schaedler broth was added into each well.

During incubation time, 30% of culture medium was replaced twice a week to supply needed nutrients for growth of bacteria.

A periodic monitoring of the biofilm state was performed after 3, 7,10,13,17,20,24,27 and 31 days of incubation. Each time 6 samples were used: two for bacterial cultivation, another two for scanning electron microscopy, one as positive control and the last one as negative control.

This procedure of biofilm formation over dentinal disks was repeated 3 times to confirm the reproducibility.

3.3.3 Infection of the Root Canals

Biofilm was developed inside the root canal by infecting samples with bacteria inside a sterile plastic container. Eleven root canals were placed in each container, then 24 ml of standardized cell suspension was added.

The containers were incubated anaerobically at 37°C on an orbital shaker (150 r.p.m). After 24 hrs, 8 ml of Schaedler broth was added in each container. The incubation time lasted 21 days.

Similar to the dentinal disk incubation process, 30% of the culture media were substituted with fresh culture media twice a week to provide required nutrients for growth of bacteria.

3.3.4 Washing

All samples were removed and washed using 0.1 M phosphate buffer saline twice, each time 3 minutes.

3.3.5 Sampling

Once the incubation procedures were accomplished (and after final treatment of the test groups), microbiological sampling was performed from the dentinal disks and canals. A #10 K-file was used to rub the dentinal surface to collect any possible viable bacteria. The sample was then cultivated in 5% sheep blood agar.

The technique of culturing was inspired by scientific publications of Bonsor *et al*. (163, 164). A design of 5 parallel lines was created on the agar plate and this was repeated 3 times more which gave a 5 growing area to the culture pattern (See part 3.2.4). During streaking the lines the K-file by which the sample was collected from root canal walls was turned clockwise to ensure that the entire sample was transferred on the agar plate.

Samples were incubated anaerobically at 37ºC. The bacterial growth was observed and recorded after 24, 48, 72 and 120 hours of incubation. At the end of the incubation period, colonies were taken from agar plates to perform gram staining.

3.3.6 Microscopy

3.3.6.1 Fixation

Those samples, including both dentinal disks and root canals that were intended to undergo imaging procedures (CLSM and SEM), were fixed with a 4% paraformaldehyde solution during 4 hours duration.

3.3.6.2 Dehydration

The fixed samples were then dehydrated using one of the two following methods:

A- For direct scanning electron microscopy, dentinal disks were dehydrated by transferring the samples through a series of ethanol washes, 70%, 80% and 100% during 5 minutes, 5 minutes and 10 minutes respectively. Samples were then placed in a desiccator at least for 24 hours before the ion sputtering process.

B- For *In Situ* hybridization, root canals were kept in 50% Ethanol solution at 4°C until use (52).

3.3.7 FISH and Confocal Microscopy

3.3.7.1 16S rRNA Probe Design

Oligonucleotide DNA probes were designed with different fluorescent dyes at 5' end as described in Table. 5 (Biomers.net®, Ulm, Germany).

	Probes and bacteria	5'-3' sequence of 16S rRNA	Dye	Reference
1	ENF 191 *Enterococcus faecalis*	GAA AGC GCC TTT CAC TCT TAT GC	ATTO488	(168-170)
2	POGI *Porphyromonas gingivalis*	CAA TAC TCG TAT CGC CCG TTA TTC	DY-405	(171)
3	Strept *Streptococcus salivarius*	CAC TCT CCC CTT CTG CAC	Cy5	(168)
4	FUS664 *Fusobacterium nucleatum*	CTT GTA GTT CCG CTT ACC TC	Cy3	(172)

Table 5. Sequence of 16S rRNAs of different members of the artificial biofilm.

To test the sensitivity, prior to any experimentation, each probe was double tested using SILVA ribosomal RNA data base (173) and BLAST software (174).

To test the efficiency of the probes, hybridization was first performed on pure culture of each bacterial species.

Bacteria were fixed on Shandon EZ single Cytofunnels® (Thermo Shandon Ltd., Cheshire, UK; Fig. 22C) using a Shandon Cytospin® Cytocentrifuge (Thermo Shandon Ltd., Cheshire, UK; Fig. 22A and B)

A

B

C

Figure 22. A and B: Shandon Cytospin® Cytocentrifuge, **C:** Shandon EZ single.

3.3.7.2 Hybridization

Probes were diluted with Tris-EDTA buffer according to data supplied by the company (Biomers.net®, Ulm, Germany). with a pH of 8. Subsequently they were stored at -20°C.

The hybridization buffer was formulated to use on pure bacterial cultures. The buffer was prepared by mixing 0.01% (w/v) Sodium dodecyl sulfate (SDS), 0.9M NaCl, 20 mM Tris/HCl. Then hybridization buffer was adjusted by adding 20% (v/v) of Formamide. Finally, concentration of added DNA probes to hybridization buffer were calculated to obtain a final concentration of 30 ng/µl.

For each sample, 500 µl hybridization buffer was prepared. Bacteria fixed on each slide were covered with 50 µl of the respective probe mixture, then remaining buffer was dispersed on a piece of tissue paper. Each slide was transferred into a 50 ml falcon tube and covered with wet tissue paper. This is to prevent evaporation of the hybridization buffer. To complete the hybridization process, the set was placed in an incubator for 90 min at 46°C.

Once hybridization accomplished, the washing step was carried out. A washing buffer composed of 0.01% (w/v) Sodium dodecyl sulfate (SDS), 88 mM NaCl, 20 mM Tris/HCl was prepared. Samples were rinsed twice by washing buffer each time for 10 min at 46°C inside a falcon tube.

Finally, all samples were cleansed with water to remove any remnant. All procedures from the very first beginning till the end were conducted in the dark. Samples were then kept away from light till confocal visualization.

After testing the sensitivity of the probes to each bacterial species, the FISH was performed for root canal control groups. Each infected root canal was blocked at the apex

and orifice and then split longitudinally in two pieces with a diamond disk (Prodont-Holliger, Vence, France) using sterile water.

The hybridization protocol was adapted from Böckelmann *et al.* (175) and Schaudinn *et al.* (52). The hybridization buffer was adjusted in following ratios: 0.01% (w/v) Sodium dodecyl sulfate (SDS), 0.9 M NaCl, 20 mM Tris/HCl. Then the hybridization buffer was diluted by adding 35% (v/v) of Formamide. Probes were added to the hybridization buffer to achieve a final concentration of 5 ng/µl.

For each sample, 500 µl hybridization buffer was prepared. The root canal surfaces were covered with 50 µl of respective probe mixture and then remaining buffer was dispersed on a piece of tissue paper. Each root canal was then transferred into a sterile 25 ml tube and covered with wet tissue paper to prevent evaporation of the hybridization buffer. To complete the hybridization process, the set was placed in incubator for 90 min at 46°C.

After the hybridization process, the washing step was performed. A washing buffer constituted of 0.01% (w/v) Sodium dodecyl sulfate (SDS), 88 mM NaCl, 20 mM Tris/HCl prepared. Samples were rinsed twice with washing buffer each time for 10 min at 46°C inside the falcon tube.

Finally, all samples were cleansed with water to remove any possible remnant. All described procedures were performed in the dark, and treated samples were kept away from light until use. The same procedure was repeated for all test groups once the clinical treatment was completed.

3.3.7.3 Confocal Microscopy

Sample observation under confocal microscope was performed by placing samples in LabTek™ chamber slides™ (Nunc, ThermoFisher Scientific, International). The chamber slides were filled with sterile water.

The microscopy was done in the Prism facility, "Plateforme PRISM – IBV- CNRS UMR 7277- INSERM U1091-UNS». The confocal microscope was a Zeiss LSM 710 on an inverted Axio Observer Z1 stand (Carl Zeiss Microscopy GmbH, Jena, Germany; Fig. 23), using objectives Plan Apo 10X dry NA 0.45 and/or LD-LCI Plan Apo 10X multi immersion (oil, glycerol, water) NA 0.8 and/or LD-C. The lasers used were a 405 nm diode laser and/or an Argon laser (458, 488, 514 nm) and/or a diode pumped solid state laser (DPSS) 561 nm, and/or HeNe 594 nm and/or HeNe 633 nm. The microscope was composed of 2 descanned Photomultiplier tubes (PMT), 1 descanned spectral PMT 32 channels, 1 binary GaAsP (BiG) descanned module equipped with 500-550 and 575-610 filter-set and with 1external PMT for transmission. The z-acquisitions were done using the microscope z-drive.

Images had a 512x512 pixels resolution in a field of 2x2 µm. Images were processed and analyzed with ImageJ an open source image analytic software developed by worldwide contributors (imagj.net).

Figure 23. Zeiss LSM 710 Confocal microscope.

3.3.7.4 Scanning Electron Microscopy

A- Coating

Samples were taken from desiccator. Then were gold coated (Ion sputter, JOEL JFC-11E LTD, Japan; Fig. 24).

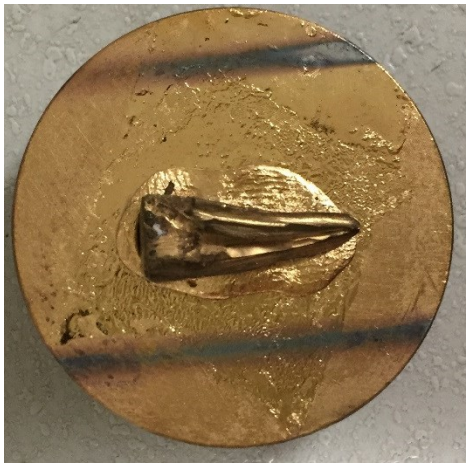


Figure 24. A gold coated tooth specimen.

B- Visualization

Samples including dentinal disks, control and treated root canals were visualized using Scanning Electron Microscope (JSM-5310LW, JEOL LTD, Japan) under total vacuum with original magnifications ranging from 75X to 7500X. Images were used to evaluate the biofilm formation on dentinal disks and walls of root canals and finally to assess the state of the biofilm and the dentinal surface after clinical treatment (See Fig. 16).

People not familiar with the study judged the SEM images obtained from the test groups in a triple blind test. To standardize the assessment, 500X images from coronal, middle and apical third of each root canal were included. A 10-cell-grid (Fig. 25) was designed over each picture and a custom indexing system from 0 to 4 was applied. Zero referred to 100% open tubules. Indexes 1, 2, 3 and 4 were representing 25%, 50%, 75% and 100% closed tubules, respectively.

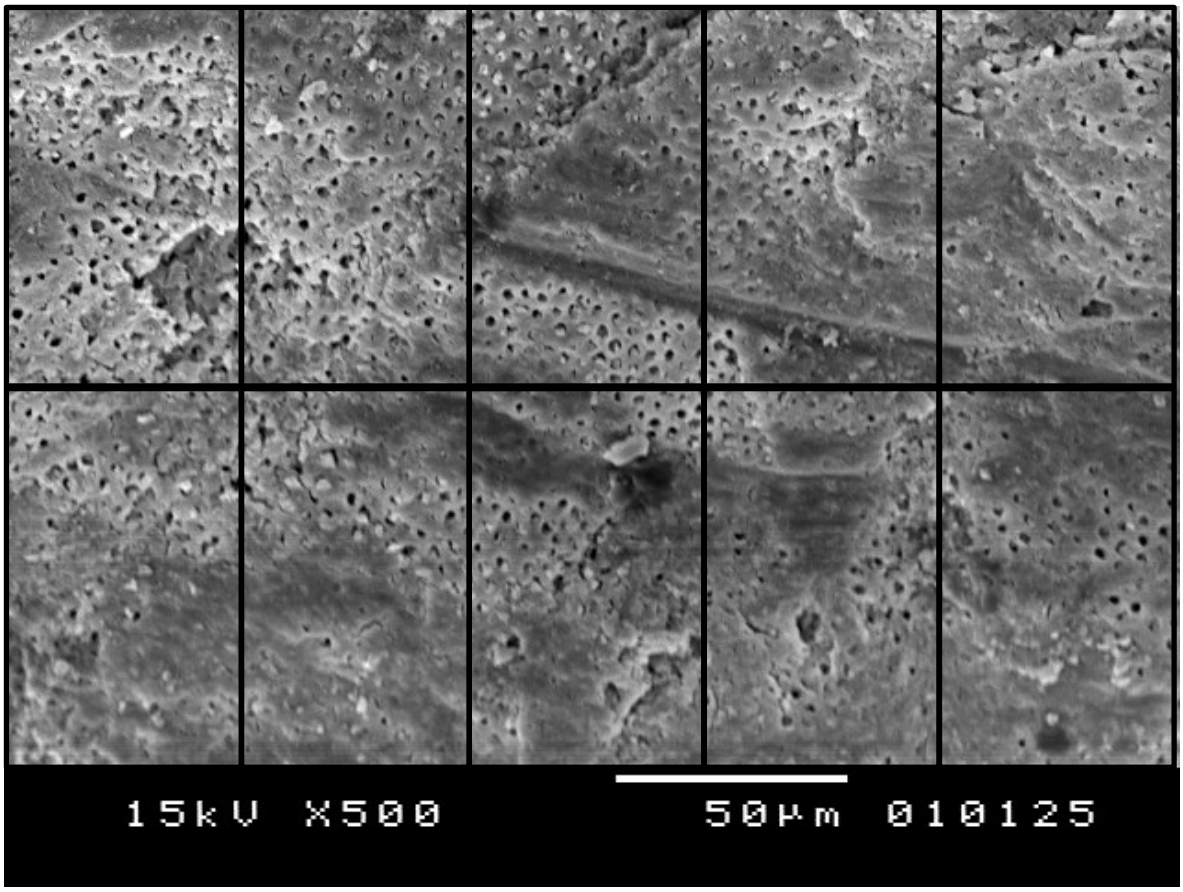


Figure 25. Grid to evaluate the state of cleanliness of the dentinal surface.

3.3.8 Test Groups

3.3.8.1 Group 1: Passive Ultrasonic Irrigation

Root canals were removed from the plastic container containing bacterial inoculum and then rinsed twice, each for 3 min in 0.1X PBS buffer on an orbital shaker at 100 r.p.m.

Next, root canals were treated using the OneShape® single file system (Micro-Mega, Besançon, France; Fig. 26). First a #10 recapitulation file was inserted to the working length (root canal length minus 0.5 μm) . Then a OneShape® was placed down to 2/3 of the WL using an in and out movement without pressure. An upward circumferential filing movement was done in order to pre-enlarge the canal. The OneShape® instrument was then removed out of the root canal and was cleansed. Canals were irrigated by 1 ml of NaOCl 2.6% (Pharmacy of CHU Saint-Roch hospital) and the canal patency was checked with a #10 K file.

Afterward, the OneShape® instrument was reintroduced into the root canal and was placed down to 3 mm from WL using an in and out movement without pressure. The OneShape® instrument was removed from the canal and was cleansed with a gauze. The canal was re-irrigated and re-checked for patency with a #10 K file.

Finally, the OneShape® file was introduced for a last time into the root canal and was taken down to the WL by performing the in and out movement. The WL was reached in one passage. An upward circumferential filing movement was performed in the last step.

Figure 26. OneShape rotary single file.

The final irrigation was performed by injecting 1 ml Salvizol® EDTA 8% solution (ACTEON, Merignac, France) in the root canals. The injected EDTA solution was agitated in the canals for one minute using an endodontic ultrasonic file, (IRRISAFE®ACTEON, Merignac, France) mounted on an ultrasonic unit (Piezon® Master 400 EMS Electro Medical Systems SA, Nyons, Switzerland). The power was set to 9 o'clock position. This device generates a fixed frequency of 28 KHz. Two ml of NaOCl 2.6% was injected in the canal and was agitated for one minute with the previously mentioned apparatus.

Another 2 ml of NaOCl 2.6% was injected again into the canals and finally the canal was rinsed with 2 ml sterile water and dried with sterile paper points. All injections were done using an open ended 26G needle.

3.3.8.2 Group 2: Rotary Instrument

Root canals were removed from the plastic container containing bacterial inoculum and then rinsed twice, each for 3 min in 0.1X PBS buffer on an orbital shaker at 100 r.p.m.

Next, root canals were treated using the OneShape® single file system (Micro-Mega, Besançon, France). First a #10 recapitulation file was inserted to the working length (root canal length minus 0.5 μm). Then a OneShape® was placed down to the 2/3 of the WL using an in and out movement without pressure (Fig. 27A-D). An upward circumferential filing movement was done in order to pre-enlarge the canal. The OneShape® instrument was removed from the root canal and cleansed. Canals were irrigated with 1 ml of sterile water (Pharmacy of Saint-Roch hospital) and the canal patency was checked with a #10 K file.

Afterward, the OneShape® instrument was reintroduced into the root canal and was placed down to 3 mm from WL using an in and out movement without pressure. The OneShape® instrument was removed from the canal and cleansed. The canal was re-irrigated with 2 ml of water for 10 seconds and re-checked for patency with a #10 K file.

Finally, the OneShape® file introduced for a last time into the root canal and was taken down to the WL by performing the in and out movement. The WL was reached in one passage. An upward circumferential filing movement was performed in the last step.

The final irrigation with NaOCl and EDTA was omitted for this group in purpose of evaluation of the instrument's effect on the biofilm and the dentinal surface of root the canal wall.

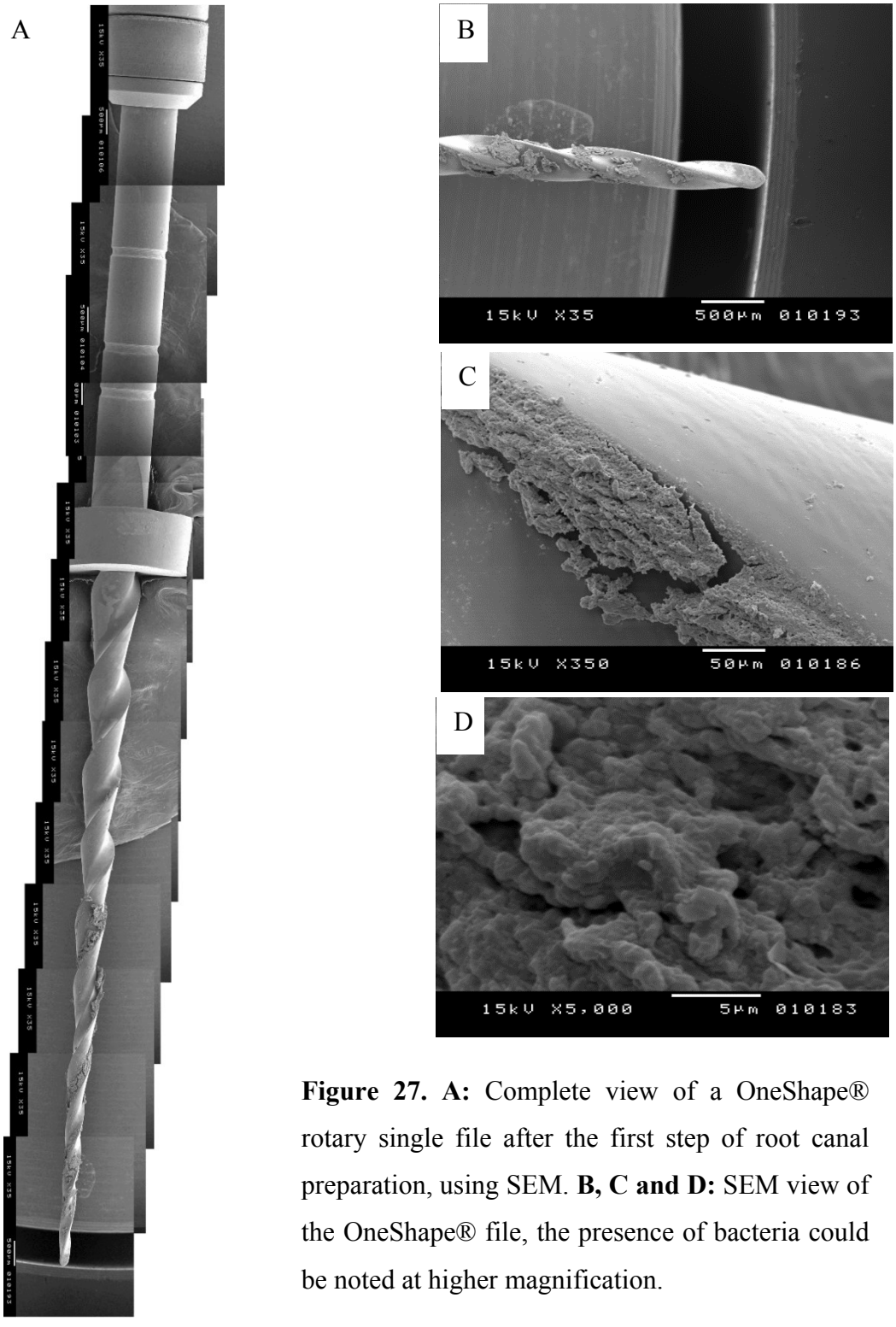


Figure 27. A: Complete view of a OneShape® rotary single file after the first step of root canal preparation, using SEM. **B, C and D:** SEM view of the OneShape® file, the presence of bacteria could be noted at higher magnification.

3.3.8.3 Group 3: Rotary Instrument and Photodynamic Therapy

Root were removed from plastic the container containing bacterial inoculum and then rinsed twice, each for 3 min in 0.1X PBS buffer on an orbital shaker at 100 r.p.m.

Next, root canals were treated with the OneShape® single file system (Micro-Mega, Besançon, France). First a #10 recapitulation file was inserted to the working length (root canal length minus 0.5 μm). Then a OneShape® was placed down to the 2/3 of the WL using an in and out movement without pressure. An upward circumferential filing movement was done in order to pre-enlarge the canal. The OneShape® instrument was removed from the root canal and was cleansed. The canals were irrigated with 1 ml of sterile water (Pharmacy of Saint-Roch hospital) and the canal patency was checked with a #10 K file.

Afterward, the OneShape® instrument was reintroduced into the root canal and was placed down to 3 mm from WL using an in and out movement without pressure. The OneShape® instrument was removed from the canal and was cleansed. The canals was re-irrigated with 2 ml of water for 10 seconds and re-checked for patency with a #10 K file.

Finally, the OneShape® file was introduced for a last time into the root canal and was taken down to the WL by performing the in and out movement. The WL was reached in one passage. An upward circumferential filing movement was performed in the last step.

Instead of final irrigation, Photo Activated Disinfection was performed.

A light emitting diode with a wavelength of 635 nm (Aseptim^{TM} Plus, Leutkirch im Allögo, Germany) with a power density of 900 mW/cm^2 was used as source of light. Light was delivered with disposable conical plastic tips (Fig. 28A).

A concentrated stock solution was prepared from Toluidine blue O powder (Sigma-Aldrich, Schnelldorf, Germany) as follows: 0.075 g of Toluidine blue O was dissolved in 50

ml sterile water to get a 1.5 mg/ml stock solution (Fig. 28B). This stock was preserved in dark. The working concentration of Toluidine blue O was 15 µg/ml and this was obtained by diluting of 0.1 ml of stock solution in 9.9 ml of sterile water.

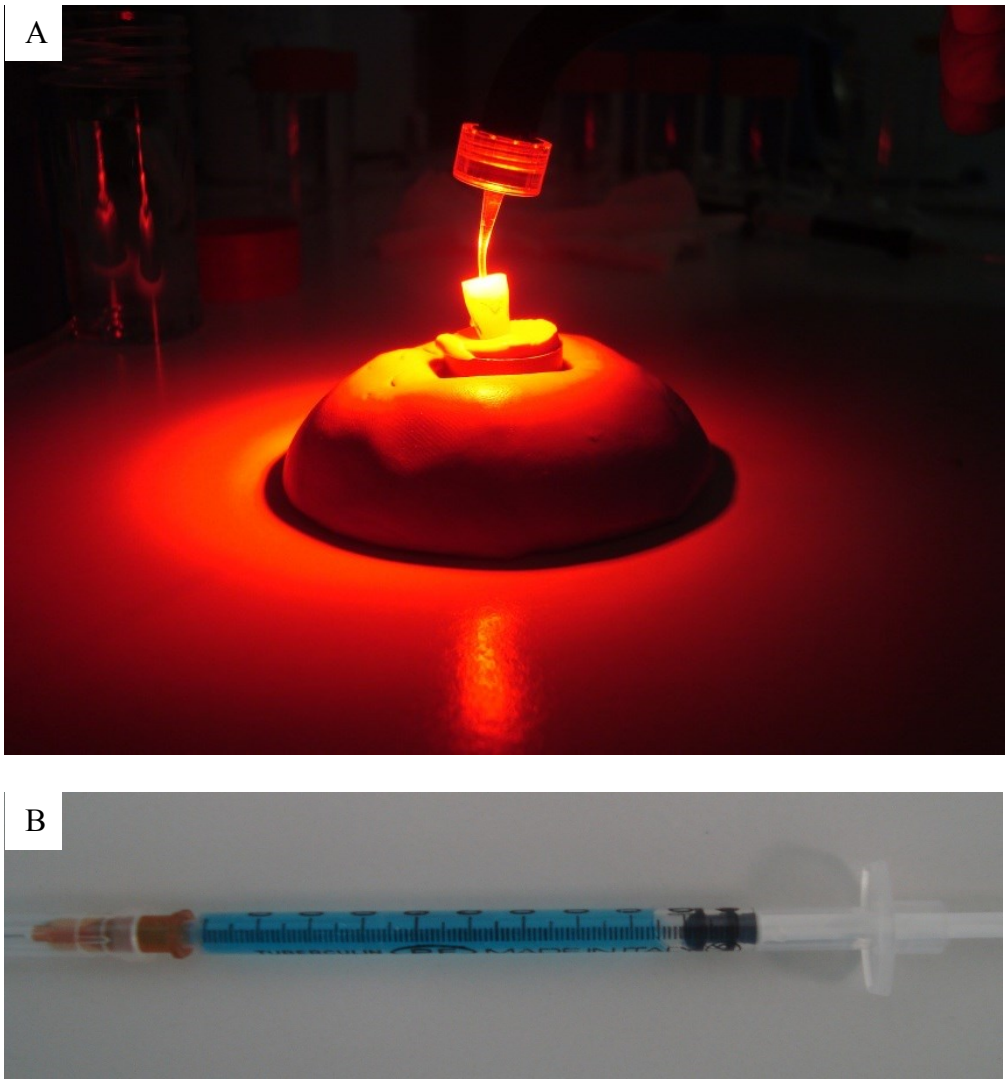


Figure 28. A: Activation of Aseptim™ solution by a LED light of 635 nm of wavelength, the sample was mounted in a putty of silicon impression material to facilitate the performance of treatment **B:** Toluidine blue O photosensitizer solution was injected into the canals using a 26G needle.

The Toluidine Blue solution was injected into the canal using a G26 needle to fill up entire volume of the canal. The excess of the product was collected during injection using a sterile pipette tip and a suction device. The procedure was continued by rubbing the solution inside the canal for one minute, using a size 10 K-file. A specially designed sterile flexible tip was attached to the LED device and was inserted into the canal space till a tug-back sensation was achieved. Photosensitizer was activated during 120 seconds. Once the procedure completed, the canal was rinsed with 2 ml of sterile water to remove the photosensitizer from the canal.

3.3.8.4 Group 4: Er:YAG Laser Delivered via Sapphire tip and NaOCl

Samples after being infected with biofilm were treated directly with an 2940 nm Er:YAG laser (FidelisII™, Fotona, Ljubljana, Slovenia) with an embarked 0.8 mm diameter sapphire tip (Fig. 29A-C).

The root canal was filled with NaOCl 2.6% and then the sapphire tip was placed at its orifice. The solution was activated during 5 sec by laser flux. The irradiation parameters of Er:YAG were adjusted to 6 Hz with a pulse duration of 300 µs (Mode SP) and 80 mJ of energy. The activation was repeated 4 times and the irrigant solution was renewed each time. The root canal space was dried and then sampled with a #10 K-file.

3.3.8.5 Group 5: Er:YAG Laser Delivered via Endodontic Fiber and NaOCl

Samples after being infected with biofilm were treated directly with a 2940 nm Er:YAG laser (FidelisII™, Fotona, Ljubljana, Slovenia) with an attached 300 µm diameter flat ended endodontic fiber (PRECISO™, Fotona, Ljubljana, Slovenia; Fig. 29D)

The root canal space was filled-up with NaOCl 2.6% then the endodontic fiber was introduced 5 mm far from working length (root canal length minus 0.5 mm). The solution was activated during 5 sec by laser irradiation through a spiral movement in an apico-coronal direction. The irradiation parameters of Er:YAG were adjusted to 6 Hz with a pulse duration of 300 µs (Mode SP) and 80 mJ of energy. The activation was repeated 4 times and the irrigant solution was renewed each time.

The root canal space was dried with paper points and then sampled with a #10 K-file.

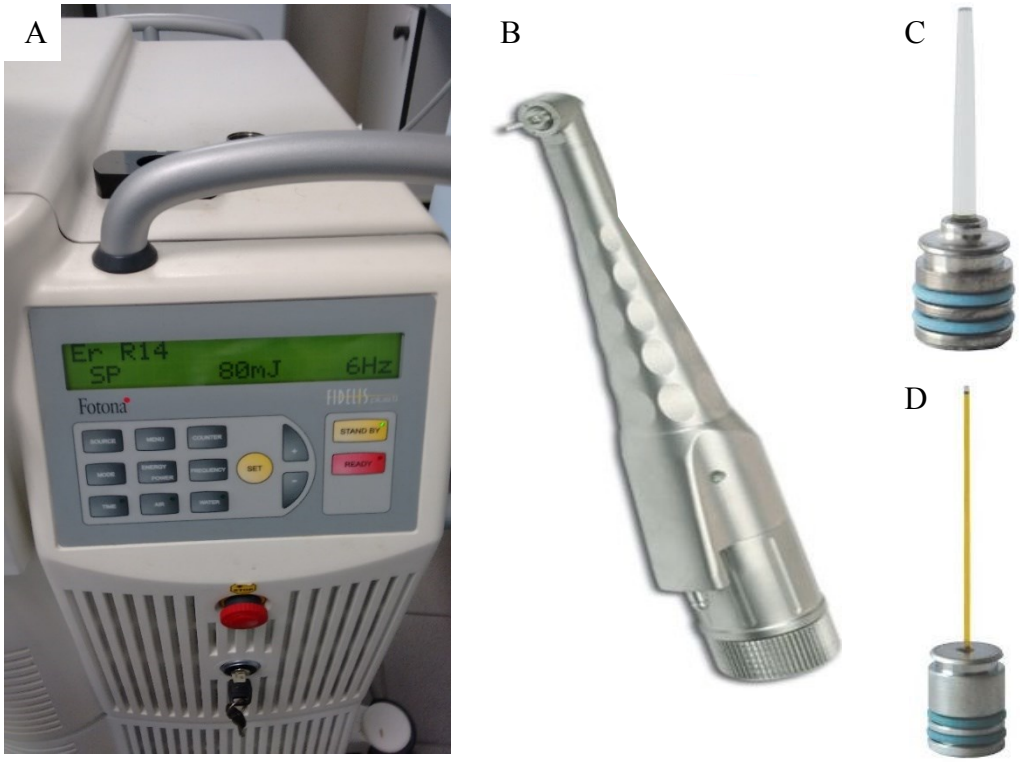


Figure 29. A: Fotona Fidelis Er:YAG Laser, **B:** R14 hand piece, **C:** Sapphire tip with a diameter of 0.8mm at the end, **D:** Endodontic fiber with a diameter 0.3 mm.

3.3.8.6 Group 6: Diode Laser Delivered via Endodontic Fiber and NaOCl

Samples after being infected with biofilm were irradiated with a 915-1064 nm double wavelengths diode laser (DeltaCube3™, Erma-Électronique, La Teste de Buch, France) delivering the laser beam through a 400 µm diameter endodontic fiber (Fig. 30).

The root canal space was filled with NaOCl 2.6% and then the endodontic fiber was introduced 5 mm from the working length (root canal length minus 0.5 mm). Irrigation solution was irradiated during 15 sec via a spiral upward movement. The irradiation parameters of the diode laser were adjusted to chopped mode, 100 Hz and 2 W of power. The activation was repeated 4 times and the irrigant solution was renewed each times.

The root canal space was dried with paper points and then sampled with a #10 K-file. After finishing all clinical procedures, 7 samples of each group were subjected to bacterial cultures and the rest were used for imaging analysis.

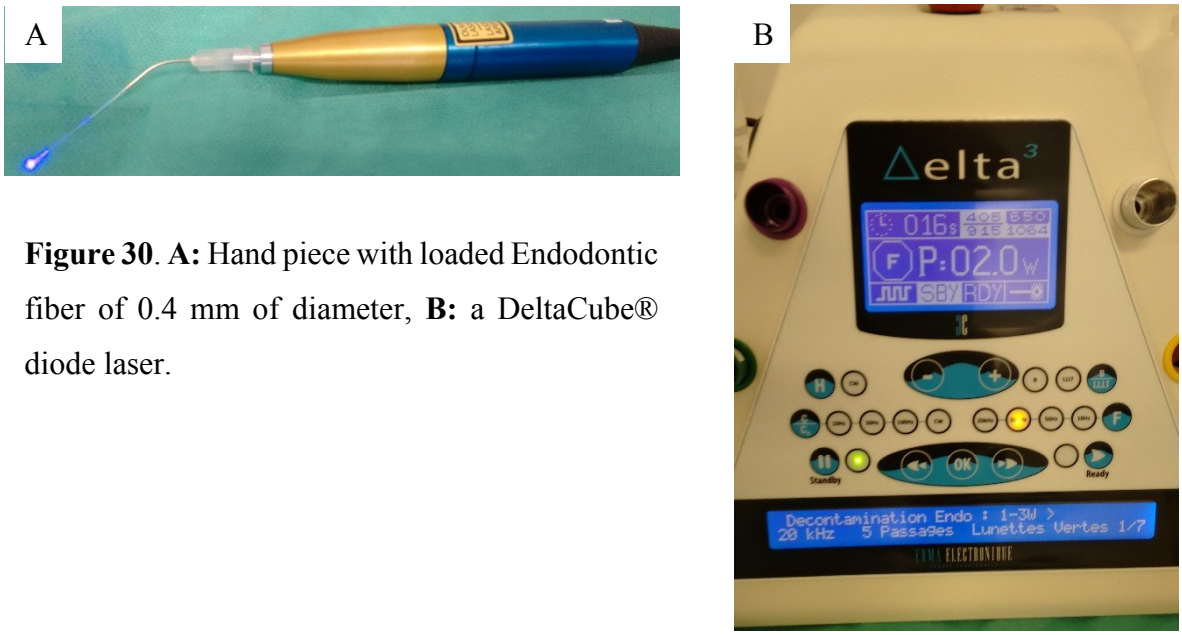


Figure 30. **A:** Hand piece with loaded Endodontic fiber of 0.4 mm of diameter, **B:** a DeltaCube® diode laser.

3.3.9 Evaluation

Bacterial culture, FISH and SEM were performed as mentioned for the aged biofilm development process. Bacterial cultures were performed to check the presence of viable cells.

3.3.10 Statistical Evaluation

Statistical analysis was carried out for test groups using BiostaTGV, Institut Pierre Louis UMR-S 1136 (http://marne.u707.jussieu.fr/biostatgv/?module=tests; Fig. 31).

Kruskal-Wallis quantitative statistical test was performed among all groups to confirm the significance of the study for both FISH and SEM images.

Significance of Kruskal-Wallis tests were confirmed by Mann-Whitney or Student's test. The Mann-Whitney or Student's T test compared the impact of treatment procedures in terms of antibacterial activity and smear layer creation among different treatment groups.

Interrater agreement test (Kappa) was performed with MedCalc® software.

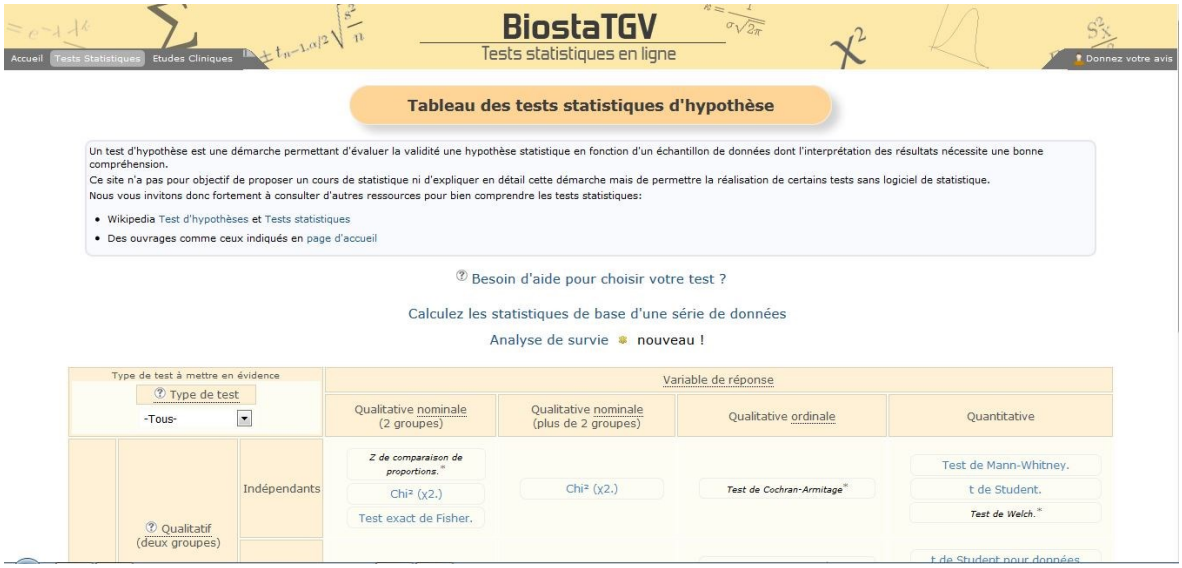


Figure 31. Schema of BiostaTGV website.

4

Results

We performed this study in order to evaluate the capability of different contemporary disinfecting system on the disruption of the biofilm attached on the walls of root canal. Our study was programmed on 4 distinct phases:

Phase I: Pilot study to verify the possible effect of Photodynamic therapy on artificial biofilm.

Phase II: Establishment of a standardized reproducible artificial biofilm.

Phase III: Characterization of established biofilm on the surface of root canal walls in orders to differentiate the presence of bacterial species and distinguish their distribution.

Phase IV: Clinical treatment of root canals infected by artificial biofilm and subsequent evaluation of efficiency of each protocol.

4.1 Phase 1: Pilot Study

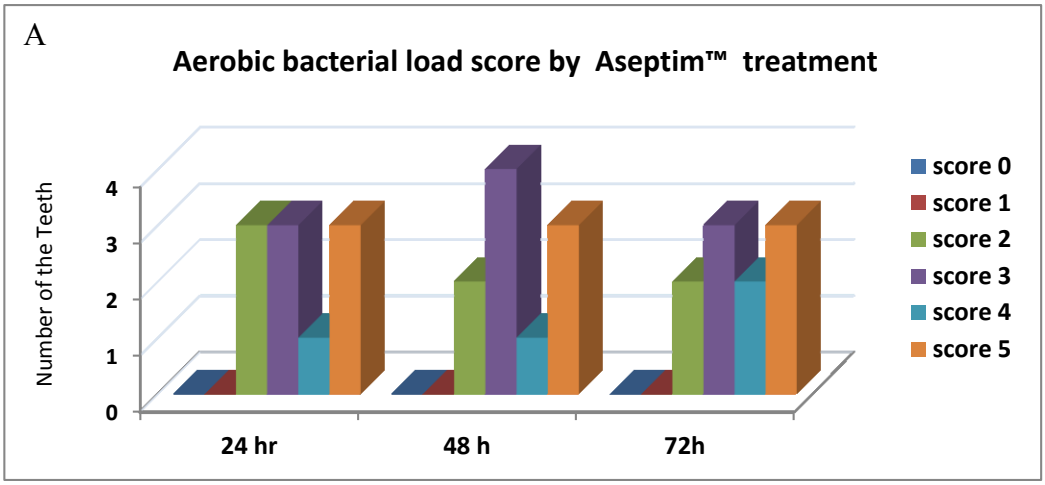
In order to evaluate the capability of different contemporary disinfecting system on the disruption of the biofilm attached on the walls of root canal, we prepared 30 root canals and inoculate them with bacterial biofilms. Then prepared root canals were treated in three different methods: a) treated with Aseptim™ photo activated disinfection system, b) treated by a Toluidine blue solution of 15 μg/ml concentration and a diode Laser, c) applying an Ultrasonic irrigation with EDTA 17% and NaOCl 2.6%. Viable bacteria after the treatments were recovered and cultivated on 5% sheep blood agar aerobically and anaerobically.

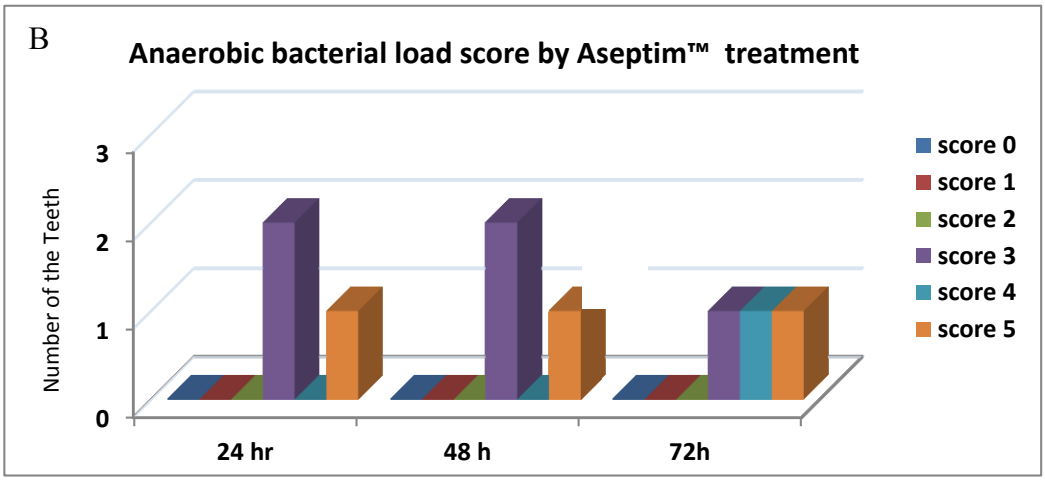
The teeth treated by Aspetim™ photosensitizing agent and LED showed different responses to photo activated disinfection upon the different recording time of bacterial growth.

Based on the scoring method, none of the root canal wall cultures had scores 0 and 1 in this group which means that in all canals there was a bacterial load after the application by Aseptim™ system.

In aerobic conditions, 24 hours after treatment, cultures taken from 3 root canals showed score 2 which was reduced to 2 canals after 48 hours and remained unchanged at final observation after 72 hours. During the first observation, there were 3 canals with a bacterial load score of 3 which was augmented to 4 canals two days after inoculation and returned to 3 canals at the end of the experiment. Score 4 was registered in only 1 culture at 24 and 48 hours after treatment, while after 72 hours 2 cultures shows the same score . Of the 10 root canals evaluated in this group, only 3 showed maximum bacterial infection with a score of 5 all during the observation (from 24 hours to 72 hours after the treatment).

The cultures taken from the root canals did not present anaerobic bacterial load of score 0, 1 and 2. At the time point of 24 hours and 48 hours after treatment, 2 root canals had anaerobic bacterial load of score 3 and one of them represented higher score of 4 in 72 hours. Only 1 root canal showed a complete anaerobic bacterial infection with a load score of 5 all during the cultivation experiment. There was no significant difference between the bacterial load score obtained from anaerobic and aerobic cultivations (Fig. 32).





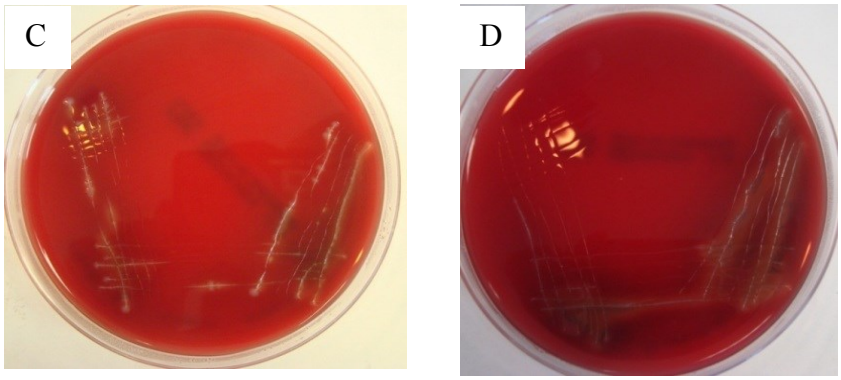
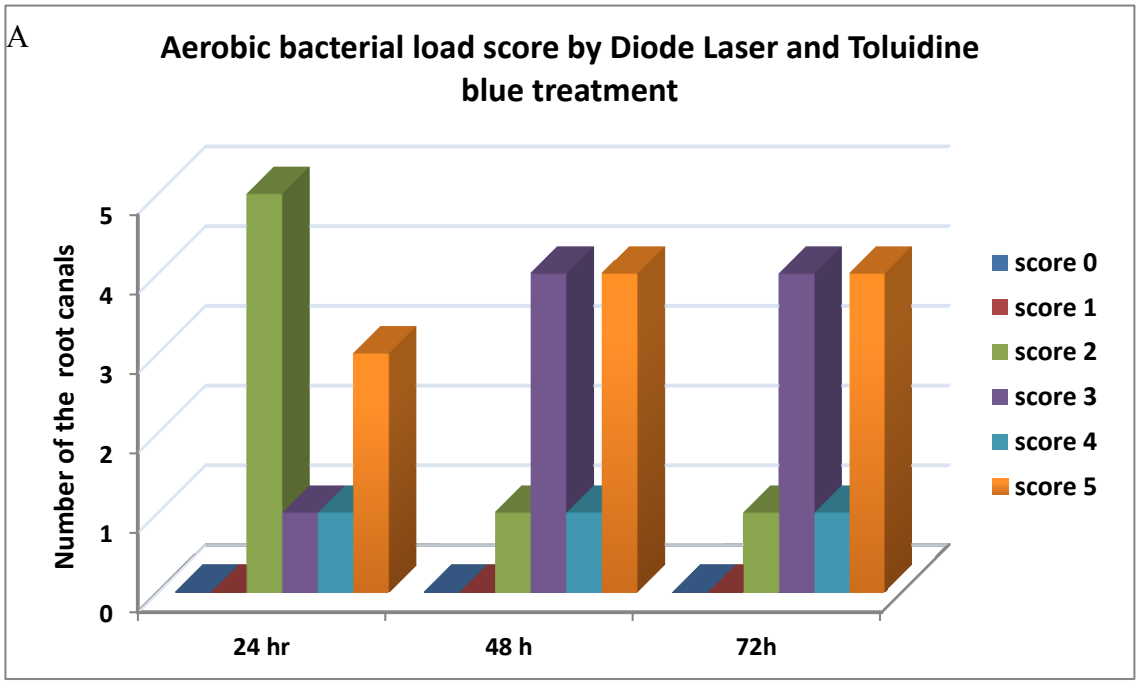


Figure 32. Bacterial load score per root canals treated by Aspetim™ and LED. Bar chart shows the numbers of the root canals with aerobic (**A)** and anaerobic (**B)** bacterial loads at 24 hours, 48hr and 72 hours after the treatment. Total number of evaluated root canals for aerobic and anaerobic cultivation was 10 and 3, respectively. Cultures showing score 4 of aerobic (**C)** and score 3 (**D)** of anaerobic bacterial load reduction.

The second group of the teeth was treated with diode Laser and Toluidine blue. The teeth in this group presented both aerobic and anaerobic bacterial load in response to the Photodynamic therapy. In aerobic condition after 24 hours, cultures taken from 5, 1, 1 and 3 root canals had bacterial load score of 2, 3, 4 and 5, respectively. There was no root canal presenting load score of 0 and 1. There was a dramatic decrease in number of root canals with score 2 from 5 to 1 and respective increase in number of root canals with score 3 from 1 to 4 at 48 hours after treatment. One root canal with score 4 and 4 root canals with score 5 were recorded in this step of observation. The bacterial load scores remained unchanged after 72 hours of incubation (Fig. 33A).

Interestingly, all 3 root canals evaluated under the anaerobic condition presented a high level of bacterial load score of 5 only after 72 hours of incubation (Fig. 33B).

Finally the third group of the root canals was disinfected by Ultrasonic irrigation in the presence of EDTA 17% and NaOCl 2.6%. Interestingly, the bacterial load score of zero was recorded for all evaluated root canals in this group all during the observation (24 hours, 48 hours and 72 hours of incubation) for both aerobic and anaerobic culture conditions (Fig. 34A-D).



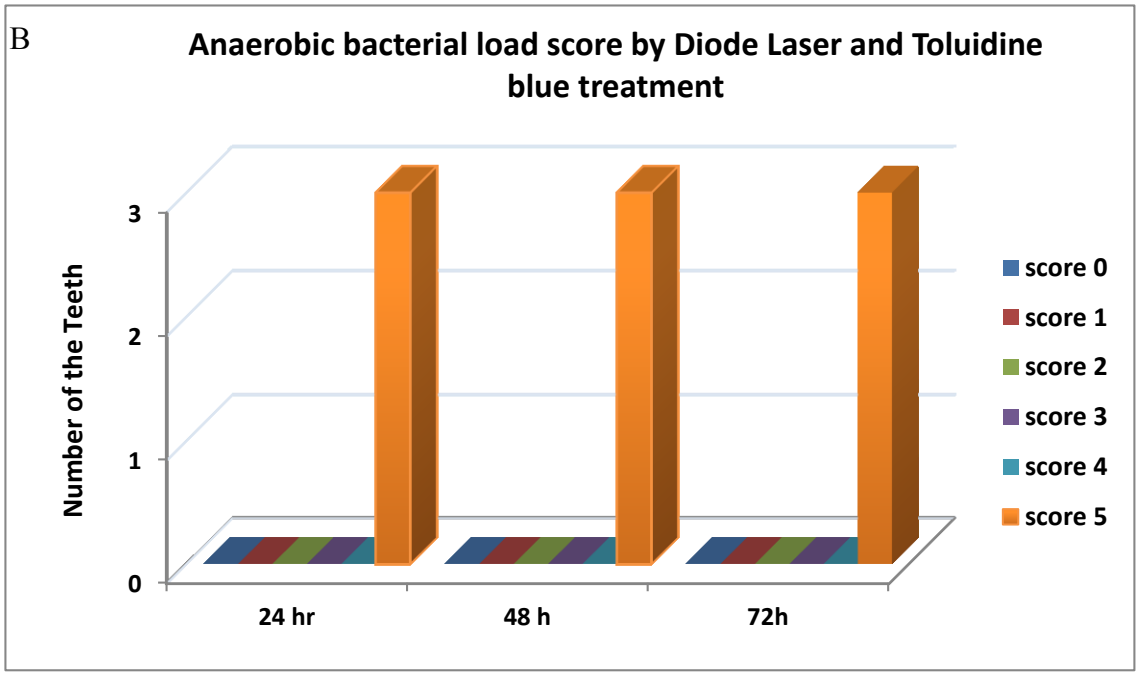


Figure 33. Bacterial load score per root canals treated by Diode Laser and Toluidine blue. Bar chart shows the numbers of the root canals with aerobic (**A**) and anaerobic (**B**) bacterial loads at 24 hours, 48hours and 72hours after the treatment. Total number of evaluated root canals for aerobic and anaerobic cultivation was 10 and 3, respectively.

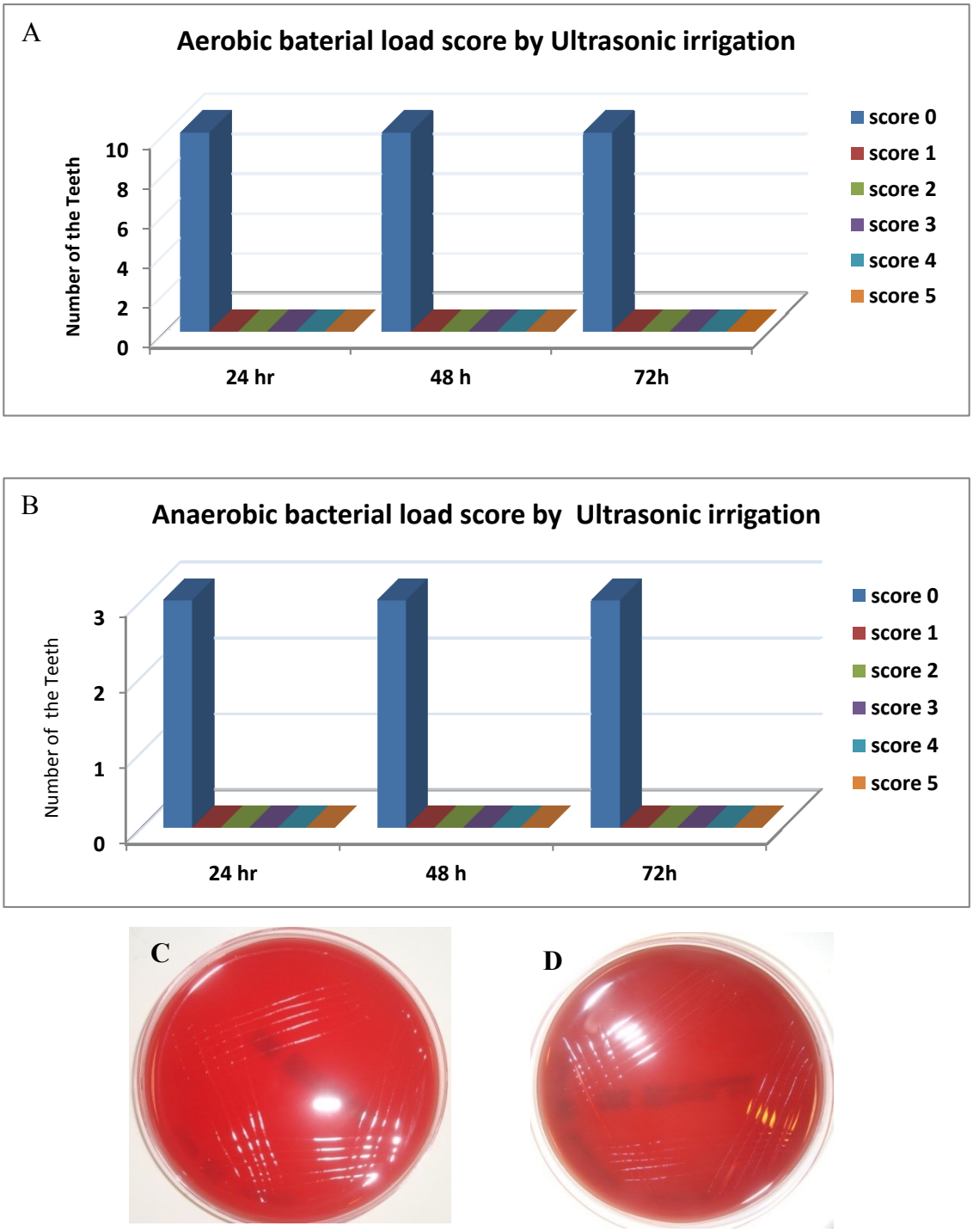


Figure 34. Bacterial load score per root canal after Ultrasonic irrigation. Bar chart shows the numbers of the root canals with aerobic **(A)** and anaerobic **(B)** bacterial loads at 24 hours, 48hours and 72 hours of incubation. Total number of evaluated root canals for aerobic and anaerobic cultivation was 10 and 3, respectively. A score 0 of aerobic **(C)** and anaerobic **(D)** culture after ultrasonic irrigations.

ANOVA (Analysis of variances) statistical analyses were done; all groups were compared by multiple two by two sample tests by Fisher's Protected Least Significant Difference test. Statistical analysis showed there is a significant difference between all three groups in the agar plates examined in aerobic condition (p <0.0001 in all observations at 24, 48 and 72 hours; Fig. 35). We observed no significant difference in results obtained from photodynamic therapy with diode laser and photo activated disinfection by Aseptim™ protocol (p<0.6267) for the final observation at 72 hours), all of these were confirmed by Student Newman-Keuls test. Ultrasonic irrigation was the most reliable protocol to disrupt the microbial biofilms (p<0.0001).

The same statistical analyses procedures were performed for the cultures incubated in anaerobic condition. A significant difference was observed in 3 groups and for all observations at 24, 48 and 72 hours (P <0.0001). The Ultrasonic irrigation had the best effects on reducing of bacterial load in anaerobic conditions (P <0.0001). However, Aseptim™ had statistically better effects than photodynamic therapy by diode Laser to reduce bacterial load in anaerobic conditions (P <0.0043) for final observation at 72 hours (Fig. 36).

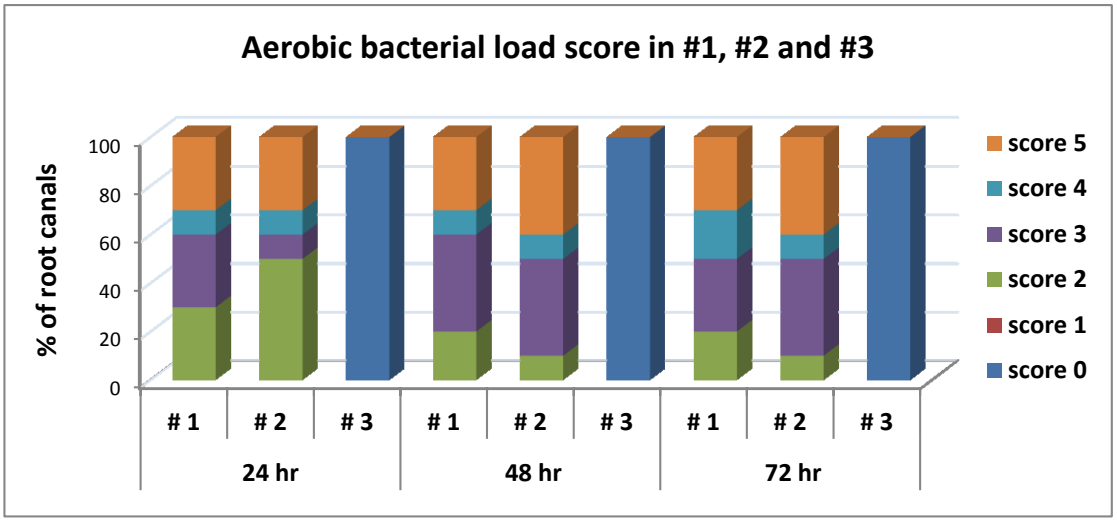


Figure 35. Comparision of Aseptim™ and Diode laser photo activated disinfection with Ultrasonic irrigation in 24, 48 and 72 hours after treatment in aerobic conditions.

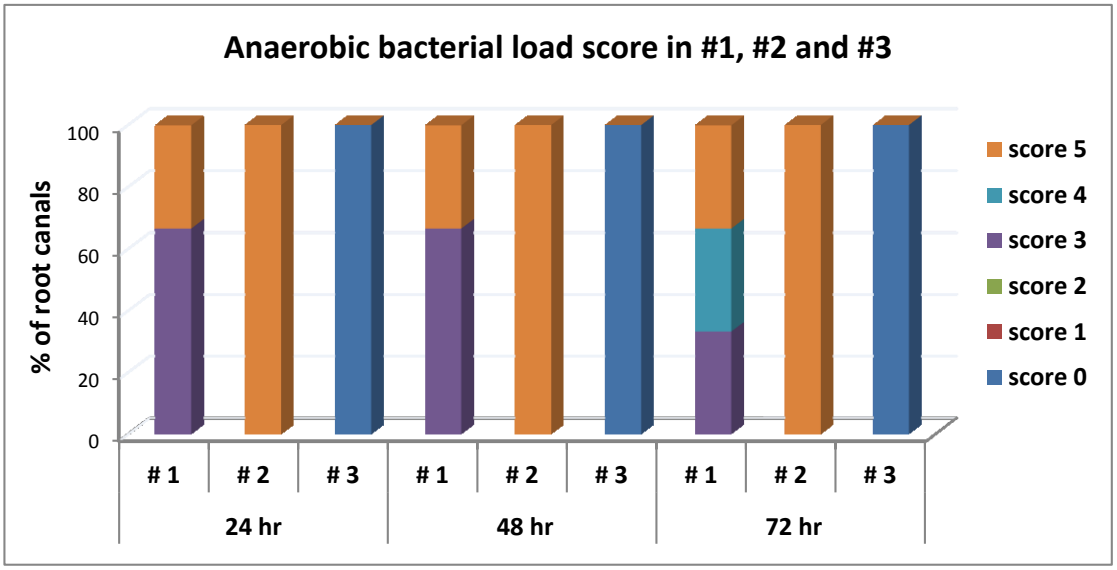


Figure 36. Comparision of Aseptim™ and Diode laser photo activated disinfection with Ultrasonic irrigation in 24, 48 and 72 hours after treatment in anaerobic conditions.

4.2 Phase 2: Establishment of Standardized Artificial Biofilm

Polybacterial biofilm was constructed by inoculation of different bacteria over dentinal disk surface. Then, dentinal disks were incubated for 31 days and during this period the periodic verifications including bacterial culturing, Gram staining and SEM observations were conducted.

After 3 days of incubation:

Gram staining and bacterial culture: gram staining revealed numerous number of gram-positive in pairs and small number of cocci chains. The β hemolysis was observed and two types of colonies were noticed: 1. Big white colonies with undefined borders, 2-3 mm in diameter, with lower number; 2. Small white colonies with well-defined borders, ≤1 mm in diameter.

SEM: Biofilm was formed and partially covered the surface of dentinal disk; it was not well matured but bacteria seemed to be firmly attached to each other. No trace of bacilli was found. The majority of present microorganisms were round species in chains and pairs (Fig. 37A-C)

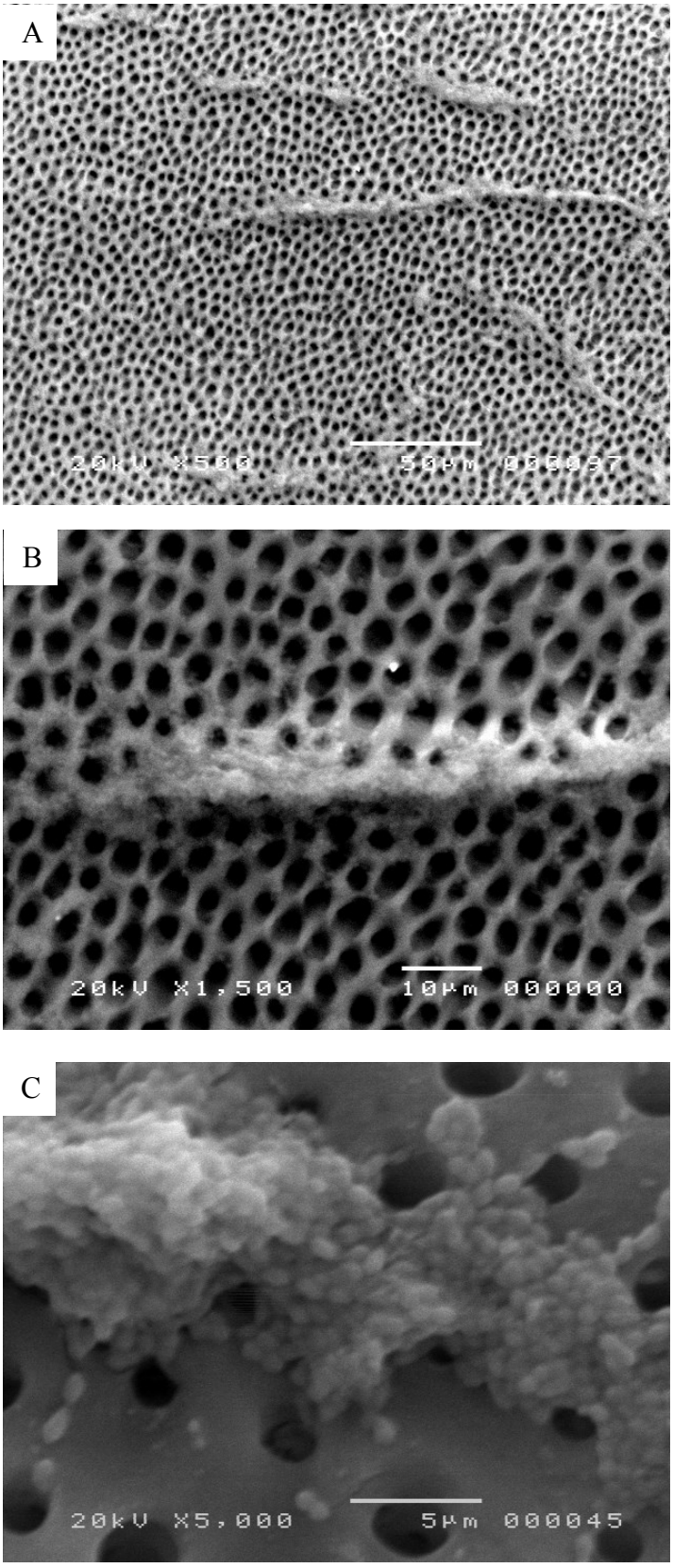


Figure 37. A, B and C: Biofilm started to be matured 3 days after incubation, dominance of cocci was clear.

After 6 days of incubation:

Gram staining and bacterial culture: the observations were identical to those after 3 days of incubation. Only gram-positive cocci in pairs and chains were detected. β hemolytic big white colonies and small whitish colonies with well-defined border were seen on blood agar plates.

SEM: The biofilm continued to maturate; the number of heaps was reduced but their size was bigger comparing day 3. Cocci in chains and pairs were distinguishable and extracellular matrix (ECM) could be perceived (Fig. 38).

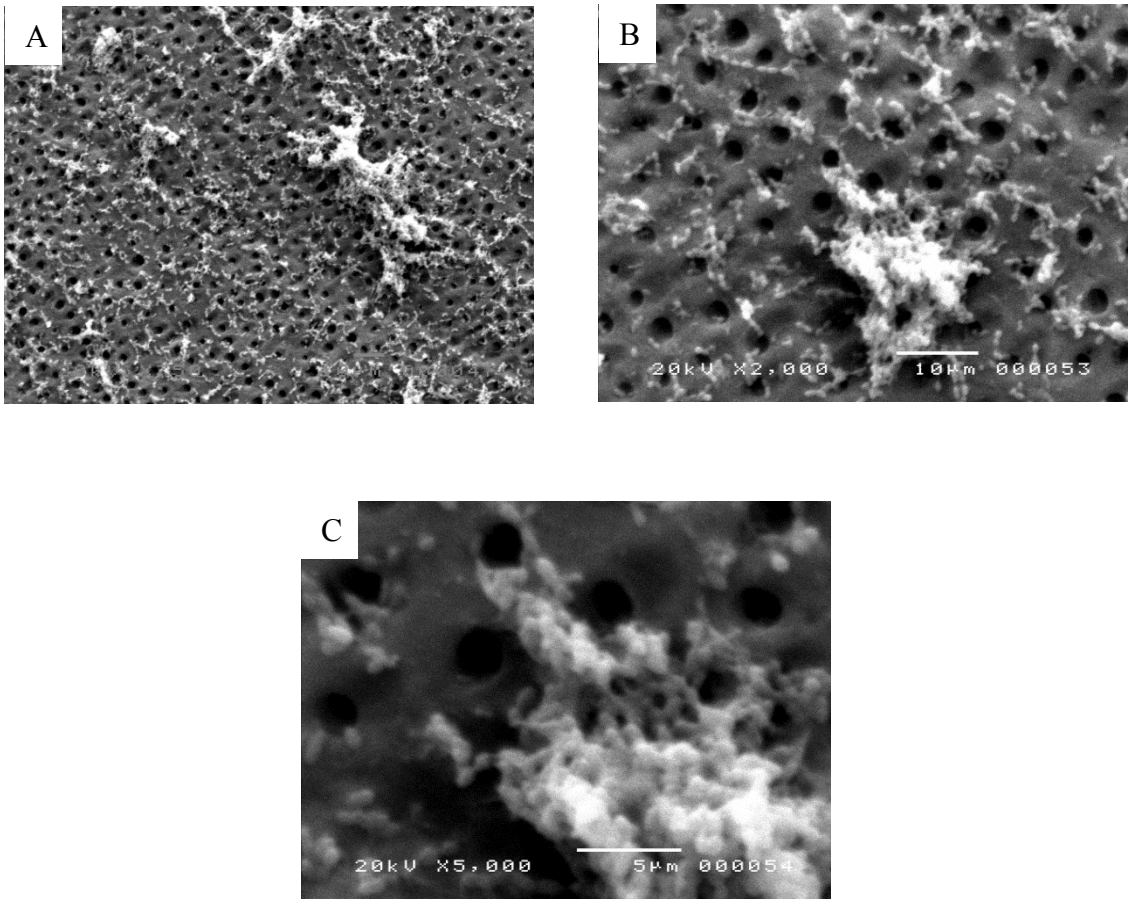


Figure 38. Coccoid bacteria are visible 6 days after incubation. ECM is started to increase.

After 10 days of incubation:

Gram staining and bacterial cultures: Gram staining showed the presence of gram-positive cocci. β hemolysis was observed. Small creamy white colonies with well-defined borders and about 1-2 mm in size could be seen.

SEM: Structure of biofilm is dispersed over larger area, just bacteria in pairs, small chains or some small aggregates could be observed. Some bacilli were also seen at the orifices of dentinal tubules in addition to the cocci (Fig. 39).

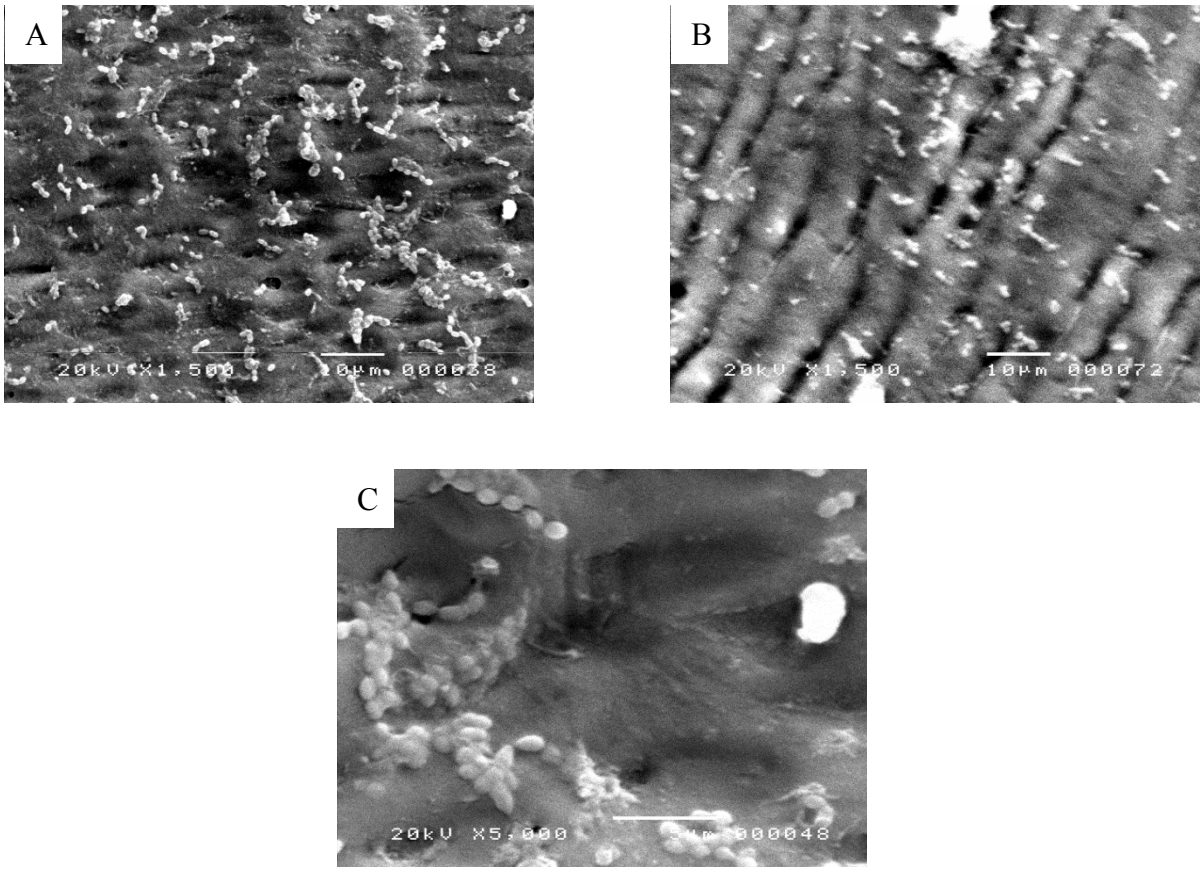


Figure 39. In addition to cocci, filaments could be seen 10 days after incubation.

After 13 days of incubation:

Gram staining and bacterial culture: Gram staining and bacterial cultures demonstrate the same images of bacteria in the previous stages; β hemolytic gram-positive chains and pairs, big colonies with undefined border and small rounded white colonies were noticed.

SEM: Biofilm was still maturating and coating a greater part of dentinal disks surface. Dentinal tubules were covered. The structure of biofilm became very dense. ECM was clear. Bacilli could also be distinguished. At larger magnifications big bacterial aggregates could be visualized (Fig. 40).

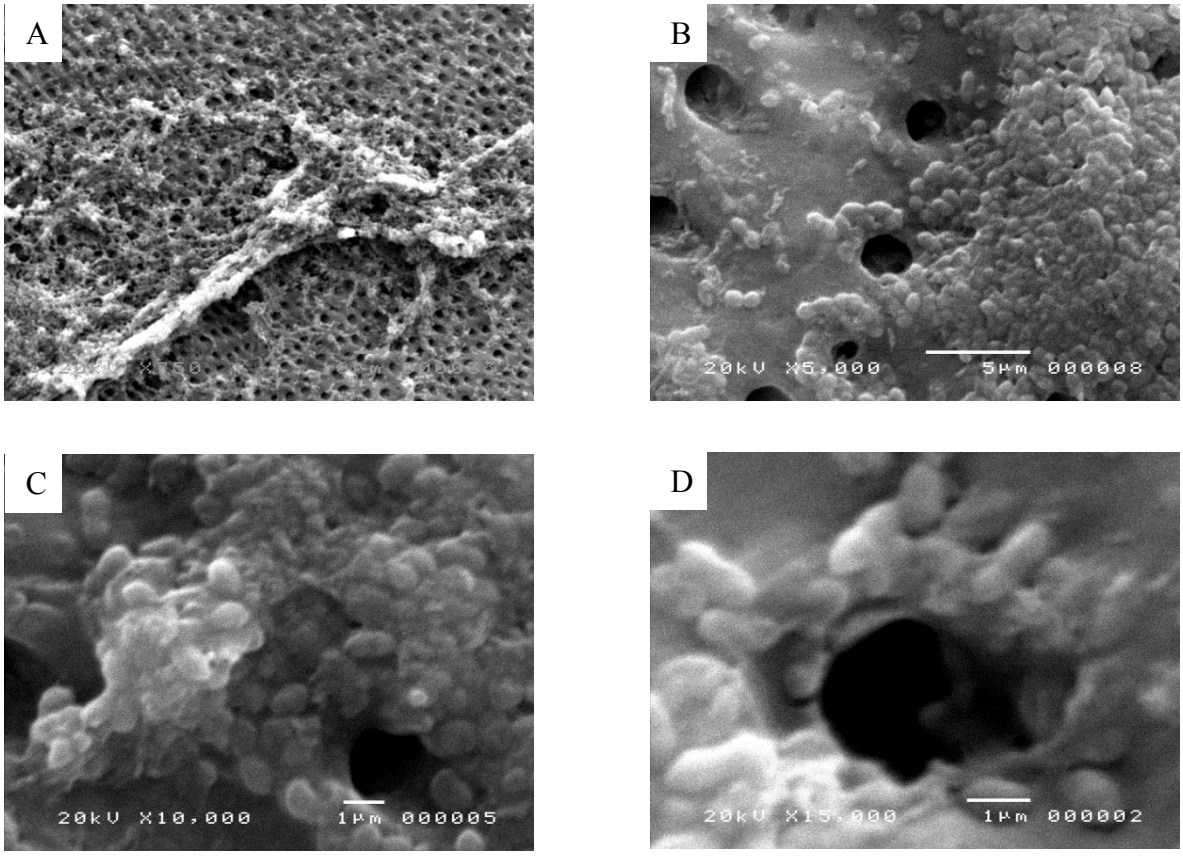


Figure 40. Different magnification of a dense bacterial aggregation. Biofilm structure was maturing, and a dense ECM was covering bacteria.

After 17 days of incubation:

Gram staining and bacterial culture: Gram–positive bacteria was observed after staining. β hemolytic colonies presented on blood agar. The agar plates were covered with big white and undefined borders and small white colonies with clear borders and a diameter less than 1 mm. The number of small colonies were more than the big colonies.

SEM: Aggregates of different bacteria but smaller in size could be observed. Biofilm covered entire dentinal surface. The rod and round shape components of biofilm could be seen in near view (Fig. 41).

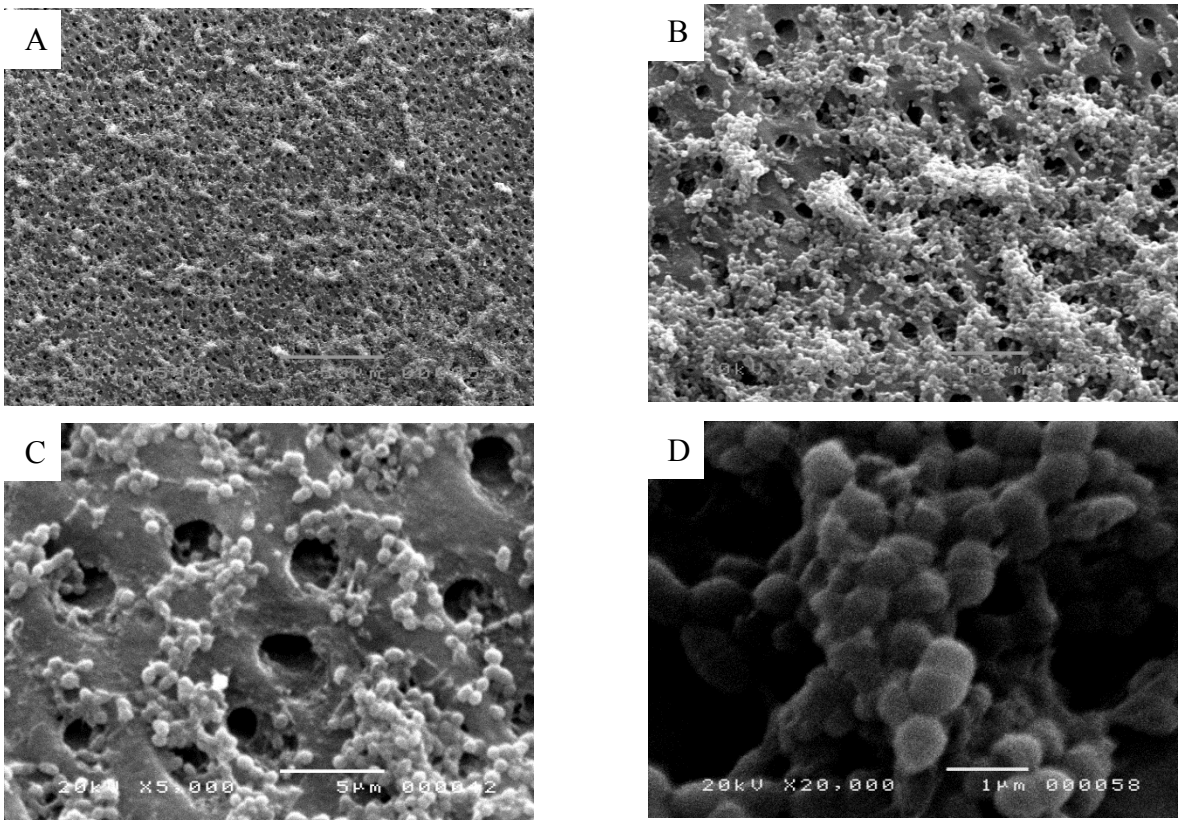


Figure 41. The number of bacterial heaps were multiplying, the coverage surface was increasing, the mixture of cocci and rods was clear after 17 days of incubation.

After 20 days of incubation:

Gram staining and bacterial culture: as it was seen in earlier days of incubation gram-positive bacteria associated with β hemolysis, big white colonies with unclear border and small well-defined white colonies on blood agar plates were noticed.

SEM: Here, the covered surface of dentin was reduced but structure of biofilm heaps were huge and matured. The biofilm communities were attached firmly to dentin and hide completely all structure beneath them. The presence of rod shape bacteria could not be confirmed because of density of biofilm mass (Fig. 42).

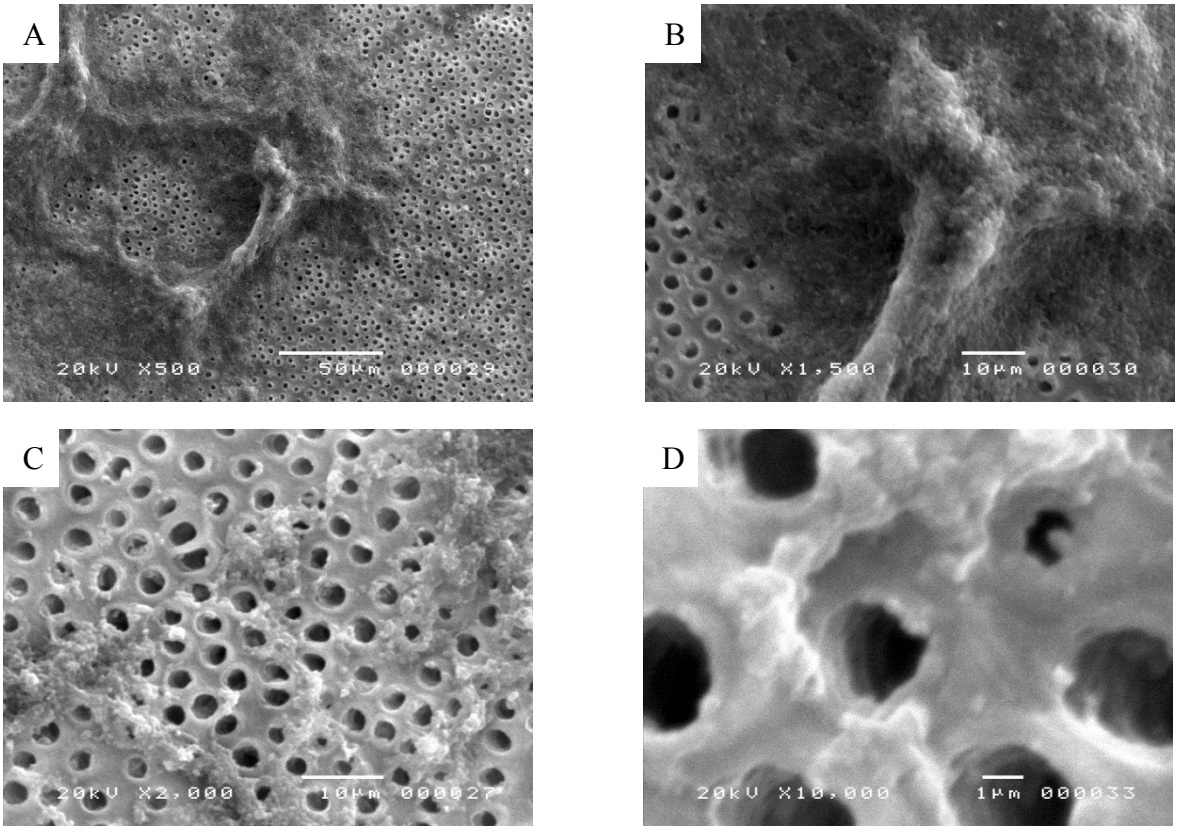


Figure 42. The structure of biofilm was dense, the bacterial communities were distinct in lower magnification, coverage surface was not huge but the structure of biofilm was growing spatially on day 20 of incubation.

After 24 days of incubation:

Gram staining and bacterial culture: Gram-positive clusters of round shape bacteria could be confirmed. The sheep blood agar represented β hemolytic process and numerous different white colonies.

SEM: Number and size of microbial aggregates were dramatically reduced, the surface covered by biofilm was samller than previous observation. However, still the dense structure of biofilm could be seen and isolated chains of bacteria could be observed (Fig. 43).

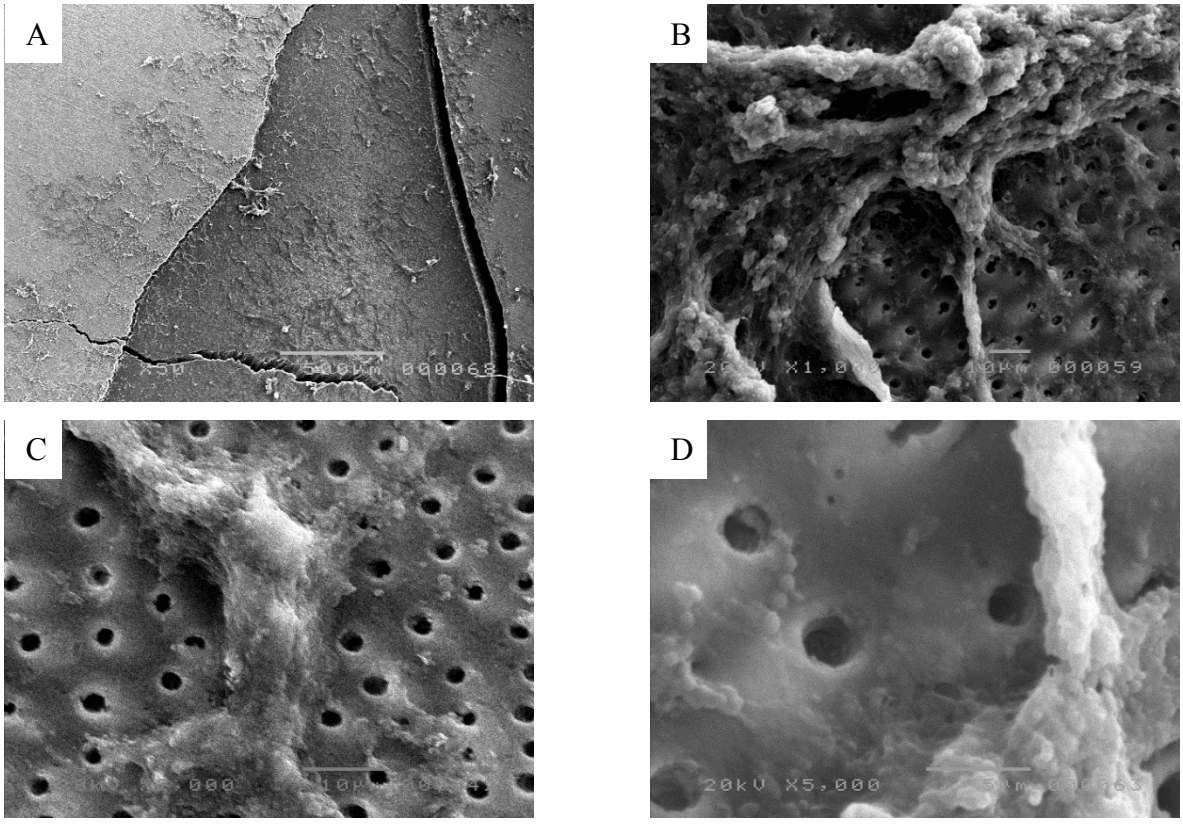


Figure 43. Biofilm was mature and dense, but surface coverage was decreasing, bacteria were embedded entirely in ECM 24 days after incubation.

After 27 days of incubation:

Gram staining and bacterial culture: Identical to previous stages (24 days after incubation) of development; Gram-positive bacteria and β hemolysis were main events to note.

SEM: Roughly no big bacterial aggregates could be recorded; only some small heaps, chains of cocci and some bacilli were represented. Traces of ECM were also presented. Dentinal tubules were visible. Bacteria penetration into dentinal tubules could be detected (Fig. 44).

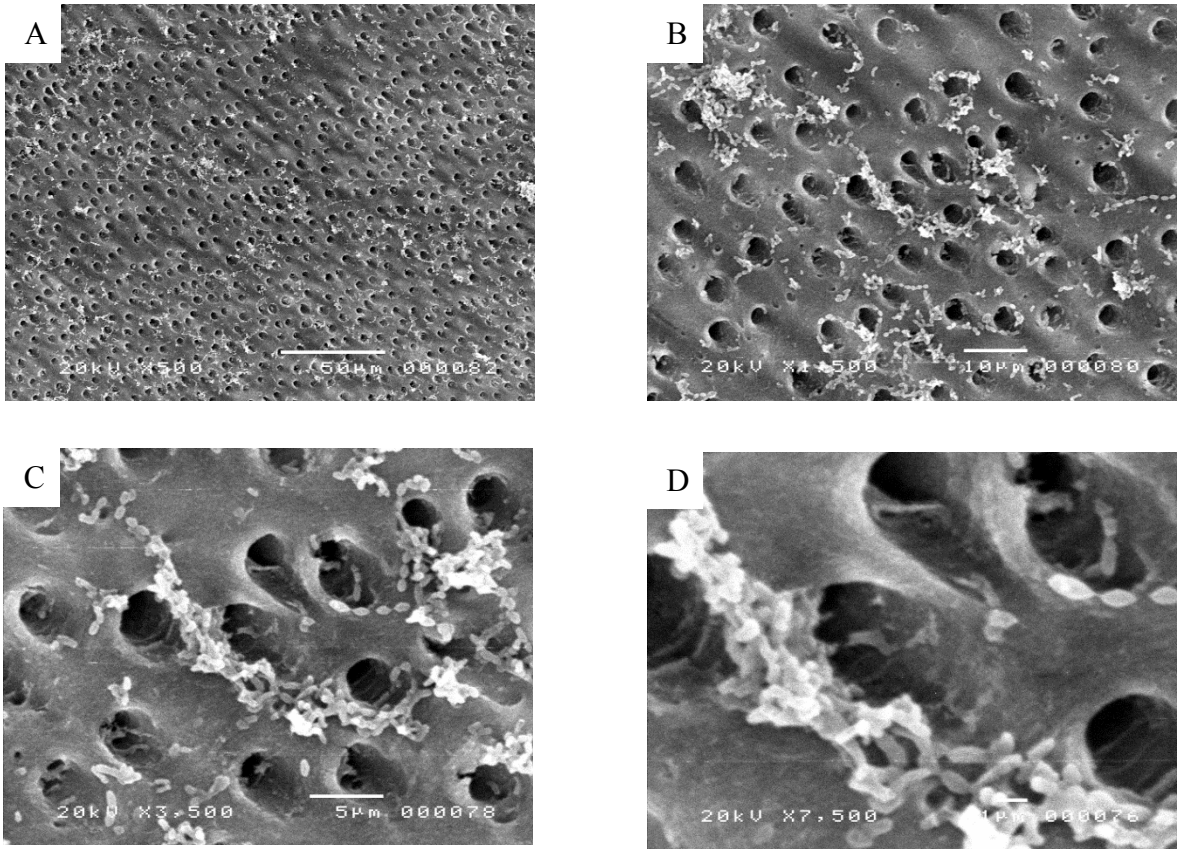


Figure 44. Biofilm structure was reducing in size and coverage on day 27 after incubation.

After 31 days of incubation:

Gram staining and bacterial culture: in the last day of programmed incubation, the observations were mostly the same as day 27. However some mixed white colonies grown over β hemolysis on agar plates could be seen. Gram staining did not reveal any gram-negative bacteria.

SEM: Bacterial aggregates started to revive all around the dentinal disk. Medium sized bacterial communities embedded in ECM could be discovered. Rod and round bacteria could be seen, over an open tubules texture (Fig. 45). Some massive well-established bacterial heaps could be found inside the cracks (Fig. 46).

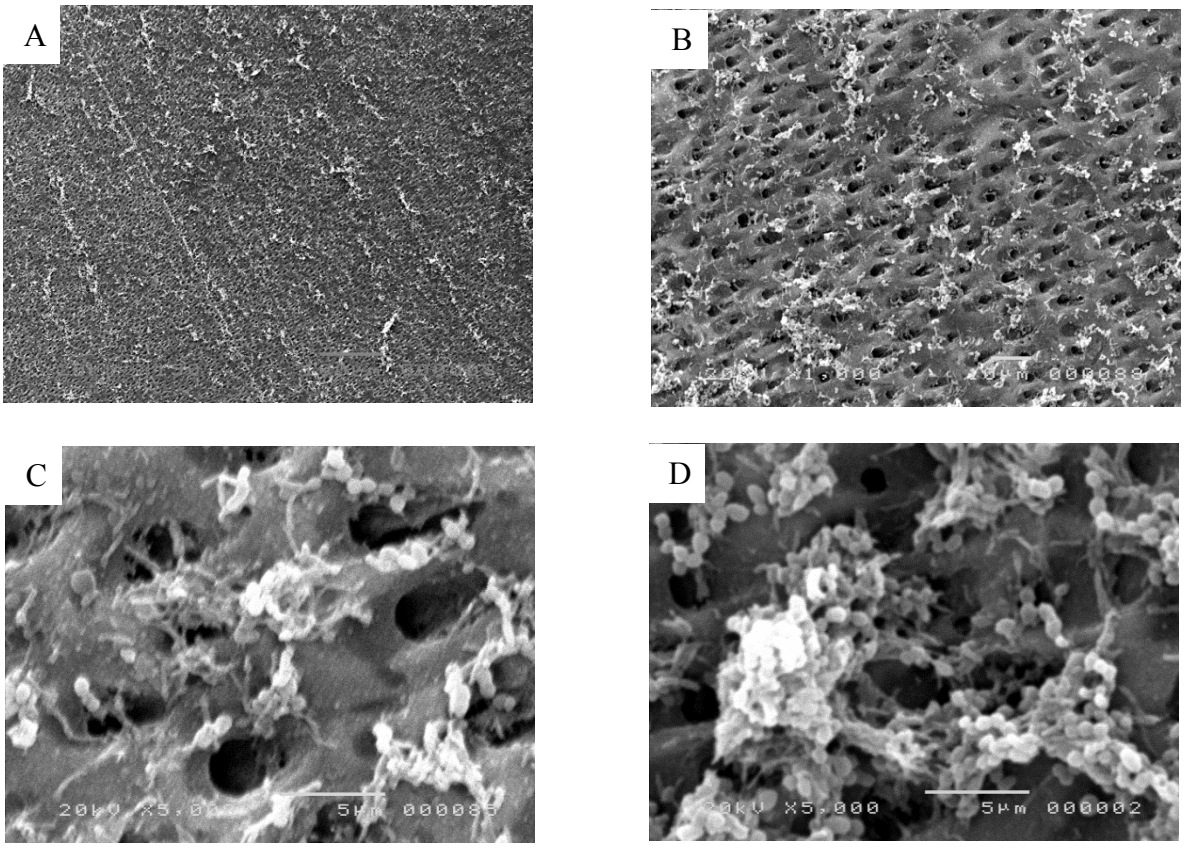


Figure 45. Biofilm started again to maturate; rods and cocci could be distinguished on day 31 after incubation..

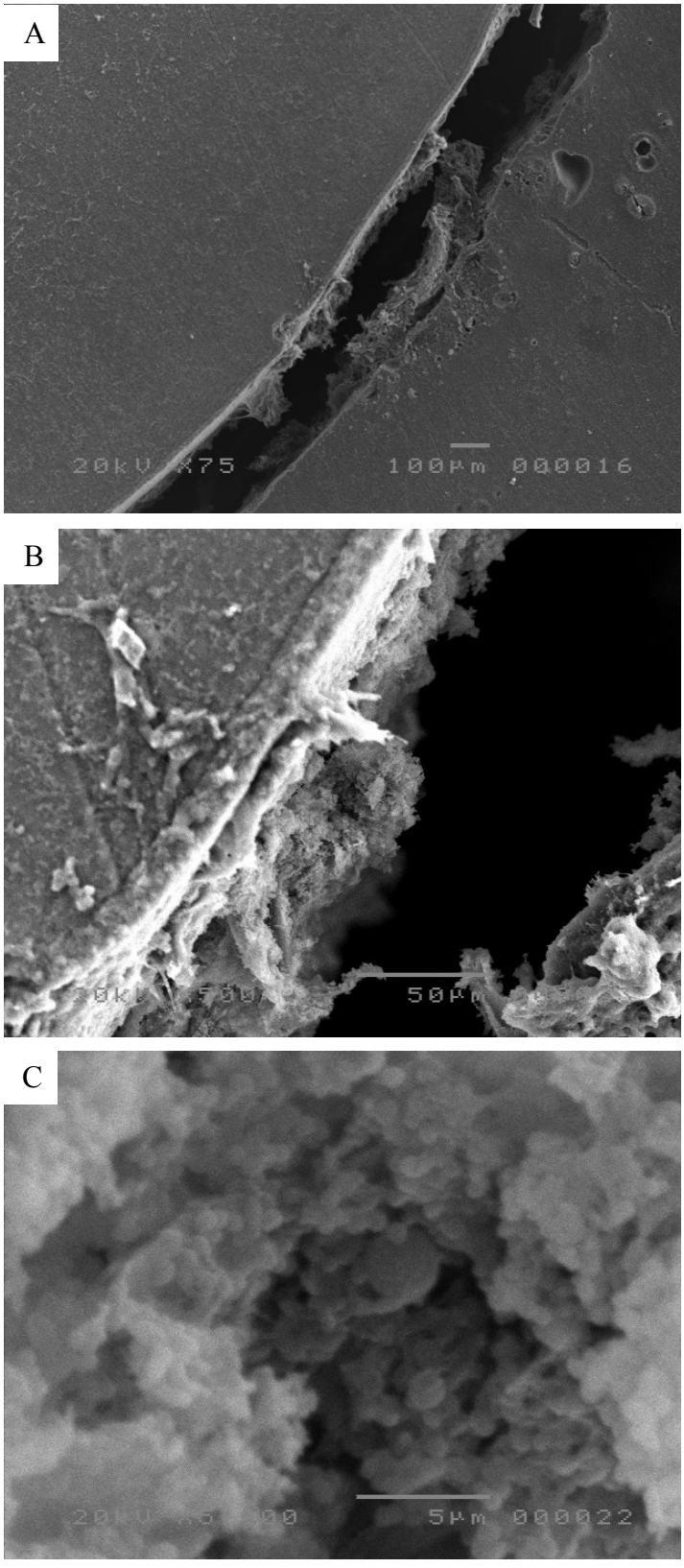


Figure 46. Huge structure of biofilm could be seen inside the cracks.

4.3 Phase 3: Characterization of Established Biofilm on Root Canal Surface

By analyzing the SEM images of phase I, we decided to keep root canals in contact with bacterial inoculum for a period of 21 days. At the day 21st of incubation, the root canals were removed from growth medium and rinsed.

Those that underwent bacterial culturing, were sampled by a #10 K-file. Rest of root canals were fixated and subjected to *in situ* hybridization. After visualization by confocal microscope, the same samples were dehydrated and coated with gold for verification by SEM.

Bacterial culturing: β hemolysis of sheep blood agar was the main evidence. There were small glossy white colonies with defined border and some creamy colonies with unclear border covering culture medium.

Fluorescent *in situ* Hybridization with Confocal microscope: In the first step, the probe EUB338 which is specific to domain Bacteria was used to detect the presence of the artificial biofilm on the root canal surface. To verify nonspecific binding of the EUB338, probe NONEUB338 was used. Hybridization with NONEUB338 gave no or some weak signals. EUB338 marked different zone on root canal walls. Scanning electron microscopy of same samples showed heavy bacterial accumulation (Fig. 47).

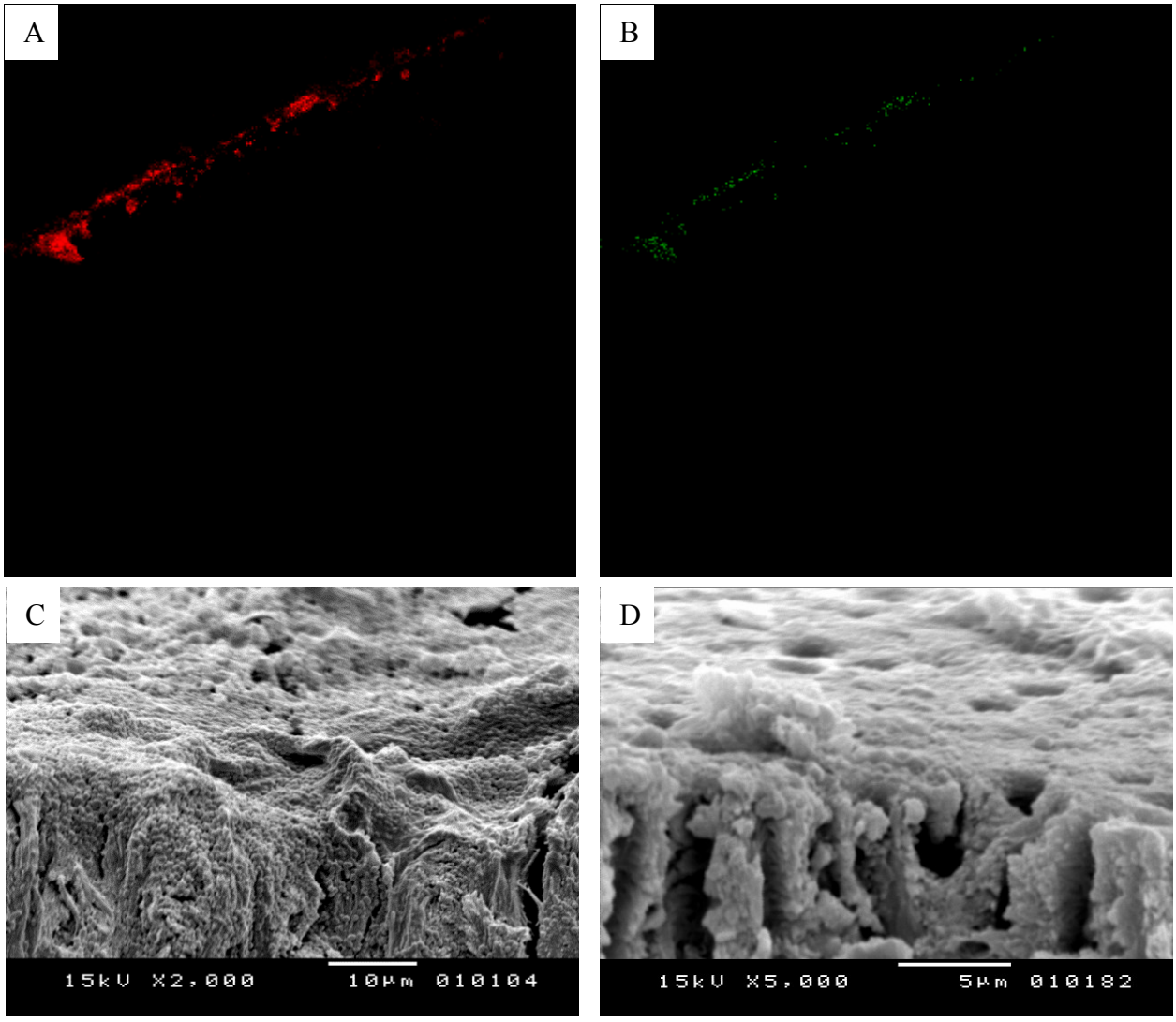


Figure 47. A: EUB338 probe demonstrated the bacterial presence over dentinal surface, **B:** NONEUB used as a negative control to show any possible unspecific binding, **C and D:** SEM image proved the bacterial presence in the zone marked by 16S rRNA probe.

Before using other probes specially designed for each bacteria, specificity of each was tested using cultured planktonic bacteria. All probes displayed the expected specificity (Fig. 48).

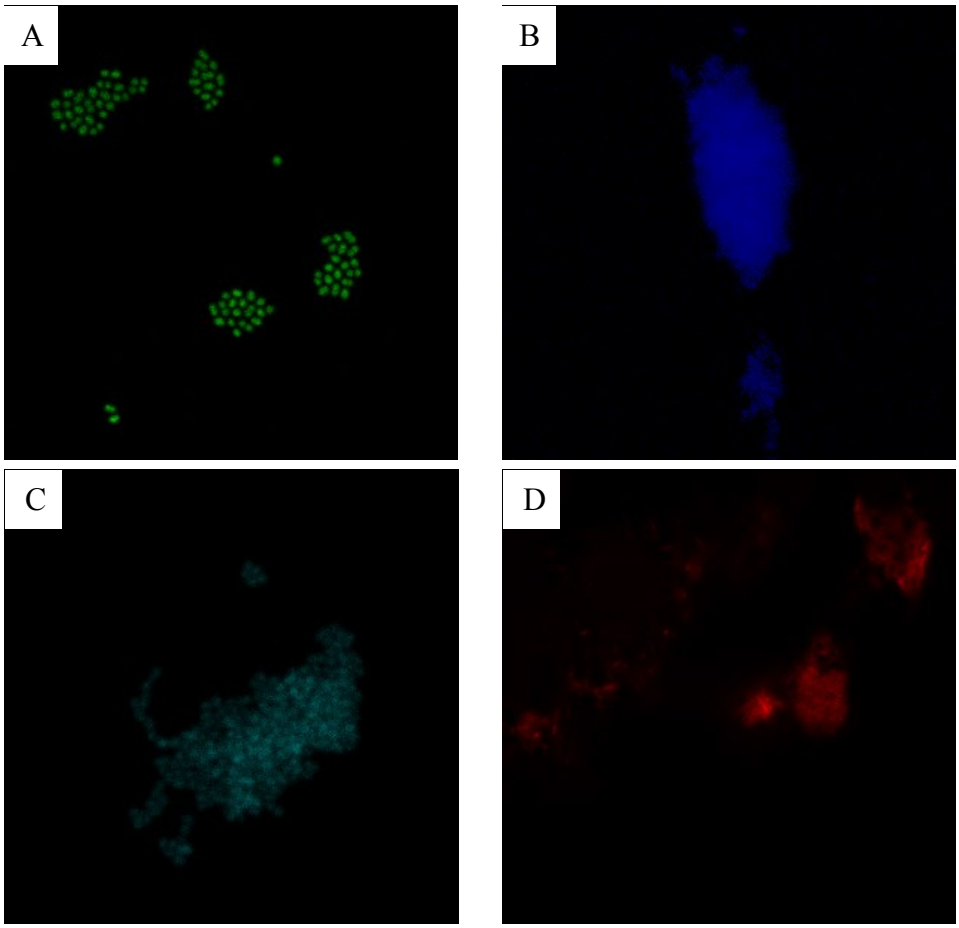


Figure 48. Specificity of all 16S rRNA was tested using pure culture of each species: *E. faecalis* **(A)**, *P. gingivalis* **(B)**, *S. salivarius* **(C)**, and *F. nucleatum* **(D).**

The signals of all 16S rRNA probes were detected in all samples which confirm the presence of all bacterial species in the artificial biofilm. Dy-405, ATTO488, Cy3 and Cy5 were used to stain respectively *P. gingivalis, E. faecalis, F. nucleatum and S. salivarius* (Fig. 49 and 50). Kruskal-Wallis statistic test showed no significant difference between bacteria incorporated into the biofilm covering dentinal surface (*p>0.05)*.

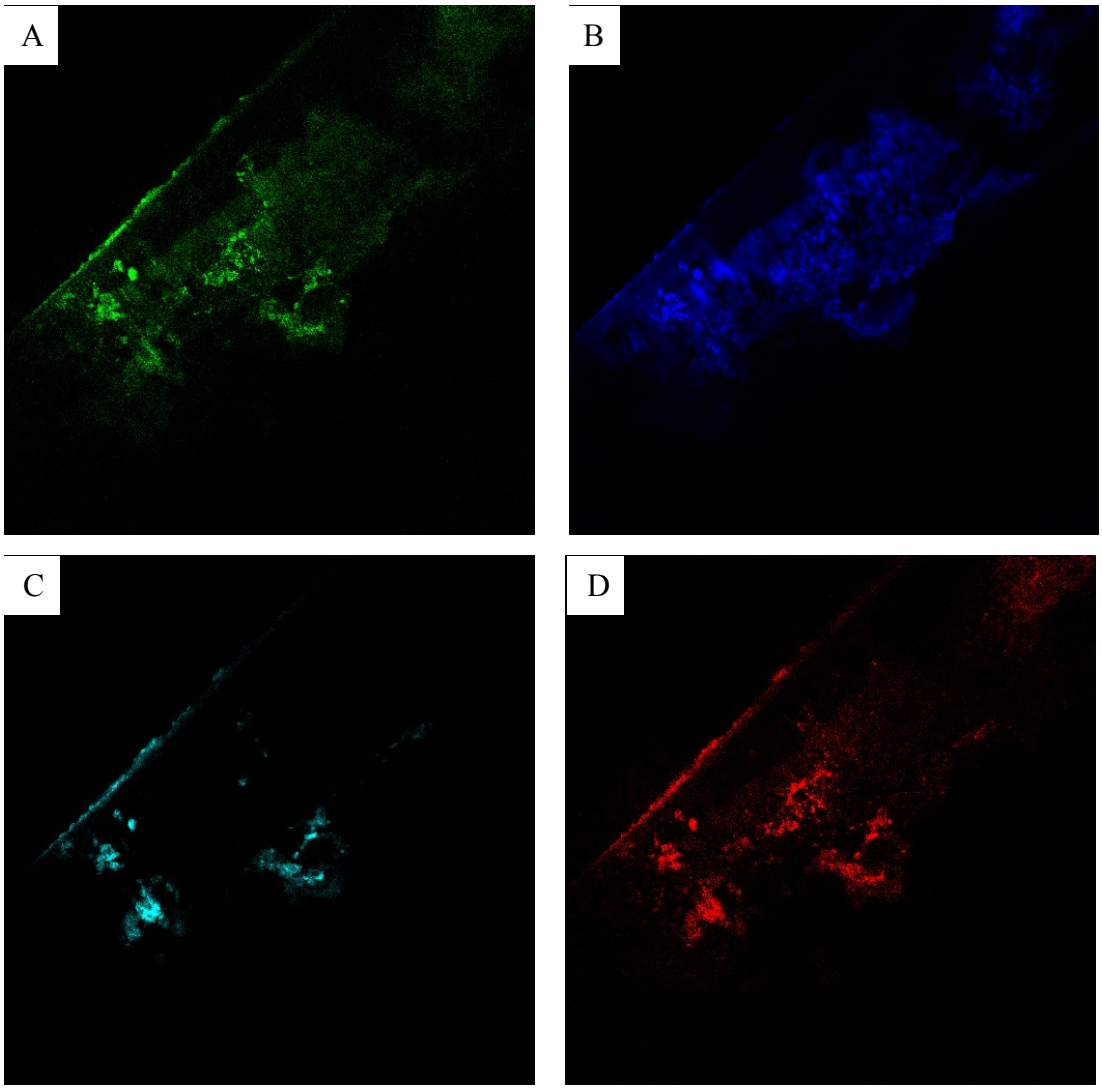


Figure 49. Using 16S rRNA, the presence of different component of the artificial biofilm over root canal walls was confirmed: *E. faecalis* **(A)**, *P. gingivalis* **(B)**, *S. salivarius* **(C)**, and *F. nucleatum* **(D).**

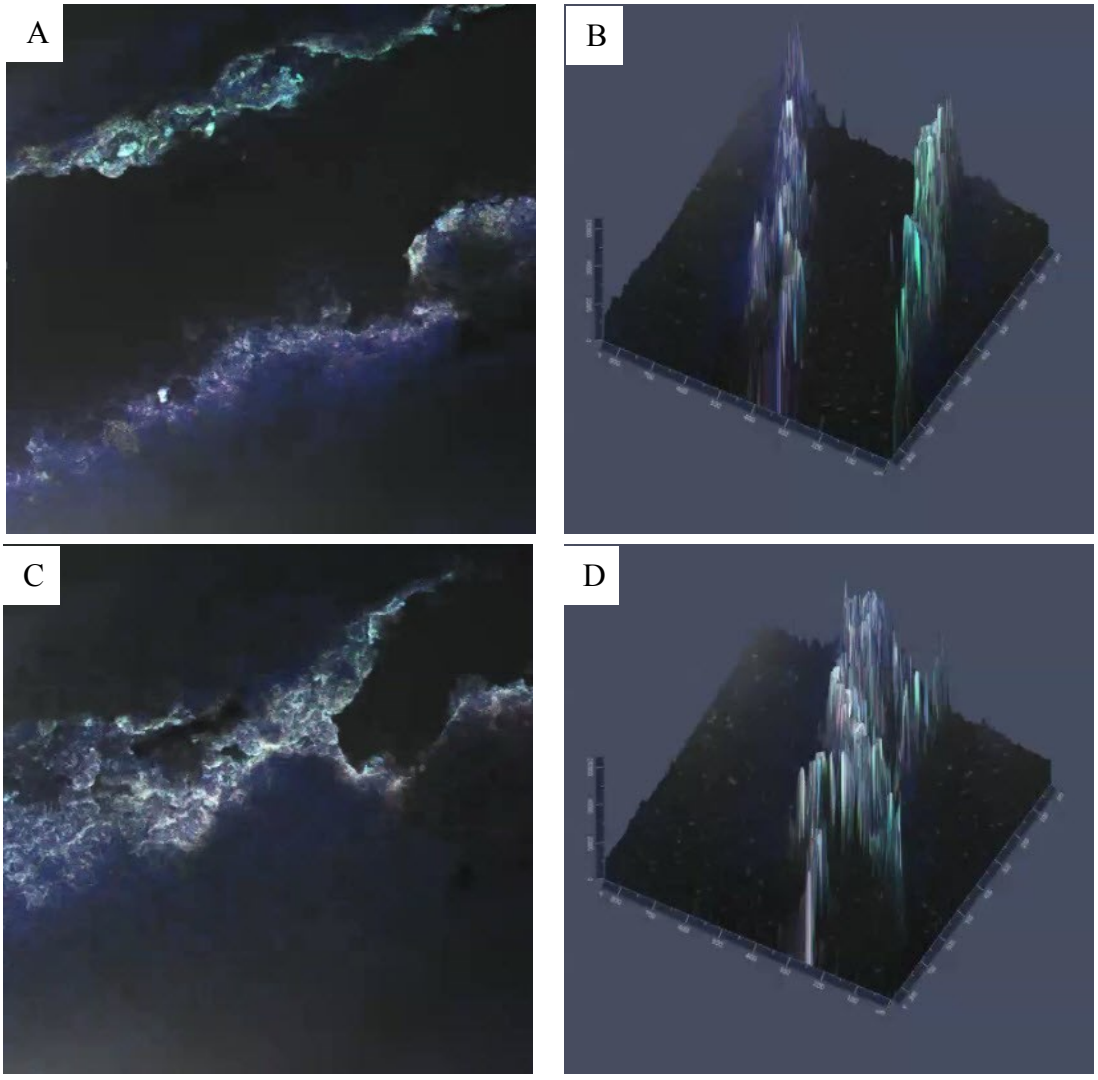


Figure 50. Z-Stack images using 16S rRNA probe signals of all 4 bacterial species present in the artificial biofilm, **(A)** 2D image of the base of biofilm, **(B)** 2,5D reproduction of the Z-Stack image of the biofilm at the level of dentinal surface, **(C)** 2D image of the biofilm surface, and **(D)** 2,5D reproduction of the Z-Stack image at the level of the biofilm surface. **(E)** Image dimensions.

However, the same Kruskal-Wallis statistic test showed that there is a significant difference among bacteria presented inside the dentinal tubules (p=0.01; Fig. 51). The Mann-Whitney statistical test revealed that the presence of *P. gingivalis* was enhanced in comparison with other bacteria (p<0.05). *S. salivarius* was the species which did not present as much as the other members of biofilm (p<0.05). Statically there was no significant difference between the intensity of *F. nucleatum* and *E. faecalis* (p>0.05).

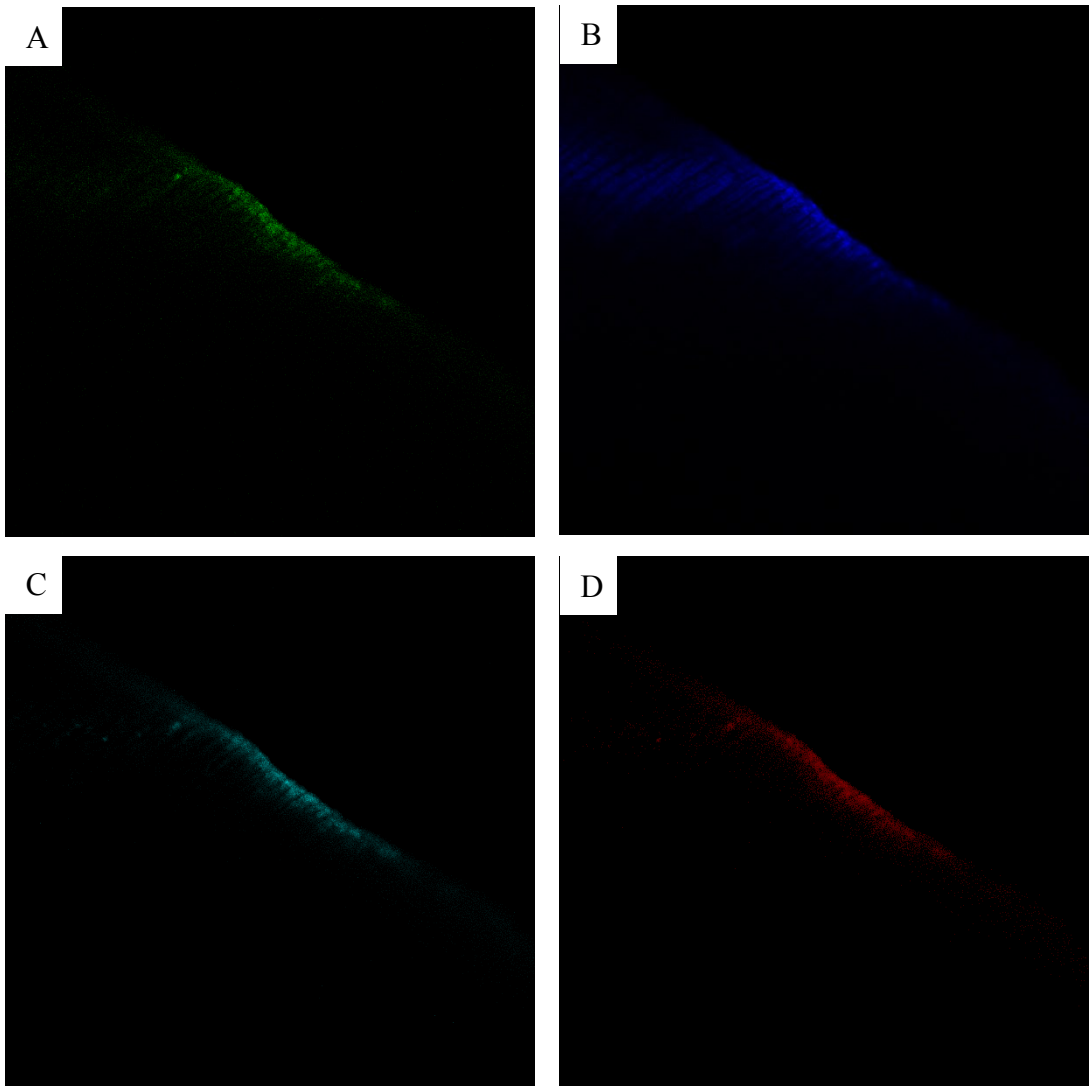


Figure 51. Bacteria were marked inside the dentinal tubules: *E. faecalis* **(A)**, *P. gingivalis* **(B)**, *S. salivarius* **(C)**, and *F. nucleatum* **(D).**

SEM: When samples were visualized at 500X, it could be noticed that the main canal of root canal was mainly covered. At this magnification, the covering layer was detectable and a dens layer of bacteria covered the dentinal tubules. At higher magnifications (1000X and 2000X) the outline of bacteria at superficial layer of biofilm could be recognized; there were round and rod shaped bacteria which were packed densely together (Fig. 52).

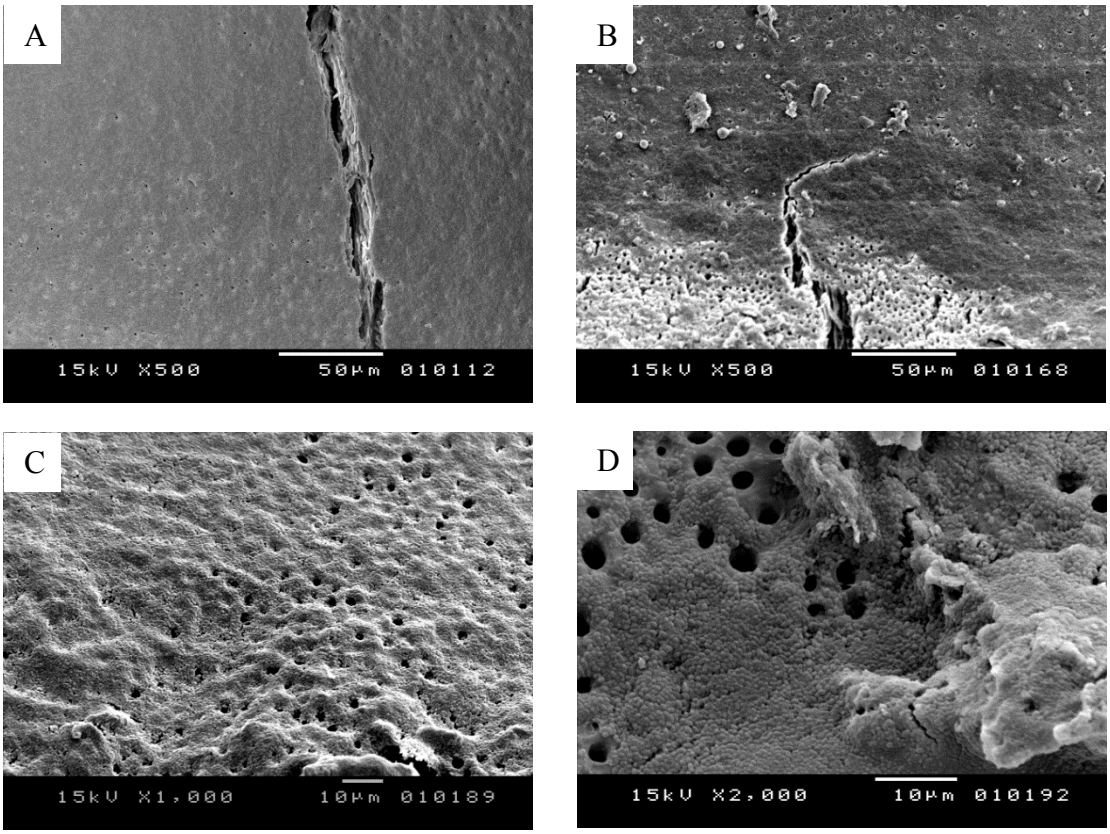


Figure 52. SEM images revealed a dens layer of bacteria covering root canal walls.

At 5000X and 7500X, it was noticed that bacteria formed the outline of dentinal tubules leaving holes at the surface of the biofilm. So, it could be speculated that the dentinal surface was entirely covered by biofilm (Fig. 53).

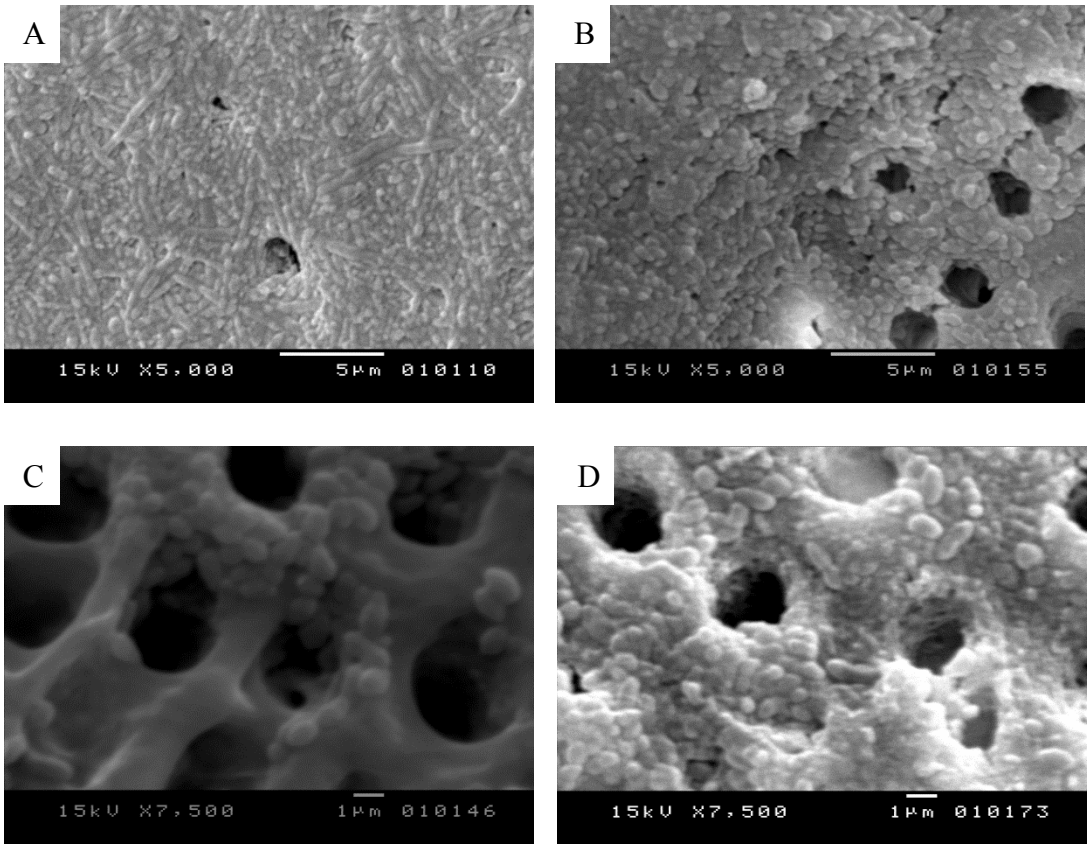


Figure 53. At higher magnifications different morphology of bacterial component of the artificial biofilm including rod and round shaped bacteria was detectable.

Once higher magnification was used, the morphology of each individual was clear (Fig. 54). The cocci (*S. salivarius)*, rod (*P. gingivalis)* and filamentous (*F. nucleatum)* bacteria could be seen easily. There were bacteria with bi-polar form, suggesting the presence of *E. faecalis* incorporated inside the biofilm. These images resemble those of each bacterium's pure culture.

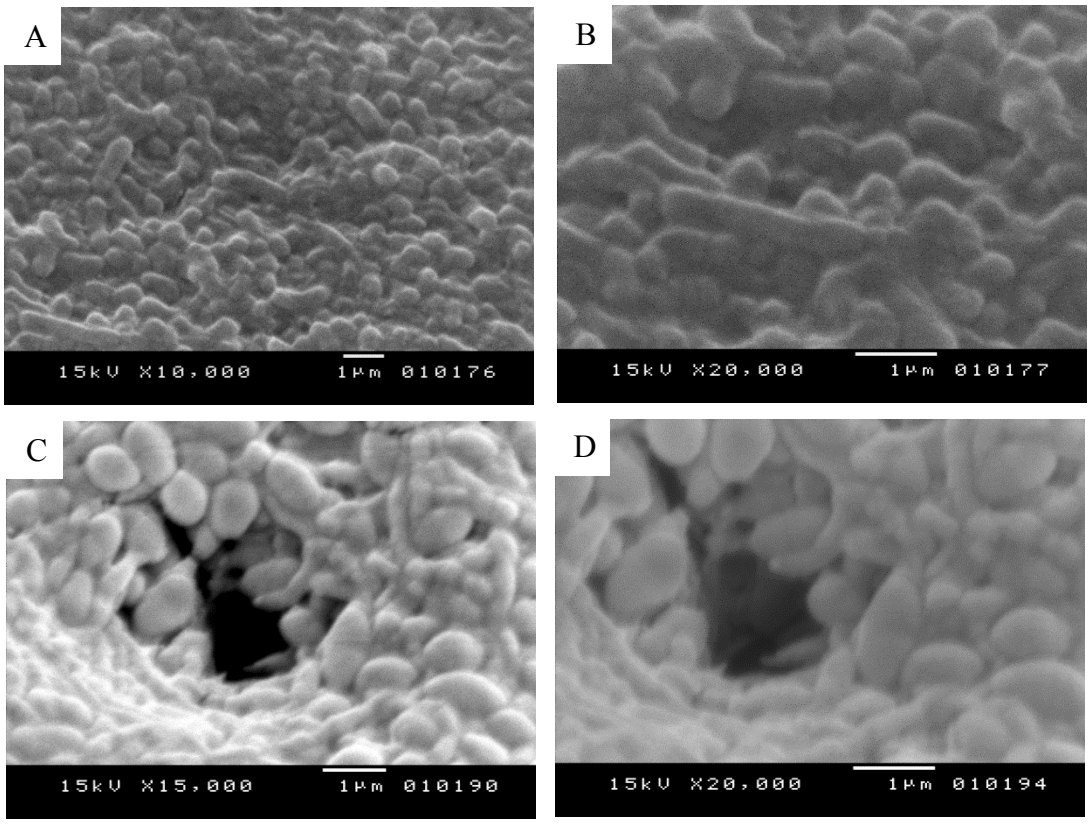


Figure 54. Morphological differentiation was distinguishable at magnification higher than 10000X. Presence of the rods was apparent.

Because of their small size (about 1 µm), bacteria could be detected inside the dentinal tubules when samples were visualized at high magnifications (2000X and more). Microorganism were found in packs near the orifices of dentinal tubules or individually more than 500 µm inside dentinal tubules far from main canal (Fig. 55)

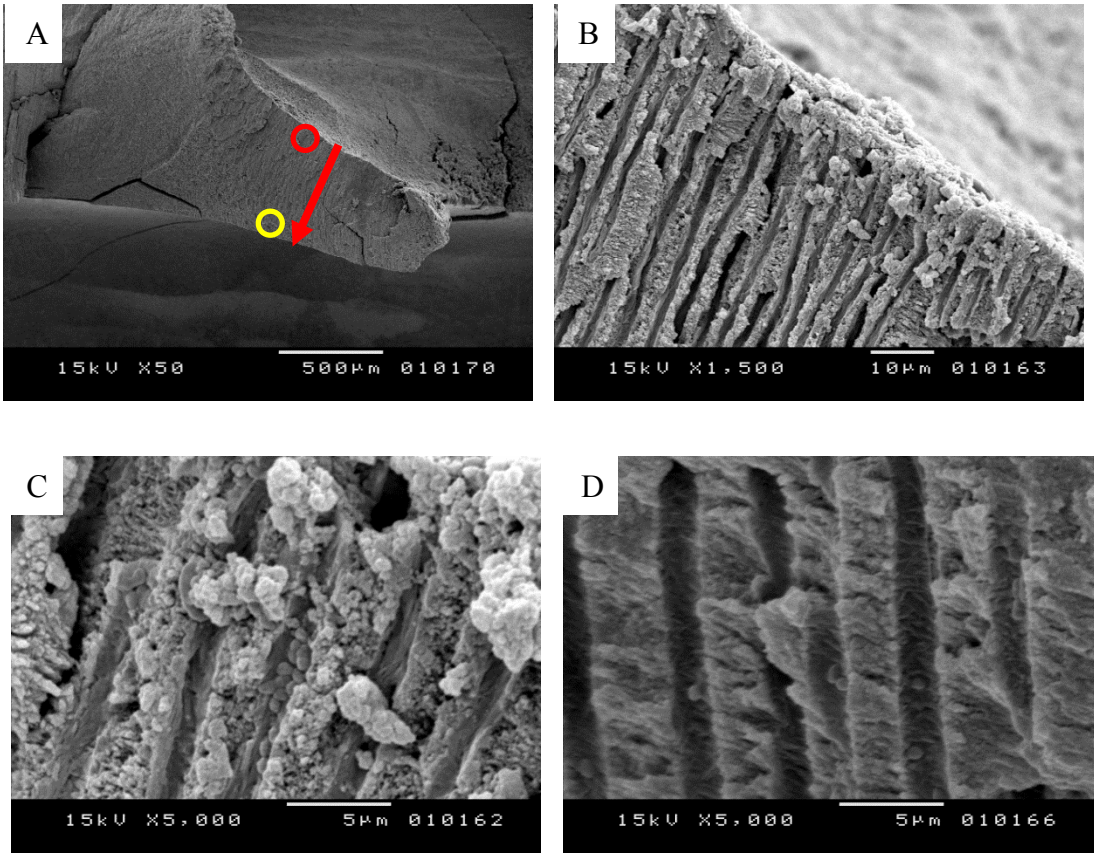


Figure 55. SEM images of zones which were previously marked by FISH technique confirmed the invasion of dentinal tubules by bacteria. By scanning distal ends of tubules we could demonstrate that bacteria are capable to travel as far as 500 µm or more from main canal. **A** (red circle)**, B** and **C** show the bacterial invasion inside the dentinal tubule near root canal surface. **A** (yellow circle) and **D** show the presence of bacteria at dentinal tubules' extremities, while arrow indicate the distance a bacterium could travel from main root canal.

4.4 Clinical Management of Root Canal Artificial Infection

4.4.1 Group 1:

Except some weak signals in the trunk of main canal, the confocal microscopy did not detect a noticeable sign of presence of bacteria in samples treated with Passive Ultrasonic Irrigation. A Mann-Whitney test indicated that PUI was efficient to remove bacterial biofilm from endodontic space (p=7.3x10^{-9}*)*. The Kruskal-Wallis statistic test suggested that the treatment was effective regardless to bacterial species (p=0.153; Fig. 56).

SEM images showed clean surfaces free from organic and inorganic debris. Student's T test confirms that PUI was effective in terms of debris removal (p=6.80x10^{-9}). Furthermore, Kruskal-Wallis test showed that the PUI was effective in all coronal, middle and apical third of root canal space (Fig. 57). The bacterial cultures of this group showed a total score of zero both at immediate reading after 24 hours of incubation and later reading after 120 hours of incubation (Fig. 69).

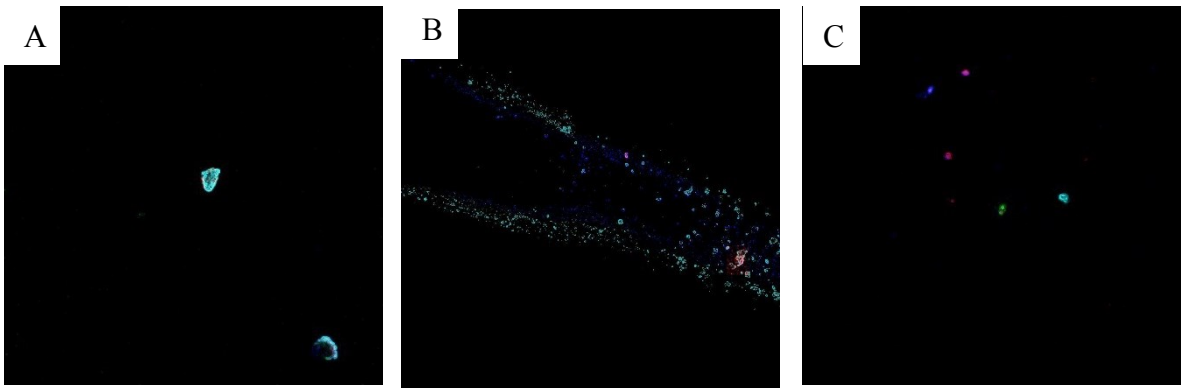


Figure 56. Confocal images of biofilm following the Passive Ultrasonic irrigation. There are no noticeable amount of bacteria detectable in coronal third **(A)**, middle third **(B)**, and apical third **(C)**.

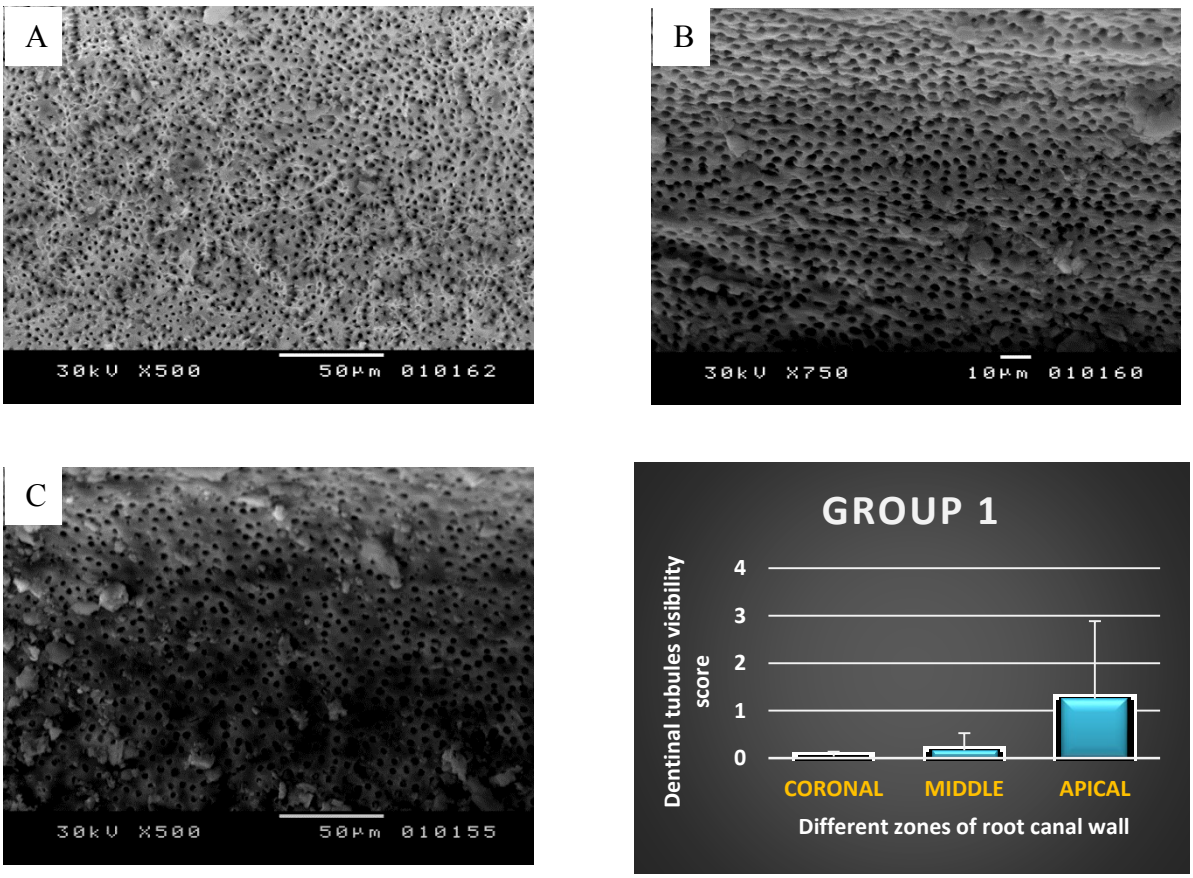


Figure 57. SEM images showed clean dentinal surface after PUI treatment: Coronal third **(A)**, Middle third **(B)**, and Apical third **(C)**; **D:** Comparison of different zones in terms of cleanliness.

4.4.2 Group 2:

We replaced the usual irrigants such as NaOCl and EDTA with H_2O to evaluate the impact of rotary instrument on endodontic biofilm. Mann-Whitney test proposed that rotary instrument could not reduce the bacterial load from root canal space (p=0.059). This finding suggests that instrumentation has an important role in reduction of bacterial load from dental canal but this treatment alone could not pretend to manage the endodontic infection. Furthermore, Kruskal-Wallis tests showed that there was no significant difference between the effects of OneShape® file on different species inside the root canal (p=0.319; Fig. 58).

The dentinal tubules were entirely closed. Student's T test showed no significant difference between group 2 and control group (p=0.298). Kruskal-Wallis test revealed that rotary instrument had the same generalized effect in all different zones of root canal space (p=0.079; Fig. 57). The bacterial cultures of this group showed a total score of 3.5 at immediate reading after 24 hours of incubation and score of 4.66 at later reading after 120 hours of incubation (Fig. 69).

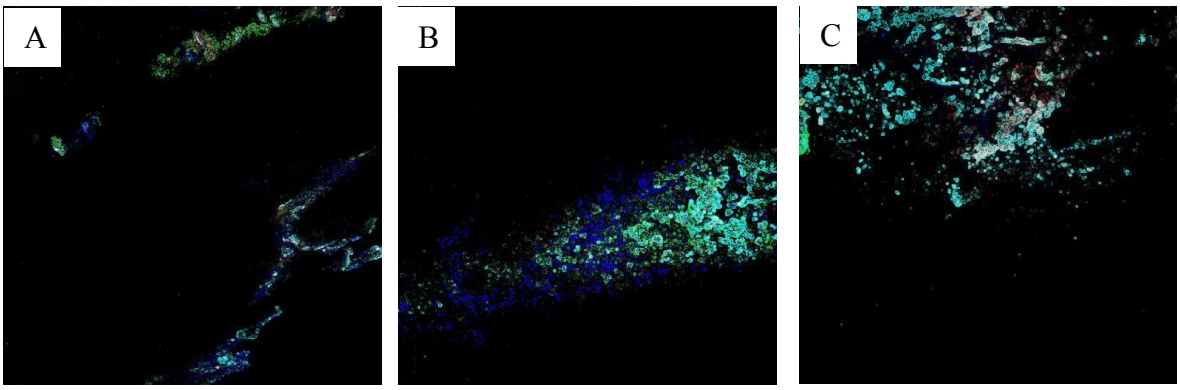


Figure 58. Confocal images of biofilm following rotary instrument application. No significant reduction in bacterial load was observed in coronal third **(A)**, middle third **(B)**, and apical third **(C)**.

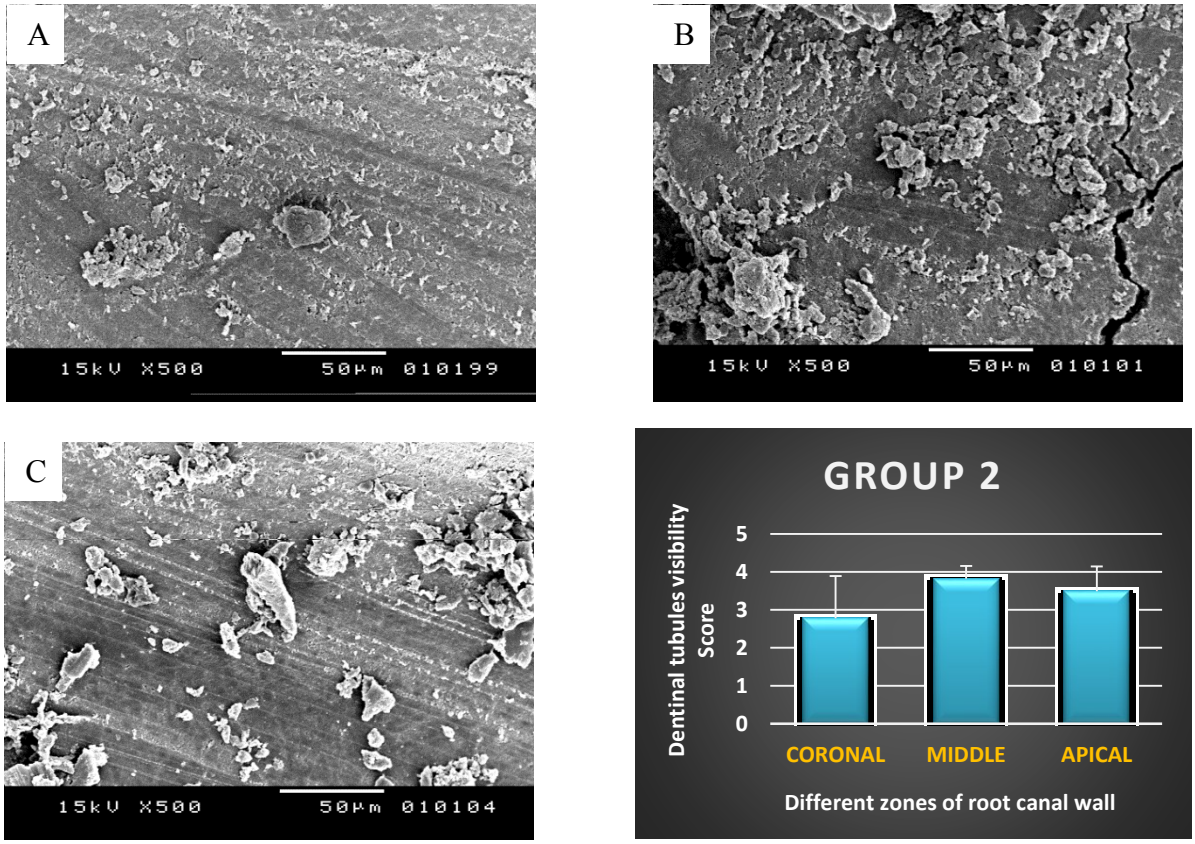


Figure 59. SEM images showed that instrument alone will make heavy smear layer in which bacteria could be trapped, **A:** Coronal third, **B:** Middle third, **C:** Apical third, **D:** Comparison of different zones in terms of cleanliness.

4.4.3 Group 3:

Mann-Whitney statistical test evoked that Photodynamic therapy using LED and Toluidine Blue was significantly active on reduction of bacterial load (p=8.87x10^{-6}). This antibacterial effect of PDT was active in the same level for all different bacteria when Kruskal-Wallis test was used to document any statistically significant difference between different groups (p=0.764; Fig. 60).

Using SEM images, in a global view PDT had no significant effect against dentinal debris (p=0.132). However, Kruskal-Wallis statistical test suggests that the outcome of PDT was more significant in coronal region (p<0.05; Fig. 61). The bacterial cultures of this group showed a total score of 1.16 at immediate reading after 24 hours of incubation and score of 1.5 at later reading after 120 hours of incubation (Fig. 69).

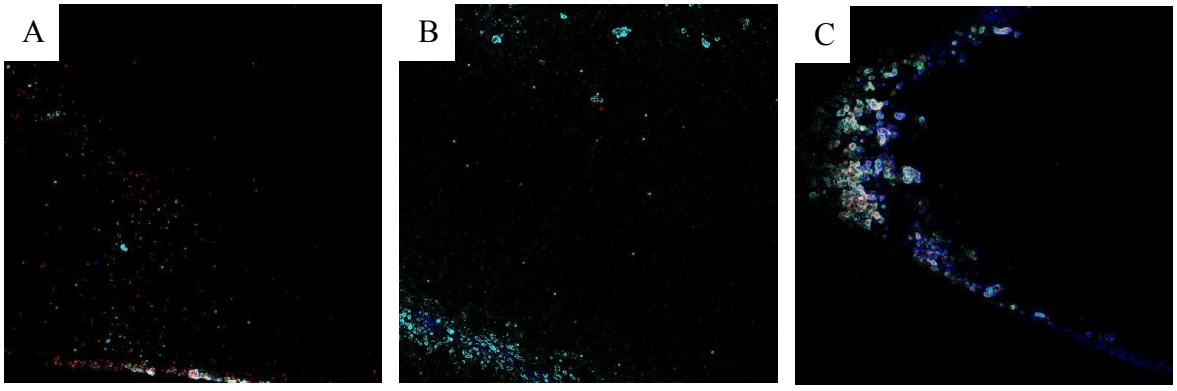


Figure 60. Confocal images of biofilm following PDT. Significant reduction in bacterial load was observed in coronal third **(A)**, middle third **(B)**, and apical third **(C)**. However, some fluorescence signals was detectable especially at the regions which biofilm was not disturbed by instrumentation.

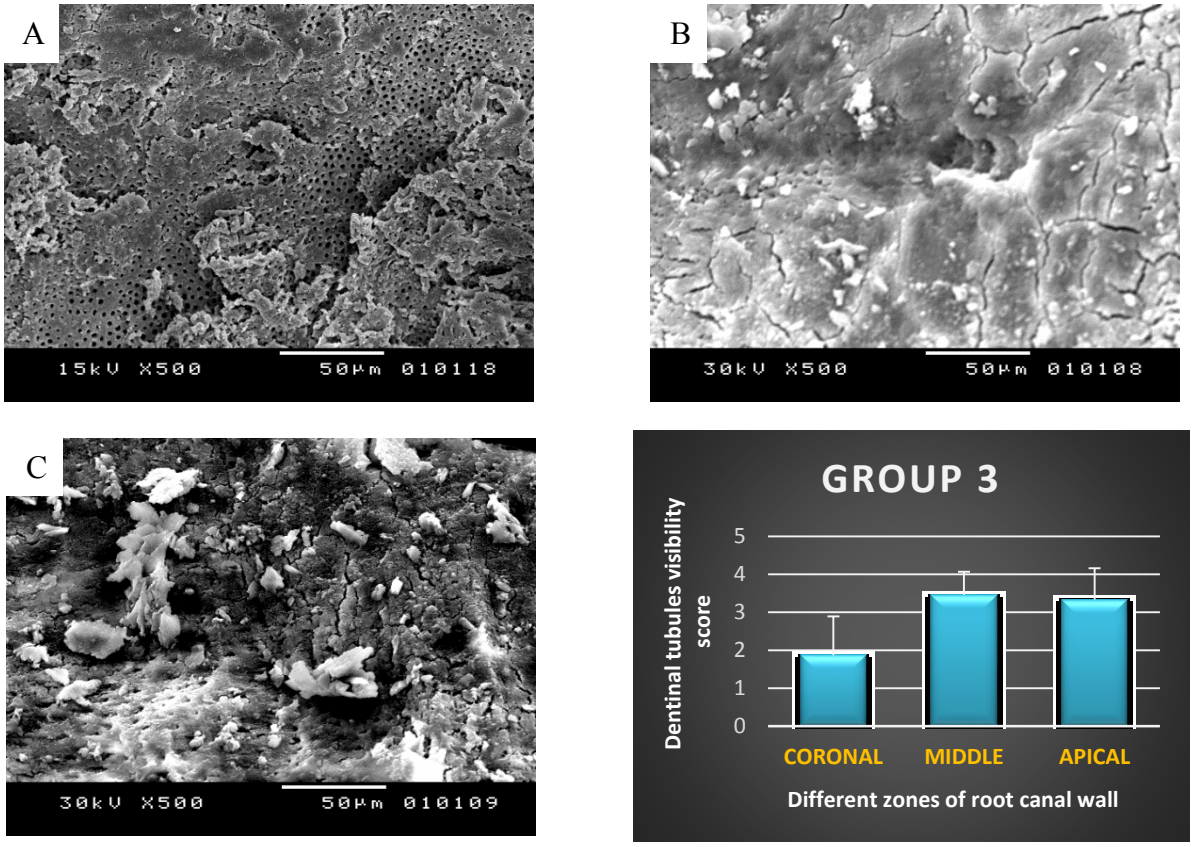


Figure 61. SEM images showed that instrumented biofilm which treated by PDT is not a clean surface, **A:** Coronal third, **B:** Middle third, **C:** Apical third, **D:** Comparison of different zones in terms of cleanliness.

4.4.4 Group 4:

The effect of sapphire tip and Er:YAG was significant on the viability of the artificial biofilm (p=7.39x10^{-7}). Testing by Kruskal-Wallis, no significant difference was observed between results obtained by this approach on different members of the biofilm (p=0.679; Fig. 62).

Referring SEM images, Student's T test showed significant difference between samples of this group compared to control group (p=0.009). Kruskal-Wallis test showed no significant effect between observations of coronal, middle and apical zones (p=0.926; Fig. 63). The bacterial cultures of this group showed a total score of zero both at immediate reading after 24 hours of incubation and later reading after 120 hours of incubation (Fig. 69).

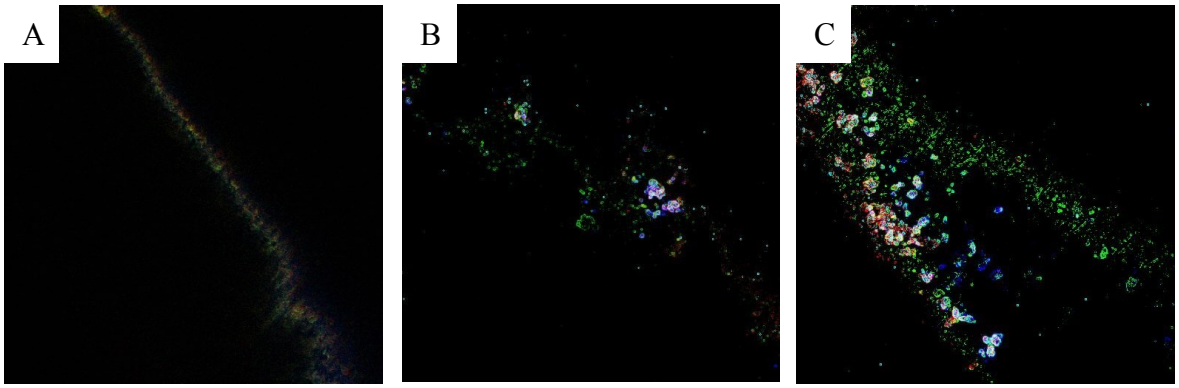


Figure 62. Confocal images of biofilm following Er:YAG delivery via sapphire tip. A significant reduction of bacterial load compared with control group was detected in coronal third **(A)**, middle third **(B)**, and apical third **(C)**.

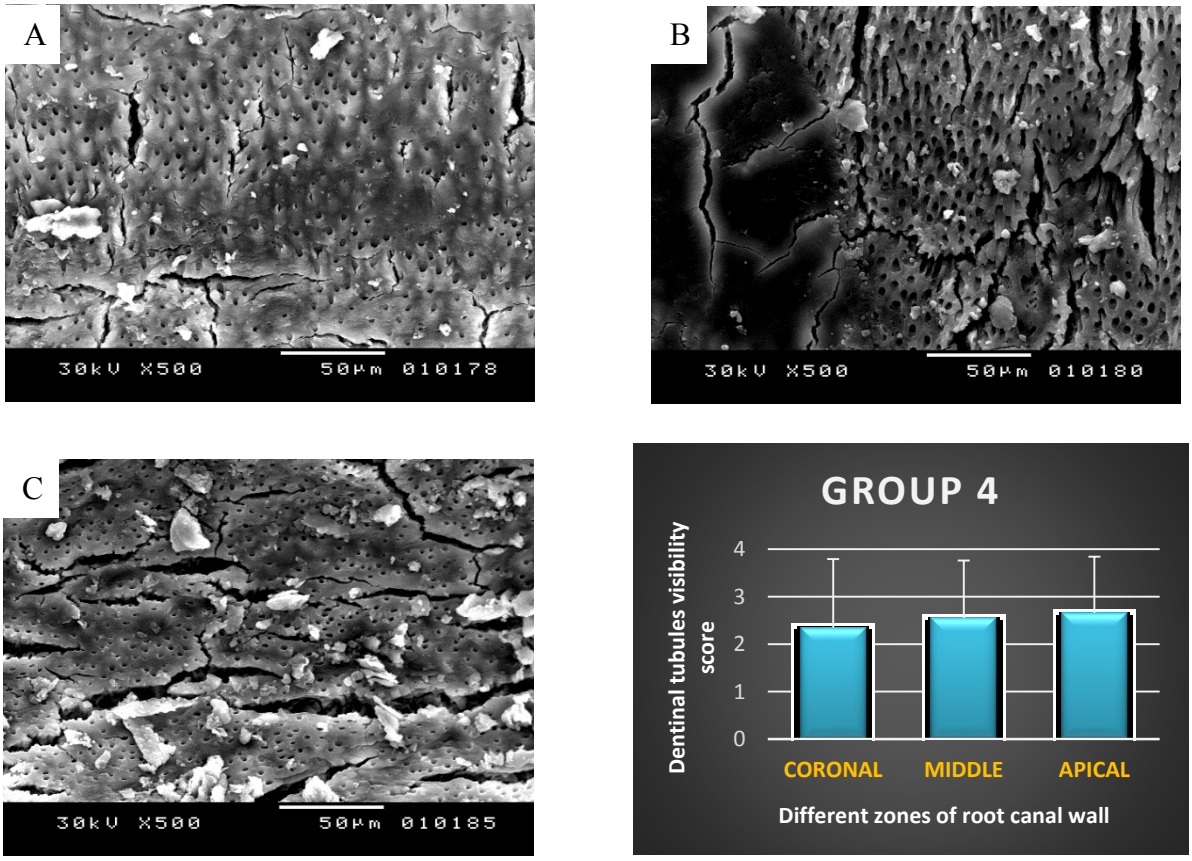


Figure 63. SEM images showed that Er:YAG laser delivered via a sapphire tip could be efficient to debride root canal walls but is not as efficient as gold standard, **A:** Coronal third, **B:** Middle third, **C:** Apical third, **D:** Comparison of different zones in terms of cleanliness.

4.4.5 Group 5:

Mann-Whitney test suggested that Er:YAG laser delivered via an endodontic fiber removed significantly the bacterial biofilm (p=3.64x10^{-5}). Not surprisingly the effect of the treatment was equal for all bacteria of biofilm (p=0.531; Fig. 64).

Regarding SEM Observations, pair comparison by Student's T test showed significant difference in terms of debris removal in contrast to the control group (p=7.58x10^{-7}). In addition, Kruskal-Wallis test displayed no difference in observations of coronal, middle and apical zones in this group (p=0.08; Fig. 65). The bacterial cultures of this group showed a total score of zero both at immediate reading after 24 hours of incubation and later reading after 120 hours of incubation (Fig. 69).

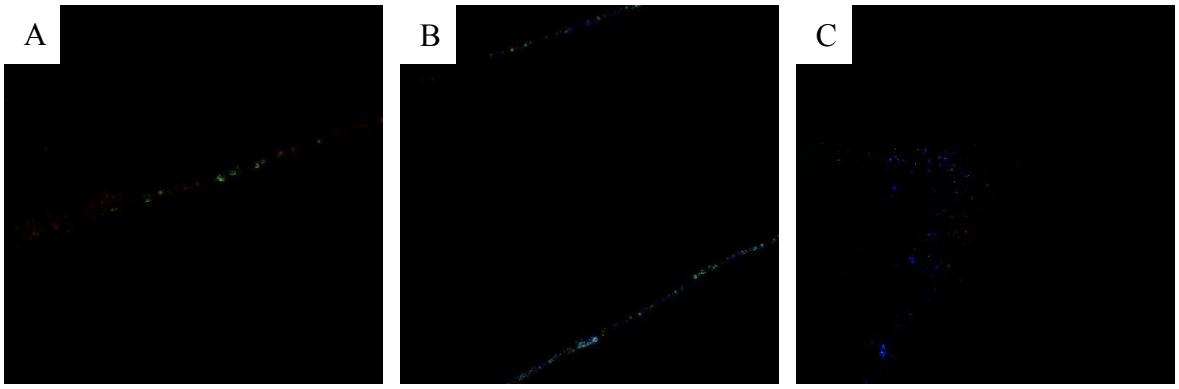


Figure 64. Confocal images of biofilm following Er:YAG delivery through endodontic fiber. Significant removal of bacteria was obtained in coronal third **(A)**, middle third **(B)**, and apical third **(C)**. However some weak fluorescence signals similar to those of PUI group was observed.

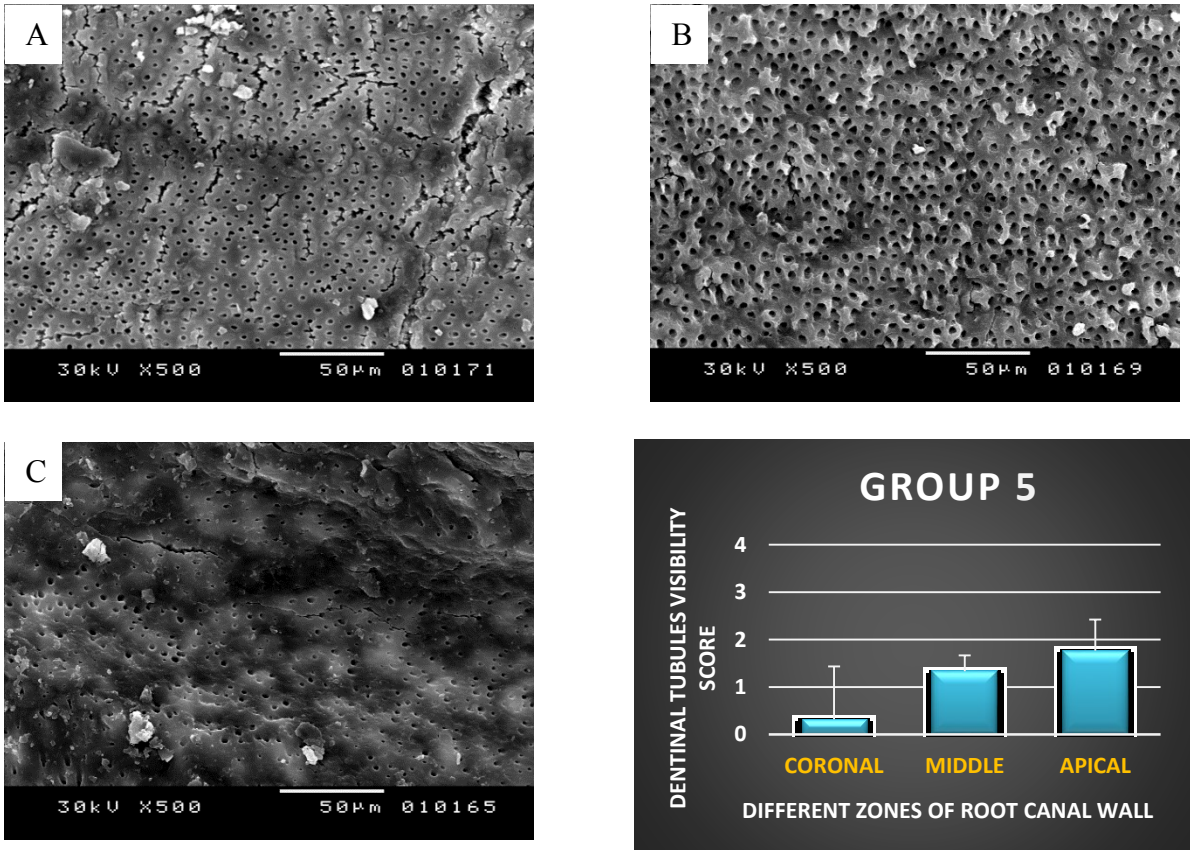


Figure 65. SEM images revealed a clean root canal surface comparable to the results obtained from PUI group, **A:** Coronal third, **B:** Middle third, **C:** Apical third, **D:** Comparison of different zones in terms of cleanliness.

4.3.6 Group 6:

According to results obtained from Mann-Whitney test, diode laser significantly eliminated the artificial biofilm (p=7.39x10^{-9}). Moreover, Kruskal-Wallis statistical test showed that the diode laser was efficient to remove different bacterial component of artificial biofilm without any significant statistic difference (p=0.2; Fig. 66).

Concerning SEM observations, Student's T test showed significant difference between diode treated samples and control group (p=3.33x10^{-12}). Besides, Kruskal-Wallis test showed no significant difference within the different zones of root canal wall of this group (p=0.194; Fig. 67). The bacterial cultures of this group showed a total score of zero both at immediate reading after 24 hours of incubation and later reading after 120 hours of incubation (Fig. 69).

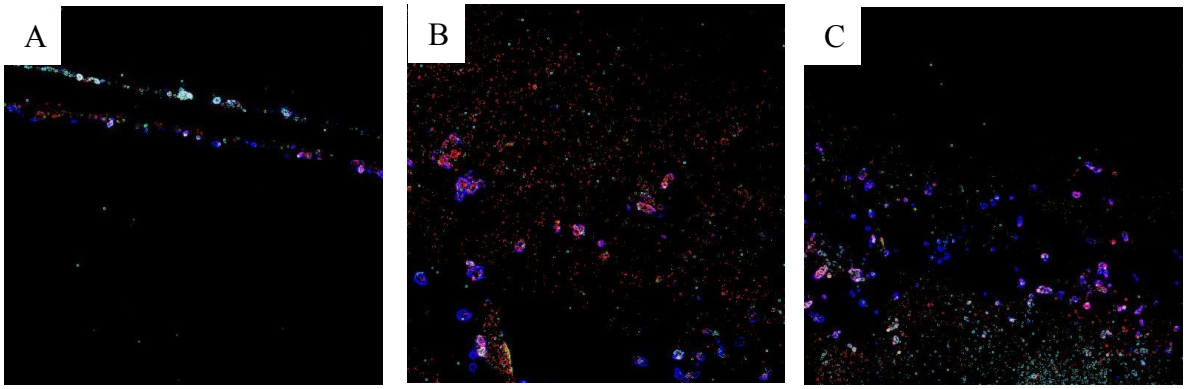


Figure 66. Confocal images of biofilm following diode laser treatment. Surprisingly, non-significant removal of bacteria was obtained in coronal third **(A)**, middle third **(B)**, and apical third **(C)** and some dispersed fluorescence signals was detectable.

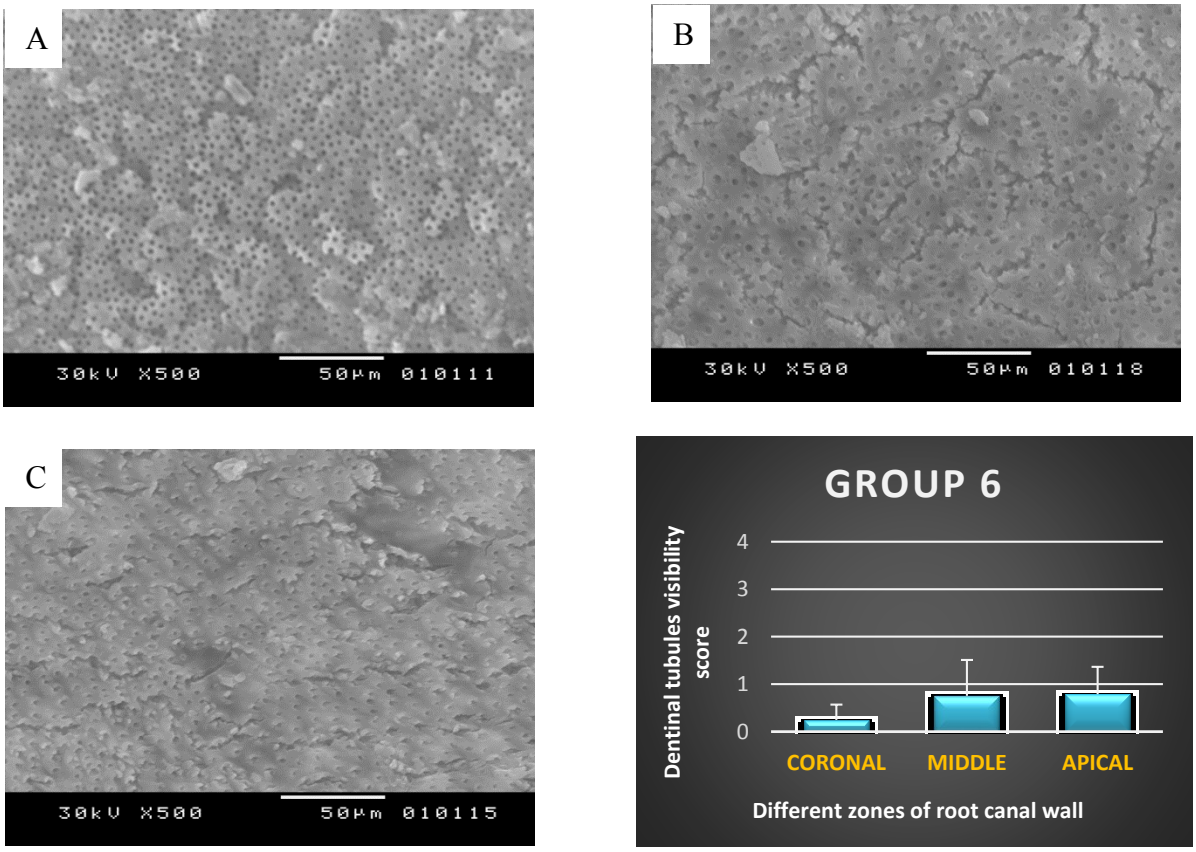


Figure 67. SEM images demonstrated the efficiency of diode laser to remove bacterial infection from root canal surface without leaving any debris behind, **A:** Coronal third, **B:** Middle third, **C:** Apical third, **D:** Comparison of different zones in terms of cleanliness.

4.3.7 Overall:

The Kruskal-Wallis statistic test of fluorescent intensity confirmed that the clinical protocols were effective to control endodontic infection (p=2.55x10^{-9}).

Intergroup comparisons were performed using Mann-Whitney statistical test. Conferring to statistic results, PUI, PDT, both Er:YAG and diode treated groups had significantly better outcome in terms of infection control compared with group treated only by rotary instrument (p<0.05). However, PUI and Er:YAG treated groups represented more effective bacterial removal than PDT and diode laser (p<0.05; Fig. 68). The Kruskal-Wallis statistical test of obtained results from bacterial cultures confirmed the outcome of different protocols (p=0.002). Er:YAG and diode lasers were as efficient as PUI in terms of bacterial load reduction and the Student's T statistical evaluation showed superiority of these groups over PDT (p=0.046). But it should be noted that the results of PDT was significant comparing to control group in terms of bacterial infection control (p=0.02; Fig. 69).

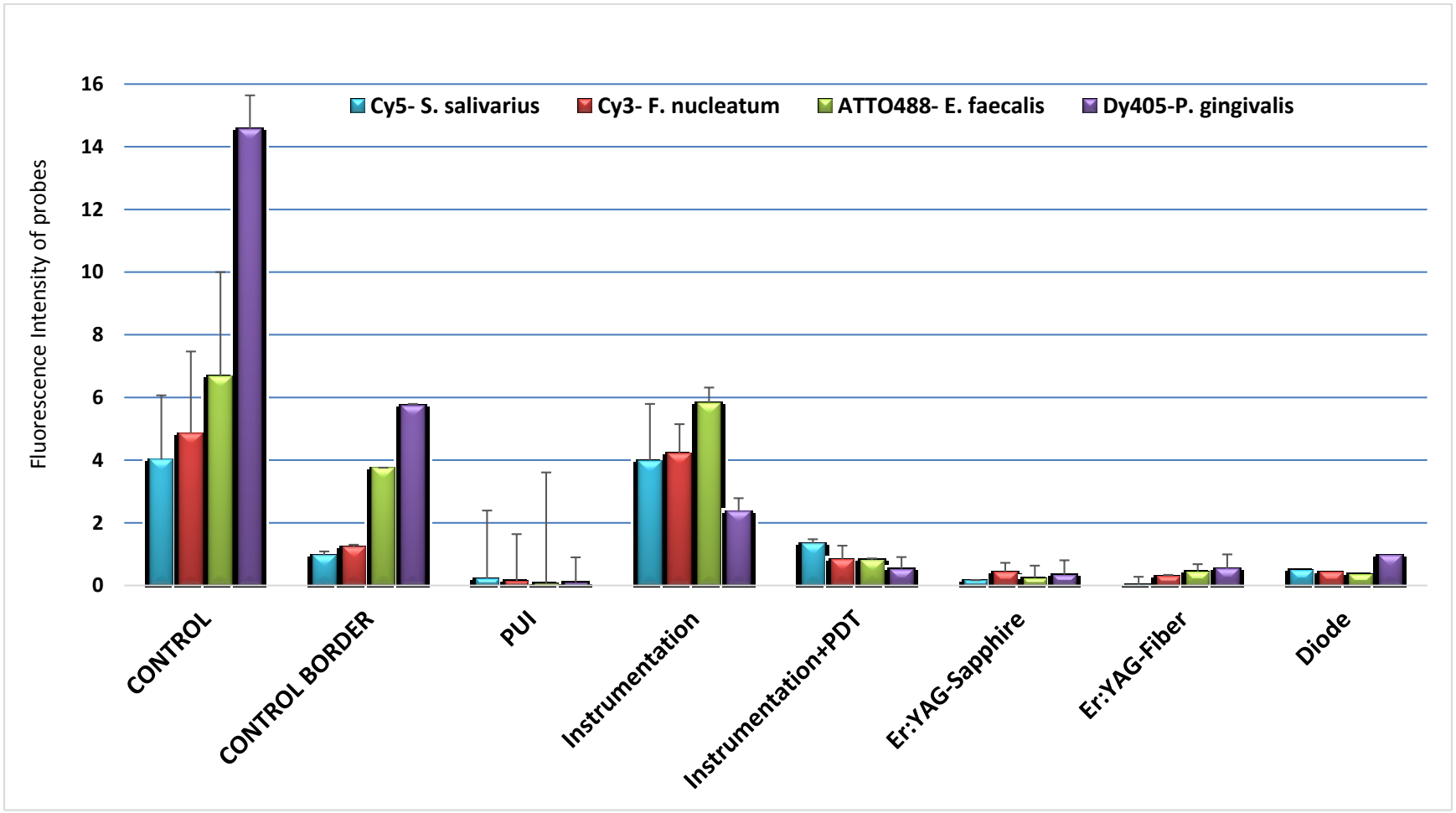


Figure 68. Comparison of fluorescence intensity of each bacterial species in control group and different decontamination protocols

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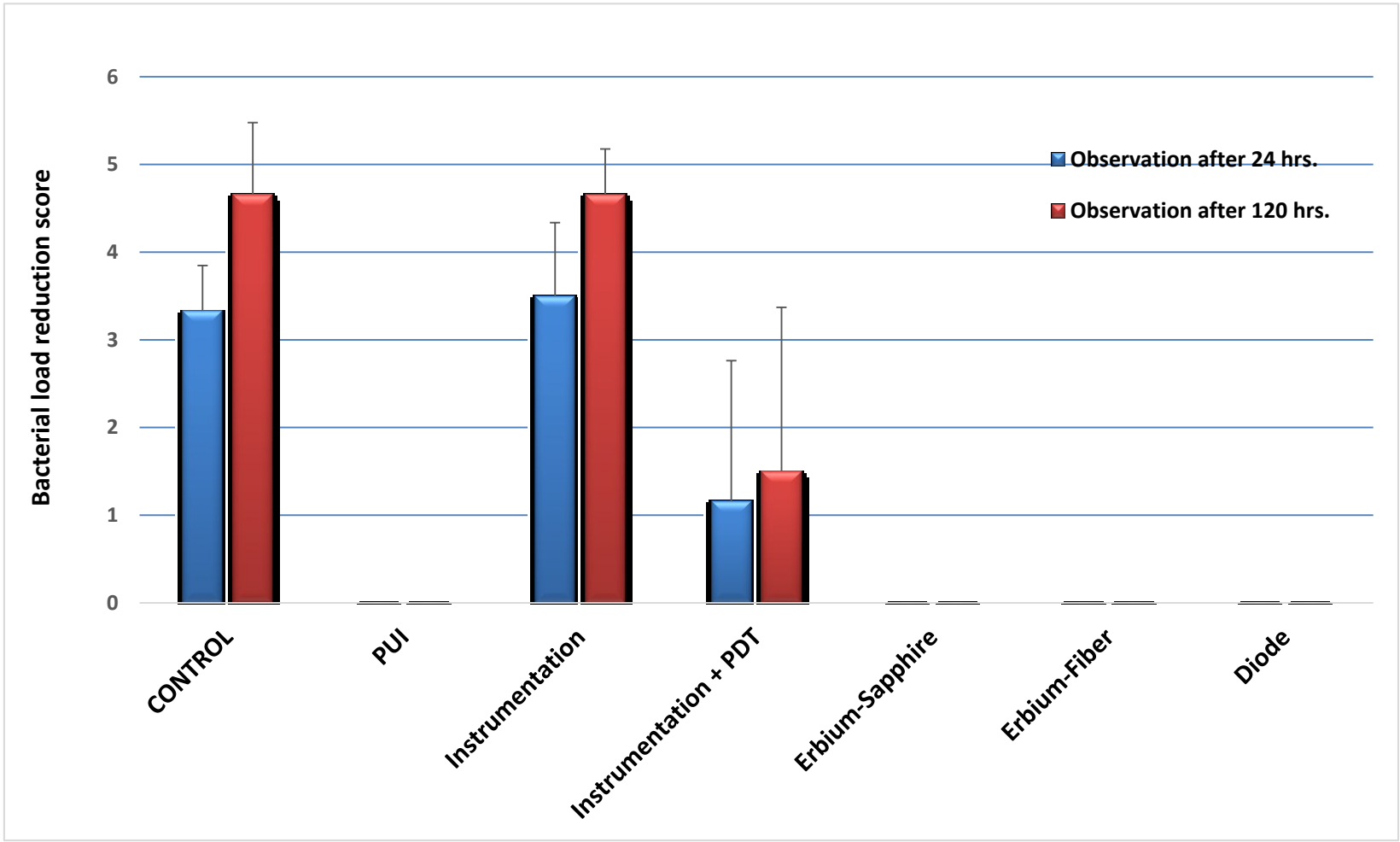

Figure 69. Antibaterial reduction efficiency of different decontamination protocol against the artificial biofilm.

Kruskal-Wallis statistical test of SEM images showed the different protocols were effective in terms of debris (organic and inorganic) removal from root canal surface (p=2.37x10^{-18}). PUI, Er:YAG delivered by fiber, diode laser had no significant difference in terms of organic and inroganic debris management (p>0.05). Student's T test displayed significant difference between results of groups 1, 5 and 6 when compared with group 2, 3 and 4 (p<0.05). However, the results of group 2 were significantly higher than those of group 3 and 4 (p<0.05). Student's T test showed group 3 and 4 had no significant difference in order to remove the artificial biofilm and inorganic debris (p=0.107).

Clearly the best results were obtained from PUI, both Erbium groups and diode in terms of bacterial decontamination. However PDT was efficient too to kill disturbed biofilm. The best results of dentinal surface debridement were obtained from PUI, Erbium delivered by endodontic fiber and diode (Fig. 70).

Kruskal-Wallis statistic test showed no significant difference among observations of all observers (p>0.05).

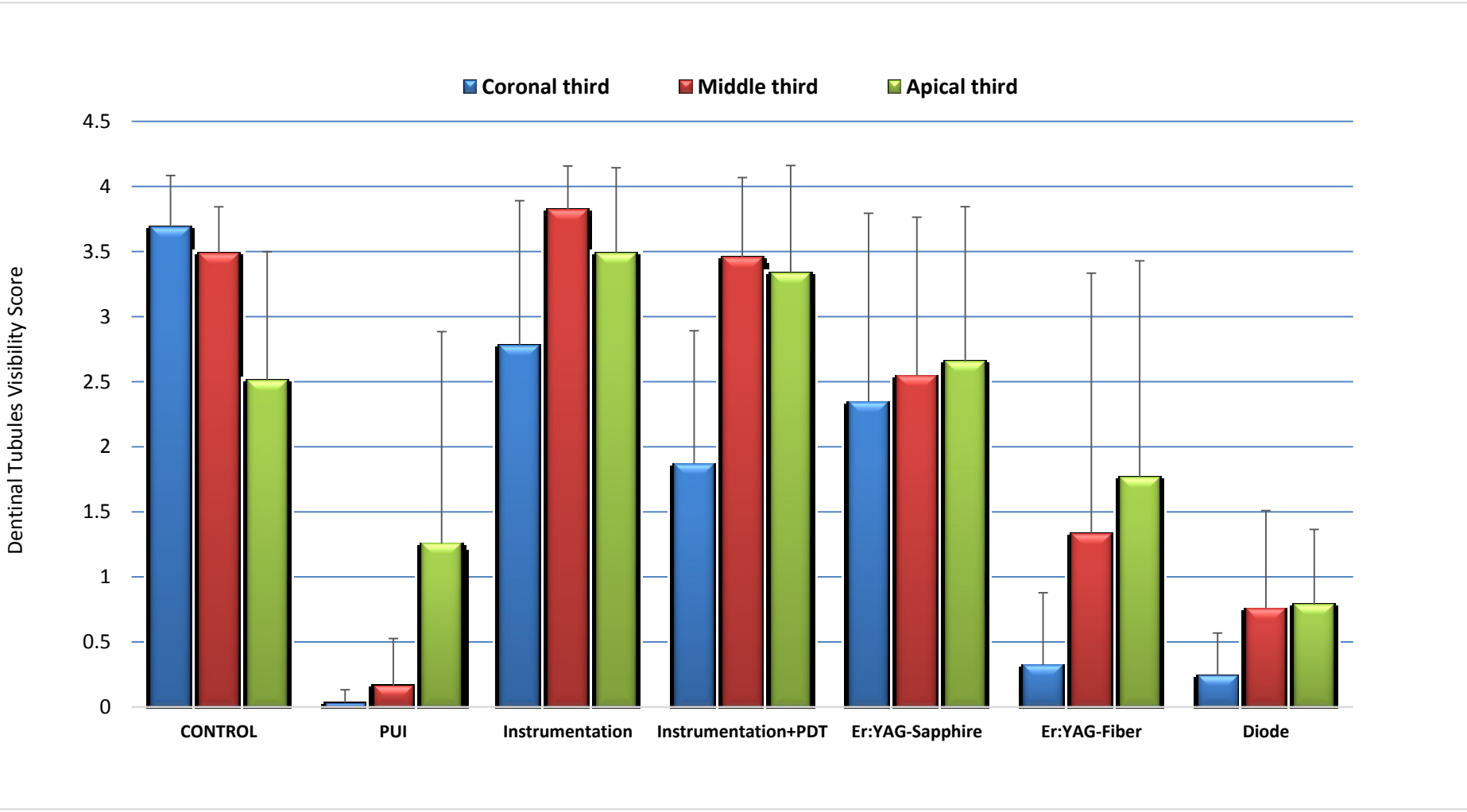

Figure 70. Comparison of cleanliness of dentinal surface after different decontamination protocol.

5

Discussion

Microorganisms have the most important role in the development of apical periodontitis. The success of root canal treatment is linked to successful elimination of endodontic infection represented generally in form of biofilm. The success rate of endodontic treatment is higher when the canal is bacteria free at the moment of filling. But clinically the conventional chemo-mechanical debridement cannot guarantee this objective. Despite a large number of studies to obtain a bacteria free endodontic space, *in vivo* "sterilization" of root canal system is not yet possible.

Testing the efficiency of treatment protocols in an *in vitro* environment before implementation in the clinic is recommended. Artificial biofilms are the most suitable targets to evaluate disinfecting capacity of different techniques.

Hence, different models of root canal infection were introduced in the field of endodontics. Among these infection study models, some are based on the presence of planktonic bacteria and others are based on bacteria attached to surfaces and organized in biofilms. Part of latter are composed of only one bacterial species or have different bacterial components. In addition, different growth conditions have been considered in the design of an infected root canal model study.

Unicellular microbial biofilms are mostly used to study root canal decontamination. (70, 71); Biofilms used in these assays are easy to develop (176-179). It has been well-established, though, that results obtained for planktonic bacteria eradication by various disinfecting agents do not reflect the same effect on bacteria in *in vivo* conditions, neither on *in vitro* biofilms. It has been demonstrated that the same bacteria in a biofilm can be 100–1000 times more resistant to antibiotic agents than their planktonic counterparts (72). Therefore, recent trials focus on studying the *in vitro* endodontic decontamination protocols using microbial biofilms instead of planktonic bacteria (180). For that reason we designed a study model which complies with these findings. In our study model, the dentinal root canal surface was largely covered with a mature biofilm that was firmly attached to its substrate. The biofilms of teeth with apical periodontitis or chronic pulpitis are multicellular. According to this ecological concept, in root canal infection, the pathogenicity is not exclusive to an individual species of microbes, but it is the entire polymicrobial unit which goes through different physiological and genetic alterations initiated by modifications in root canal environment and will lead to pathogenicity (6, 7). The major bulk of the organisms present in the biofilm is a collection of cocci, rods, filaments and more rarely spirochetes (74). This diversity of constituent bacteria in oral biofilms is difficult to reproduce in *in vitro* and laboratory conditions (20). Nevertheless, the biofilm model should mimic the natural pathogenic bacterial structure both at the level of morphology and distribution of bacteria. We selected one bacteria from each colonizing group of oral biofilms to form a simple but mature polybacterial biofilm that morphologically resembles the wild type biofilm. In our study, we also aimed to form a polybacterial artificial biofilm which could adhere to dentinal surface and penetrate into dentinal tubules as it happens in *in vivo* conditions.

Pilot Study:

In our pilot study the bacteria were chosen from a previously established research project of MICORALIS (former LOHA) laboratory. Through our study we intended only to verify the feasibility of formation of the artificial biofilm over dentinal substrate inside root canals. SEM images of root canals after 7 days of incubation revealed the presence of biofilm heaps over the dentinal surface. These images showed bacteria at the opening and inside dentinal tubules. These results confirmed that the possibility to use root canals as the *in vitro* habitat for a artificial polybacterial endodontic biofilm. In addition, because of different publications with contradictory results associated with various protocols (will be discussed in detail in the discussion of the treatment protocols) we decided to choose a PDT protocol to integrate into the main study. Many factors like duration, type of photosensitizer, quality of delivered light and its wavelength influence the outcome of PDT. For this reasons we tried 2 different protocols to initiate the photochemical reaction against the biofilm in a clinically acceptable time. The first one was using a 650 nm diode laser with its unidirectional light beam to activate toluidine blue and the second one was a commercially available PDT system (ASEPTIM®) comprising of 635 nm LED light and its specially designed photosensitizing agent. To verify the direct effect of PDT on the biofilm, we decided not to instrument the artificially infected root canals before treatment. The results showed that LED light could enhance the effect of the photosensitizer against the microbial biofilm. However with these preliminary result, the importance of the mechanical impairment of the biofilm structure has been recognized.

Biofilm design and characterization:

The artificial biofilm of the pilot study was not quietly representing the wild type endodontic biofilm as *Prevotella intermedia* and *Porphyromonas gingivalis* are both from late colonizers of oral biofilm. In our model we aimed to develop an artificial endodontic biofilm, which can structurally resemble its wild type counterpart. For this reason we chose to keep *P. gingivalis,* which is one of the main colonizers of dentinal tubules (181). For the main study, *Fusobacterium nucleatum* was replaced by *P. intermedia. F. nucleatum* is an intermediate colonizer and has the capacity to co-aggregate with both primary and late colonizers and acts as bridge between them (182). This bacterium is an anaerobe but it can tolerate the presence of oxygen in the biofilm. Furthermore, *F. nucleatum* can provide the growth conditions for strict anaerobic bacteria such as *P. gingivalis* (182). *Enterococcus faecalis* does not participate usually in primary endodontic infection (183). However, this bacterium is trending up as an *in vitro* model for endodontic disinfection studies. Frequent recovery of *Enterococcus faecalis* in root canals with a persistent infection made this bacterium as a suitable organism to test different decontamination methods (85, 176) and brought us to integrate this facultative aerobe in the structure of our biofilm model. *Streptococcus salivarius* is a facultative aerobic bacterium and an early colonizer of oral biofilm (184). This bacteria has the ability to attach the acquired pellicle and initiate the biofilm formation (185).

The first trials on dentinal disks allowed us to study the growth of artificial biofilm in a periodic aspect. The very first SEM images showed a continuous bacterial growth over dentinal surface, which was visualized in other observations too. According to Hall-Stoodley *et al.* (186) dynamic growth of bacterial biofilm leads to formation of tower- and mushroom-

shaped structures sized more than 100 µm. In our study the biofilm structure needed 20 days of incubation to show features of mature bacterial community. In our study, we observed an inverted progression of biofilm size increasing and distribution after 24 days. Dispersal or detachment is an important phase of biofilm lifecycle which plays role in transmission of microbes to new habitats from their reservoir to host or within the host (187). This could be an active process linked to individual bacteria present inside the biofilm structure following the environmental changes or could be a passive phenomenon which happens due to physical stresses like movement of substrate and liquid flow. Passive dispersal may happen also due to enzymatic reactions such as matrix lysing enzymes or intercellular relationships like competition, mutualism, phagocytosis and etc (187).

We noticed that the biofilm resumed to grow and to cover dentinal surface after 31 days of incubation. We believe this might be due to detachment phase of biofilm lifecycle to adapt its behavior to resist environmental changes and to contaminate a larger area. It has been shown by Shen *et al.* that 3 weeks or older biofilms are more resistant to disinfection techniques (69). Therefore, the prepared sterilized root canals were infected with bacteria for 21 days, which seems to be enough to obtain an artificial biofilm. At this age, despite firmly attached and mature structure, our biofilm could not cover the entire surface of dentinal disks (Fig. 42) which might be due to their flat surface and brutal movements caused by the orbital shaker (150 rpm) inside growth media. The same biofilm can colonize the entire dentinal wall of the root canal space and cover dentinal tubules (Fig. 51). Further FISH-confocal observation of the biofilm formed inside the root canal space revealed the complexity of deep biofilm structure with channels and circulatory system. These structures are described to be essential for nutriment and waste exchange of biofilm (187).

We decided to characterize the artificial biofilm with FISH visualized by confocal laser scanning microscope (CLSM) and scanning electron microscope (SEM). The FISH gave us the opportunity to obtain semi quantitative results about the presence and distribution of bacterial species before and after treatment. Furthermore the specificity of fluorescent probes to each species led to identification of bacteria inside the biofilm structure. In addition, we were able to quantify the presence of each microorganism and their distribution through our artificial infectious community in situ (into the root canal). This localization of biofilm inside the root canal space could not be confirmed by PCR techniques. By using CLSM we could retrieve information of deep layers of the biofilm structure. By measuring the fluorescence intensity of different 16s rRNA signals at the moment of hybridization we could indirectly evaluate the efficiency of different disinfecting protocol. For instance absence of fluorescence signals represents the removal of bacteria following treatment protocols. This could be interpreted that the treatment protocol was efficient to remove bacterial biofilm from the root canal space. Furthermore, to confirm these results we performed scanning electron microscopy. SEM provided topographic images of the biofilm's morphology and displayed the cleansing efficiency of treating protocols. By scanning of root canal surfaces, we retrieved the zones that previously were observed with confocal microscopy and verified the nature of those tissues with fluorescent signals. It could be concluded that the combination of FISH/ CLSM and SEM identification techniques provided a comprehensive vision of biofilm.

Interpretation of data obtained from confocal images helps to confirm the presence of all microorganisms inside a mature artificial biofilm. The micro-organisms were distributed evenly inside the biofilm structure ($p>0.05$) confirming our choices that the

selected bacteria could coexist together and form a well-developed biofilm. As expected, the presence of *P. gingivalis* was statistically higher inside dentinal tubules than the other bacterial species which normally happens as a biofilm gets older and thicker when the anaerobes become dominant (181).

SEM images showed that the artificial biofilm covered the dentinal surface like a carpet or a pavement of compacted microorganisms. It was possible to determine the morphological differentiation of each species using higher magnifications (about 7000X to 10000X).

Regarding the tubular infection, Ma *et al.* (188) suggested that a better bacteria penetration into dentinal tubules could be achieved with centrifugation of bacterial inoculum and root canal samples together. However, we observed the simple injection of the microbial inoculum into the root canal space may result in a good penetration of bacteria into dentinal tubules. By scanning in the regions marked previously by fluorescent probes we could identify packs of bacteria inside dentinal tubules as far as 500µm from the main canal. FISH and CLSM techniques have been used in many scientific experimentations targeting dental and more precisely endodontic infection. Confocal microscopy is a non-invasive technique by which the *in situ* comparison of the neutralizing and eradicating effects of different methods on bacterial infection becomes possible (188). However, in the field of endodontics except some pioneering and distinguishable studies (52, 171), the use of FISH technique is limited to a straightforward Live/Dead test(189-192). This test aims to show the presence of viable biofilm before treatment and for measuring the ratio of dead and alive cells after application of disinfection root canal treatment procedure. So when using a polybacterial biofilm, the results of Live/Dead tests may illustrate the presence and distribution of bacteria

over its substrate but it cannot demonstrate the sensitivity of different bacteria to a specific treatment. Hence, using 16S rRNA species-specific probes of each bacterial species of artificial biofilm allowed us to assess the outcome of disinfectant protocols more accurately.

During the visualization of our samples with CLSM, the background noise was present even in negative controls. It could be due to difficulties we encountered because of the curved form of root canal halves during the washing phase of the hybridization process to avoid biofilm distortion. So, presence of some unbounded probes is most probable. Another inconvenience which arose during characterization methods was the difficulty to focus on the borders and the dentinal surface of root canal walls at the same time. To overcome this problem, we had to take an image for each segment, this is in addition to some blurriness caused by water in LabTek® chamber.

The protocol for sample preparation may lead to artifacts due to dehydration, such as ECM evaporation (see part 1.6.3). However, it should be reminded we did not attempt to study in anyway the ECM characteristics of the artificial biofilm, which might be disturbed during dehydration and drying procedures. Still, this did not influence the treatment results, as they were applied before any processing procedure and of course the presence or absence of bacteria was confirmed with fluorescent signals.

Treatment protocols:

Once the biofilms were standardized, they were subjected to the different experimental treatment protocols. To measure the efficiency of the different disinfection protocols on removal of the artificial biofilm from the root canal, we characterized the bacterial composition of our biofilm after each treatment.

The effect of ultrasonic irrigation on both disinfection and debridement aspect is well established in literature (193). According to Van der Sluis *et al.* Passive Ultrasonic Irrigation improves debridement capacities of endodontic irrigation solutions (194). NaOCl dissolves organic tissue such as bacterial biofilm and EDTA cleans dentinal surface from residual inorganic material (88, 93, 94). In our pilot study, we applied PUI for the purpose of enhancing the antimicrobial activity and debris removal of NaOCl and EDTA. This group exhibited the best results. Thus, based on the literature and regarding our pilot study, we have selected PUI as gold-standard.

The second group was treated only with rotary instrumentation and H_2O to evaluate the sole effect of mechanical debridement on the biofilm. For the third test group the PDT was carried out after mechanical instrumentation the root canal space. We used Er:YAG to treat the fourth and fifth groups in which the laser beam was delivered respectively with a sapphire tip and an endodontic fiber. The last group was irradiated with a diode through an endodontic fiber.

In terms of bacterial load reduction, we compared the intensity of fluorescent probes signals retrieved by confocal microscope. Except for group 2 that had been treated only by rotary instrumentation and showed no notable difference with the control group, all other treatments resulted in significant bacterial killing. However, as shown in figure 63, PUI expressed better results than PDT and diode groups. The Er:YAG laser in both delivery methods could reduce bacterial infection as efficient as PUI. Confocal FISH imaging revealed the absence of fluorescent signals. According to Olivi and De Moor (135), LAI using Er:YAG laser works in different manners regarding laser energy delivery protocol. When Er:YAG laser is delivered through a tip in the pulp chamber and/or at the orifice of

the root canal, the interaction with dental tissue and irrigants is photomechanical. But, the intracanal irradiation with endodontic fiber showed to initiate cavitation effect via a photothermal phenomenon inside irrigation solution. Laser Activated Irrigation (LAI) has the advantage to excite the irrigants using very low power and this protects the dental tissues from any iatrogenic effects including thermal and explosive damages. We used nearly the same parameters described by De Moor *et al*. which are sufficient and safe to perform LAI (195, 196). Both studies showed Er:YAG and Er,Cr:YSGG efficiently removed root canal debris with an energy of 75 mJ and a total irradiation time of 20 sec (4X, 5 sec) without the need to move the endodontic fiber, We used 80 mJ during same application time for both sapphire and endodontic fiber. As mentioned before, confocal images obtained from both groups showed maximum bacterial eradication; but, SEM showed that in group treated with sapphire, the dentinal tubules were covered entirely with debris. However, it is notable that Er:YAG delivered by endodontic fiber was as efficient as PUI in terms of cleanliness of root canal surfaces (Fig. 68). Our results are in line with those of Peeters *et al.* (197) and Guidotti *et al.* (198) in terms of number of opened dentinal tubules after LAI. When taking the energy fluence into account, there is a considerable difference between both sapphire and endodontic fiber tips. The energy delivered by the endodontic fiber was 116 J/cm^2 but the sapphire delivered an energy fluence of 15.92 J/cm^2. In addition, the sapphire tip was kept steady at canal orifice and had no contact with root canal walls while endodontic fiber was moved spirally along root canal and close to dentinal surface. It could be speculated that our parameters for Er:YAG delivered with sapphire enhanced the effect of NaOCl to kill bacteria but its energy was not enough to remove the residual debris of microbial biofilm from dentinal surfaces of the root canal. .

The results of group 5 were obtained only by irradiation of NaOCl solution by the endodontic fiber with a helical upward movement. We demonstrated that an irradiation of the root canal filled of NaOCl for 4 cycles of 5sec is sufficient to eradicate bacterial community from root canal space. But it should be mentioned that confocal microscope still exhibited some weak and statistically negligible fluorescence signal in very small dentinal debris which remained on dentinal surface. Nevertheless, this suggests that some small number of viable bacteria still might be found in dentinal debris. Guidotti *et al.* demonstrated the best results in terms of root canal debridement with LAI can be achieved when double irradiation of the root canal is performed to activate both NaOCl and EDTA (198).

Regarding the diode laser, Moritz *et al.* examined an 810 nm diode laser's ability to kill root canal bacteria in *in vitro* and *in vivo* studies (199, 200). Afterward it was demonstrated that this laser is able to decontaminate deep layers of radicular dentin which is an important factor against the tridimensional structure of root canal space (201). The explanation is that the diode laser is not absorbed in water or in the superificial layers of dentin which results in diffusion of laser beam into deeper layer of dentin (119). Same results were obtained by 830 nm (202), 940 nm (203) and 980 nm (204) diode lasers. These findings demonstrate that different wavelengths of diode laser were effective in terms of reduction of the bacterial load. We applied a diode laser with a combination of 915 nm and 1064 nm wavelengths to confirm this concept that near infra-red lasers are efficient against bacterial infection. Confocal images of the treated biofilm with diode laser showed significant reduction of bacterial infection from root canal space, which was as efficient as other groups treated with Er:YAG laser, but obtained signals were stronger than those recovered from PUI. The SEM images did not exhibit presence of any microorganisms over dentinal surface.

However, SEM images gave evidence of some roughness produced over inter-tubular dentin. It could be hypothesized that the state of root canal walls after diode laser application. This might have lead to stuck some unbound fluorescent probes to dentinal surfaces, which could not be removed during washing procedure. Bacterial culture revealed presence of no survived bacteria after treatment of root canal.

da Costa Ribeiro showed photo-thermic damage of direct application of diode laser on dentinal walls is negligible when "reasonable" parameters are used (205). They showed thermal elevation caused by this laser is up to 8.6°C in continuous mode and between 1.2 to 3.3°C in pulsed mode which is crucial to prevent any harm to periodontal tissue. But this little thermal change could still result in closure of dentinal tubules. The morphological changes caused by diode lasers on root canal dentin are power dependent. Diode Lasers remove smear layer at 1.5W, whereas increasing power leads to extreme changes in dentin like melting of the dentinal surface (206-208). Despite any morphological alteration, diode lasers have no adverse effect on structural characteristic of the mineral matrix of dentin (208). To prevent any cumulative thermal effect, it is mandatory to consider recovery time during diode laser irradiation intervals. For this reason we used an output power of 2 W with frequency of 100 Hz and we irradiated the canal for 15 sec and renewed each time the NaOCl solution. According to Gutknecht *et al.* and Alfredo *et al.* (209, 210) by respecting needed resting time, diode laser could be considered as a safe device and even raise up to 3 W. To prevent overheating and melting of dentin and further thermal damages to surrounding tissues we continuously moved the endodontic fiber during irradiation as previously described in literature (209-211).

Smear layer elimination could be achieved using diode in conjunction with some irrigation solutions. However, type of irrigation solution determines the outcome of treatment (207). According to Alfredo *et al.* synergy of diode laser and 17% EDTA solution promote the smear layer removal whereas activation of 1% NaOCl solution with the same wavelength produces smear (132). In our study, we intended to examine the effect of laser on biofilm and smear layer; thus, we did not use EDTA to exclude its chelating effect. Contrary to results of Alfredo *et al.* we noted that coupling of 915 nm and 1064 nm diode laser combined with NaOCl 2.6% leads to a satisfactory removal of bacterial infection from root canal space without any additional debris creation over dentinal surface. The SEM image analysis represented significant removal of smear layer similar to PUI . These pictures showed DeltaCube® laser efficiently cleaned root canal surface from bacterial infection in apical as well as coronal and middle regions and left dentinal tubules open.

Diode lasers might contribute to activation of irrigation solutions due to their high frequency that reaches to 20-50 KHz. Hmud *et al.* reported that two different diode lasers (940 nm – 980 nm) resulted power dependant formation of impulses and water vapor (140). This possibly results in a better debridement of the root canal space It should be recalled, Hmud *et al.* demonstrated in another study that the thermal rise inside the irrigants during irradiation with diode laser was about 30°C (141). Neelakantan *et al.* demonstrated the diode laser is as efficient as Er:YAG to activate irrigants in the root canal and disturb microbial biofilm specially inside dentinal tubules (212). However, according to Olivi and De Moor (135) the vaporization could be explained by heating action of diode lasers inside the irrigation solutions. They also described the effect of diode on the NaOCl, EDTA and other irrigants as a synergistic effect of near infra red lasers with these irrigants.

George *et al.* there are some differences in quality of explosive vapor initiated by diode and Er:YAG. The peak of cavitation and bubbles formation with diode happens with a delay of approximately 5 sec after irradiation starts. Due to slower fluid movement during irradiation by diode, the possibility of irrigants extrusion beyond the dental apex is less than that with Er:YAG (142). There is a proportional relationship between irrigant volume in root canal space and the power needed to activate it. However the form of the fiber may enhance the outcome too. The factor of power is playing an important role and cavitation occurs always in a power level more than 2 W (142). In this perspective, we observed with an ultrarapid camera the events happening during irradiation of NaOCl 2.6% solution with the DeltaCube® diode laser inside a capillary of 1.5 mm diameter. This observation was performed only to determine whether the air bubbles are formed inside solution or not. We used the same parameters of treatment protocol (pulsed mode 100 Hz with 2-3 Watt). Because of the large diameter of the capillary, with 2 Watt, the diode laser did not initiate bubble formation. By increasing the power to 3 Watt the bubbles were observed to form and to explose during irradiation (Fig. 71). These images and by considering our results obtained against the artificial biofilm brought us to hypothesize that this bubble formation resulting either from a possible cavitation phenomenon or the direct photothermal nature of diode laser-target interaction may enhance root canal debridement without any adverse effect on dentinal tissue.

The laser frequency probably influences the outcome of LAI with a diode laser. Deleu *et al.* demonstrated a 980 nm diode with a frequency of 25 Hz needs an output power of 7.5 W to initiate the bubble formation (213). They demonstrated that this protocol resulted in a carbonization on the dentinal walls. In our study, we demonstrated that the bubble

formation can be initiated with the output power of 2 W and the frequency of 100 Hz without any undesirable effect on the root canal walls.

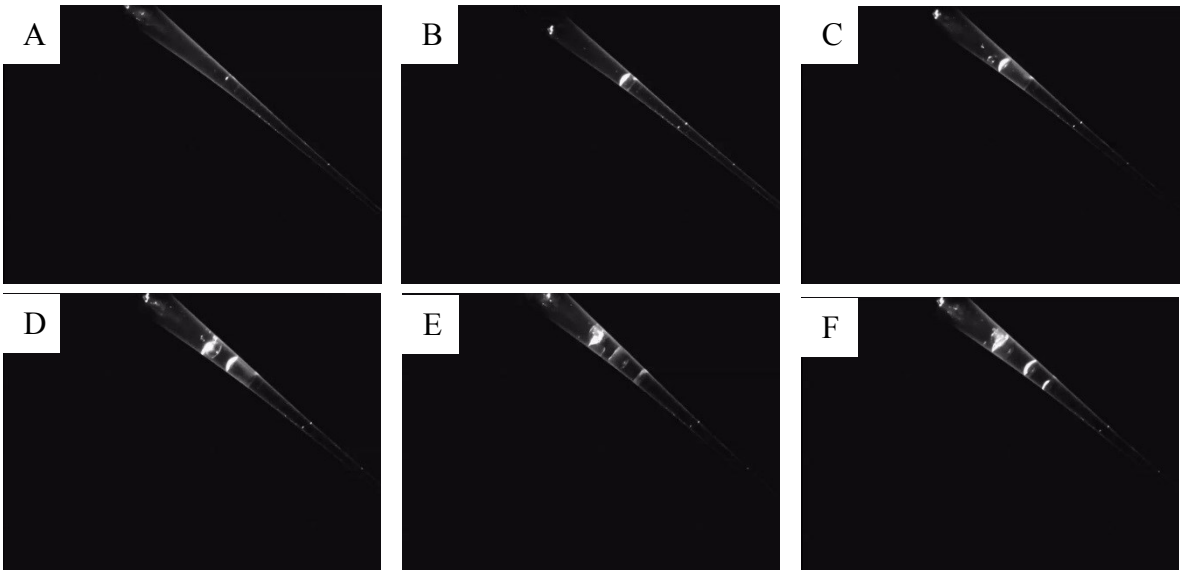

Figure 71. **A-F)** A timelaps view of the events happening during irradiation of NaOCl with DeltaCube® laser,

There are different studies about the efficiency of PDT on root canal disinfection that reported controversial results. It has been demonstrated that conventional photo-activated disinfection (PAD) could not disrupt polybacterial plaque but it might reduce a mono-species biofilm made of *Enterococcus faecalis* (214). Yao suggested that PDT is more effective on planktonic form of bacteria than their biofilm state inside root canals (215). Thus, the conventional disruption of intracanal biofilm before PDT is critical for success of treatment (150). Clinical trials of Jurič demonstrated very well the application of PDT after conventional debridement of root canal space to obtain a bacterial free canal, albeit using a monobacterial biofilm (216) . Hence we planned to examine the effect of PDT on an only mechanically disrupted biofilm. From our pilot study (217) we knew that PDT could not eradicate the bacterial infection in a clinically acceptable working time. Data obtained from this trial showed diode laser with its coherent unidirectional emission and LED laser with its diffusive light could not contribute to decontaminate a root canal space invaded with a mature polybacterial biofilm. However the PDT performed by LED (Aseptim®) could significantly reduce the bacterial load (217).

According to Soukos *et al*. (158) and Bonsor *et al.* (218) emitters or light diffusing optic fiber could scatter unidirectional light of diode laser to ensure maximum reach even in the most apical zones of root canal. Nowadays light emitting diodes (LED) showed to be promising in terms of activating the Photosensitizers (219). Using simulated root canal models, we also showed in our pilot study that LED light travels easily in all direction effortlessly with no need to move the optic fiber, which lead to better results in terms of bacterial load reduction rather than PDT utilizing a unidirectional diode laser (217). Recently, another study by Sabino demonstrated different effects of the same light source on

a bioluminescent species of *Candida albicans* (220). When micro-organisms were irradiated with laser using a light diffuser fiber, the reduction in bacterial load is 100 times more than using a normal optic fiber. Therefore to treat our samples we decided to use LED light of Aseptim® system with an output power of about 900 mW in synergy with Toluidine Blue which its antimicrobial ability is well demonstrated in literature (148, 149).

Looking at the results of Souza et al. (221) and as demonstrated in our pilot study (217), different PDT protocols could not be totally competent without pretreatment of biofilm with routine root canal debridement methodologies (instrumentation and irrigation). Promising results are reported especially when more incubation time with Photosensitizer and longer irradiation is applied (158, 222). In an *in vitro* study by Komine and Tsujimoto to measure the amount of produced singlet oxygen after 300, 600 and 900 seconds of irradiation, it was exhibited that the highest amount of singlet oxygen generated through Photodynamic therapy could be achieved after longest time of irradiation (151). Regarding working time, these conditions seem to be difficult to apply in daily clinical procedures particularly when treating multi rooted teeth. To overcome this problem, PDT in two visits could be beneficial (223, 224), however treating infected root canal in two or more sessions is disputable because of the need to use an intra-canal dressing between the sessions.

There are controversies about irradiation time; interestingly, Yildirim reported that there is no difference between 1 minute and 4 minutes irradiation of Photosensitizers (225). Keeping in mind these controversies, we supposed that the longer irradiation of photosensitizer in a clinically acceptable duration only happens by using a LED. The LED light is diffusing in all direction. The LED light is in contact with photosensitizer during all irradiation time with no need to move the tip. This phenomenon could not happen with a

laser light since it is unidirectional; thus to activate the chemical solution inside the root canal space the fiber has to be moved in all direction. This could be the reason to obtain better results of LED over the diode activated PDT. In this study, we showed that PDT is efficient to kill disturbed infectious communities. Confocal images of non-instrumented biofilms revealed that the bacteria are still detectable in deeper layers indicating the need for efficient disruption of endodontic biofilms before PDT application.

SEM images demonstrated that PDT cannot be a substitute for chemo-mechanical debridement of the root canal space due to its chemical nature of action, thus both organic and inorganic debris should be cleaned by routine gold standards. However, PDT has no selective action and resistance to this treatment is rare (223). Consequently it seems logical to consider PDT as an adjuvant to the conventional root canal decontamination techniques in order to neutralize those bacteria that could still be detectable inside endodontic system (main and accessory canals plus dentinal tubules).

6

Conclusion

Management of endodontic infection is the key for successful treatment. We intended to elaborate a biofilm model structurally similar to the wild type endodontic biofilm. After construction of our model we used FISH-confocal (using species-specific probes against 16S rRNA) followed by SEM imaging technique to characterize the artificial biofilm *in situ* before and after various decontamination procedures. To our knowledge this protocol has never been used for *in situ* characterization of an endodontic artificial biofilm that could open a new avenue to investigate the different endodontic disinfection methods.

Concerning the elaboration of the *in vitro* biofilm, the choice of four different bacterial species (*S. salivarius, E. faecalis, F. nucleatum and P. gingivalis*) used to build this biofilm was functional. These species could coexist and form an *in vitro* polybacterial biofilm. The artificial biofilm could effectively infect the root canal space by covering dentinal surfaces and the invasion of the dentinal tubules. The age of the biofilm (maturation) was 21 days. This seems enough to obtain a mature and structured biofilm that could resist against antimicrobial protocols and provide a vision of future outcome of these protocols against *in vivo* endodontic infection. The FISH-confocal technique using species-specific probes against 16S rRNA is beneficial to illustrate the *in situ* presence and distribution of bacteria

deep inside the biofilm structure over the dentinal substrate and inside the dentinal tubules. This characteristic of the biofilm gives a 3-dimensional aspect to endodontic infection. Combination of FISH-confocal using species-specific 16S rRNA probes and SEM imaging technique provided a broad perspective of biofilm inside root canal space. This original protocol allows us to confirm localization, migration and evolution of different species into the root canal and dentinal tubules.

Concerning the efficacy tests of disinfection procedures- mechanical bacterial removal with only rotary endodontic instrumentation is not an efficient procedure. This step plays certainly an important role in disrupting the biofilm, but an additional procedure (final irrigation and agitation of disinfection irrigants) is needed to remove biofilm and obtain clean dentinal surfaces. As the gold standard, PUI demonstrated the best results in terms of bacterial eradication from root canal space and a clean dentinal surface (absence of debis). Regarding the SEM results, the kappa inter-rater test showed a moderate agreement between the raters (0.4-0.6) and the large standard deviation in different groups could be a consequence of the low number of samples. However, the apical region seems to be the most difficult zone to clean whatever the protocols. The capacity of Er:YAG and diode lasers delivered with an endodontic fiber to remove bacterial biofilm is comparable to the results obtained with PUI. The differences are not statistically significant.

The mode of action of Er:YAG laser is expressed through a photomechanical phenomenon when the laser beam is delivered in the pulp chamber or at the root canal orifice via the sapphire tip. The parameters used in this study seem not to do be sufficient to initiate this photomechanical action of Er:YAG through irrigants and subsequently to remove the biofilm from the root canal space. However, using the endodontic fiber to irradiate the root

canal space, with the same parameters, can produce cavitation effect inside NaOCl solution during laser-activated irrigation . The cavitation phenomenon promotes the efficient removal of the artificial biofilm from the root canal walls.

In the conditions of this study, the diode laser can enhance the effects of NaOCl irrigation solution to remove the artificial biofilm and improves the outcome of irrigation procedures in terms of debridement of the dentinal walls.

PDT is active to reduce bacterial load when the root canal space is mechanically instrumented when the biofilm structure is disturbed. LED light should be favored over the unidirectional beam of lasers to activate photosensitizers. LED diffuses inside the root canal space and provides more contact with the photosensitizing agent during irradiation period with minimum effort.

Finally it should be mentioned that PUI remains the most reliable technique to remove artificial biofilm. In addition to its simplicity, PUI is more affordable as compared to photonic procedures which need special equipment and training. Lasers have their place in endodontic decontamination. They improve the outcome of root canal irrigation procedures when they are used to activate the irrigation solutions. PDT could contribute to kill the disrupted biofilm bacteria and cannot be considered as a substitution to chemo-mechanical disinfection.

Traduction Française

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Abréviations

ARNr	Acide Ribonucléique ribosomale
ATCC	American Type Culture Collection
CHX	Chlorhexidine
E.	*Enterococcus*
EDTA	Acide Ethylenediaminetetraacetic
F.	*Fusobacterium*
FISH	Hybridation *in situ* en Fluorescence
LT	Longueur de Travail
MCBL	Microscope Confocal à Balayage Laser
MEB	Microscope Électronique à Balayage
MET	Microscope Électronique à Transmission
NaOCl	Hypochlorite de Sodium
P.	*Porphyromonas*
PDT	Thérapie Photodynamique
PS	Photosensibilisateur
PUI	Irrigation Ultrasonore Passive
S.	*Streptococcus*

1

Introduction

1.1. Qu’est-ce que c’est le biofilm?

Les biofilms peuvent être définis comme des populations sessiles de micro-organismes attachés à une surface et noyés dans une matrice extracellulaire autoproduite de polysaccharides et de protéines. La matrice représente 85 % du volume d'un biofilm (8). La majorité des microorganismes dans la nature se retrouvent dans les biofilms. La possibilité de s’attacher à et de rester sur une surface, constitue la stratégie fondamentale de survie pour des organismes procaryotiques. L'expression des gènes peut s’altérer sensiblement lorsque les cellules forment un biofilm. À l'intérieur de biofilms, les systèmes de communication intercellulaires sont utilisés par certaines bactéries pour échanger et synchroniser l'expression des gènes (2). Ainsi, de nombreux organismes ont un phénotype radicalement différent après fixation sur une surface et constitution du biofilm (10).

Les bactéries dans les communautés de biofilms renforcent leur capacité de défense contre les agents antimicrobiens, les stress environnementaux et les systèmes de défense de l'hôte. Le biofilm protège ses composants (les bactéries) des micro-organismes compétiteurs et augmente leur pathogénicité (10). Les maladies aigües causées par des bactéries pathogènes planctoniques ont été éliminées, car les agents infectieux de ces maladies ont été identifiés et peuvent être neutralisés

par des agents antimicrobiens. Les nouveaux pathogènes microbiens sont fréquents et abondants dans la nature ; ils vivent en communautés protégées au sein desquelles ils résistent aux antibiotiques et aux nombreux systèmes de défense de l'hôte. Ils peuvent provoquer des infections suraigües chez l'hôte surtout quand le système immunitaire de ce dernier est fragile (17). Le système immunitaire humain peut ne pas combattre les bactéries incorporées à l'intérieur du biofilm en raison de leurs antigènes cachés ; cela peut conduire à la suppression de l'expression des cellules phagocytaires. La matrice du biofilm peut agir comme un bouclier contre les agents physiques et la proximité des organismes au sein du biofilm peut permettre des interactions métaboliques et promouvoir le transfert des facteurs génétiques de virulence (18).

1.2. Le biofilm oral

Le biofilm formé sur les surfaces dentaires, gingivales et muqueuses est généralement appelé plaque dentaire. La cavité buccale se compose de différents tissus durs et mous; ces tissus pourraient servir de substrat pour la formation de biofilms. Toutefois, le taux élevé de nettoyage physiologique (salive, mastication, déglutition, mouvements divers) des tissus mous (sauf la face dorsale de la langue) perturbe l'accumulation importante de plaque dentaire (19). Chez l'adulte, la plaque dentaire mature se compose d'environ 500 espèces différentes de bactéries (20), enfermées dans une matrice d'origine bactérienne et salivaire. Les premières bactéries se fixent à la surface des dents au moyen de la pellicule acquise ; cette pellicule contient des molécules salivaires telles que les protéines riches en proline, les histatines, les stathérines qui adhèrent à la surface de la dent (21). La pellicule acquise se forme peu de temps après le nettoyage des dents et la colonisation bactérienne est détectable en quelques minutes (22).

Les *cocci* (principalement les *Streptocoques*) sont identifiées comme les premiers colonisateurs ou pionniers. La colonisation se réalise en deux étapes : la première étape est l'adhésion bactérienne à la pellicule au moyen des adhésines sur la surface cellulaire d'un tissu et les sites de liaison spécifiques sur la pellicule acquise. La deuxième étape est la croissance bactérienne par division et l'attachement à d'autres bactéries à travers le processus de co-agrégation (23).

Les caractéristiques de la communauté bactérienne commencent à changer. Par exemple, les bactéries Gram-négatives du genre de *Fusobacterium* agissent comme un pont entre les colonisateurs pionniers et les colonisateurs tardifs. Autrement dit, les *Streptocoques* en tant que les colonisateurs primaires ne peuvent pas s'associer avec les colonisateurs tardifs directement,

mais ne peuvent le faire que par l'intermédiaire de leur capacité d'agréger avec l'espèce *Fusobacterium*. Parmi les colonisateurs tardifs se trouvent les espèces *Propionibacterium*, *Prevotella*, *Veillonella* et l'espèce *Selenomonas flueggei* (Fig1.A) (24).

Les organismes aérobies comme *Neisseria* et *Nocardia* voient leur proportion s'inverser avec la progression du développement du biofilm. Cependant, les organismes anaérobies comme *Fusobacterium* et *Veillonella* croissent en nombre dès lors que le biofilm grandit. Malgré ces réorganisations, la croissance des organismes anaérobies est tributaire de la croissance préalable des organismes aérobies et aéro-anaérobies facultatifs. Ces derniers permettent l'augmentation de l'épaisseur de plaque et permettent de réunir les conditions nécessaires pour la croissance anaérobie (25).

La phase finale de la formation du biofilm est le processus de détachement. On ne connait pas exactement le rôle du « détachement » des bactéries (elles redeviennent planctoniques) au cours de la formation d'un biofilm. Mais il est raisonnablement possible de penser que ce détachement contribue à la colonisation des nouveaux sites (Fig1.B) (26).

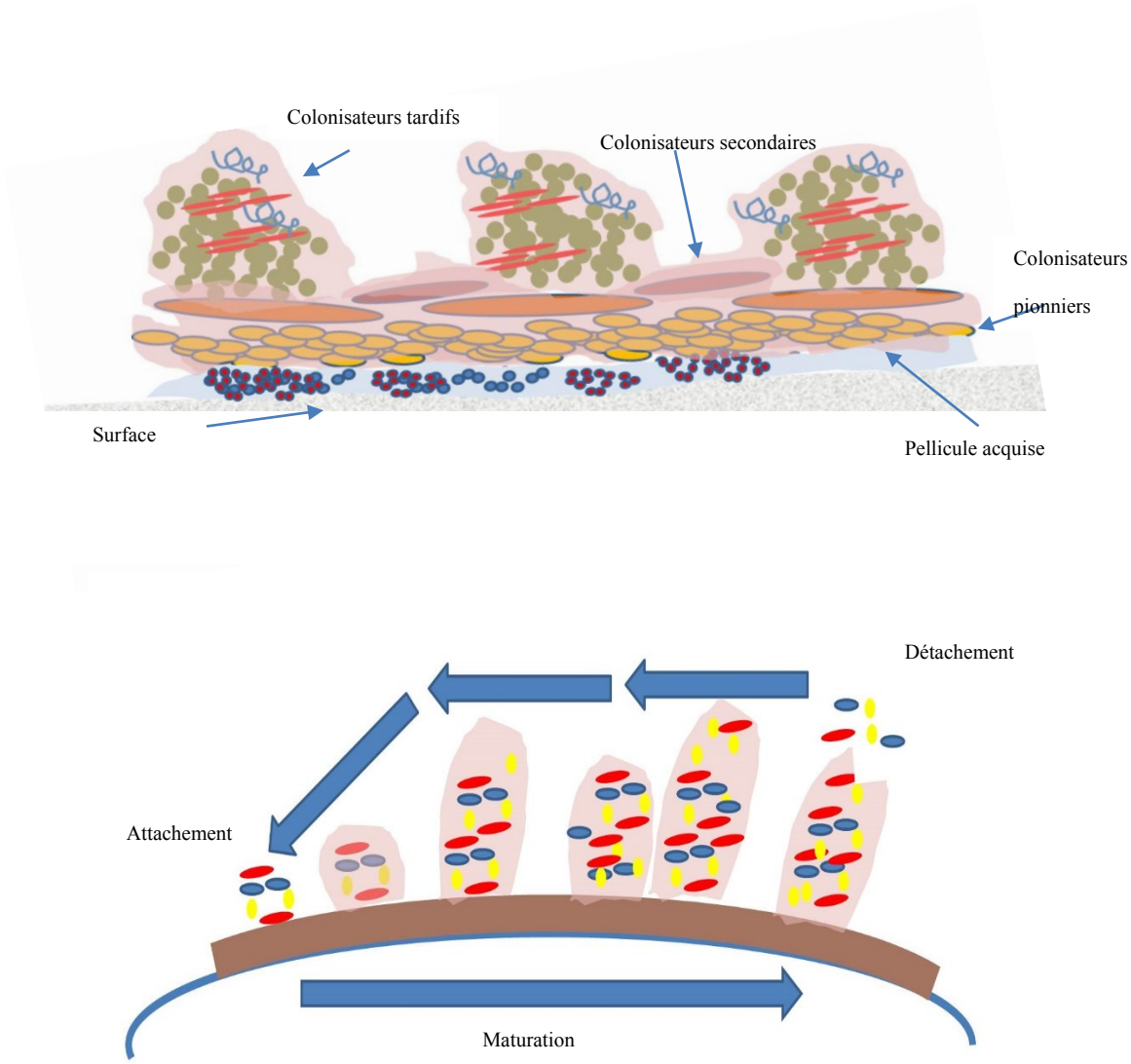

Figure 1. A- La structure du biofilm composée par des colonisateurs pionniers, secondaire and tardifs. B- Le cycle de la formation du biofilm : 1. Adhésion, 2. Colonisation and 3. Détachement

1.3. Réseau canalaire

La complexité de l'endodonte, formé de la chambre pulpaire et du réseau canalaire ne facilite pas la tâche du praticien. Macroscopiquement, le réseau canalaire est formé d'un canal principal et de canaux latéraux et accessoires. Au plan microscopique, la dentine radiculaire en constitue ses parois minéralisées. La dentine est parcourue de tubuli dentinaires. La dentine, composée principalement d'hydroxyapatite (en particulier localisée dans la dentine intertubulaire) et de collagène de type I et d'eau (70%). D'autres types de collagène coexistent (types III, V, VI) ainsi que des protéines non collagéniques et des protéoglycanes sont présents aussi sous forme d'éléments mineurs (27). Les tubuli dentinaires contiennent les prolongements cytoplasmiques (fibres de Tomes) des odontoblastes située sur la couche périphérique de la pulpe. Ces tubuli s'étendent de la pulpe à la jonction amélo-dentinaire au niveau coronaire et à la jonction amélo-cémentaire au niveau radiculaire (28) avec un parcours sinueux, et des anastomoses entre eux.

D'autre part, plus on s'éloigne de la pulpe, plus le diamètre des tubuli diminue (0.9µm contre 2.5µm proche de la pulpe), comme diminue leur nombre depuis la chambre pulpaire vers la zone apicale (55000 tubule/mm^2 contre 15000 tubule/mm^2). Il n'en reste pas moins vrai que le diamètre des tubuli, que l'on soit proche de la pulpe ou non, que l'on soit à l'apex ou au niveau coronaire permet largement la pénétration bactérienne. (27).

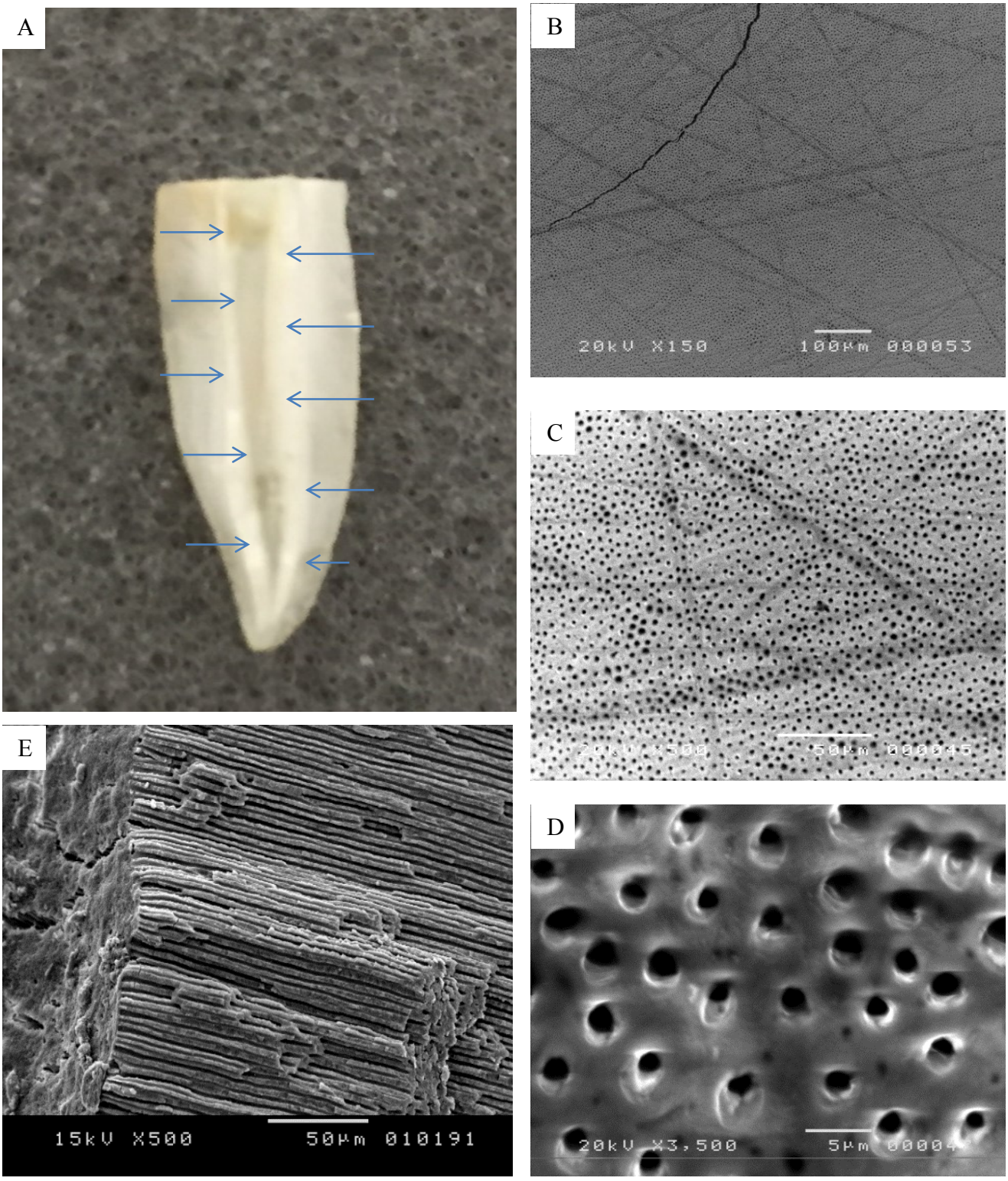

Figure 3. Anatomie macro- et microscopique du canal radiculaire, A- Espace canalaire, B,C,D,- La surface dentinaire avec différent grandissements, les tubuli dentinaires sont visible , E- Vue latérale des tubuli dentinaires

1.4. Infection endocanalaire

Les bactéries à l'état planctonique ou organisées en biofilm constituent la principale cause d'infection endodontique conduisant à la nécrose pulpaire et ses complications périapicales. Le succès du traitement radiculaire est lié à l'élimination complète des bactéries et du biofilm de l'espace canalaire (29, 30). Miller (31) pour la première fois a démontré la capacité des microcoques et des filaments à envahir les tubuli dentinaires. Kakehashi et al. (32) ont illustré le rôle pathogène des micro-organismes dans la pulpite et les maladies périapicales en exposant chirurgicalement la pulpe des dents de rats axéniques. La contamination de cet espace stérile est observée après déminéralisation de l'émail, de la dentine et du cément le plus souvent suite à une carie. Cette déminéralisation ouvre un passage pour les bactéries vers le tissu pulpaire (Fig. 4).

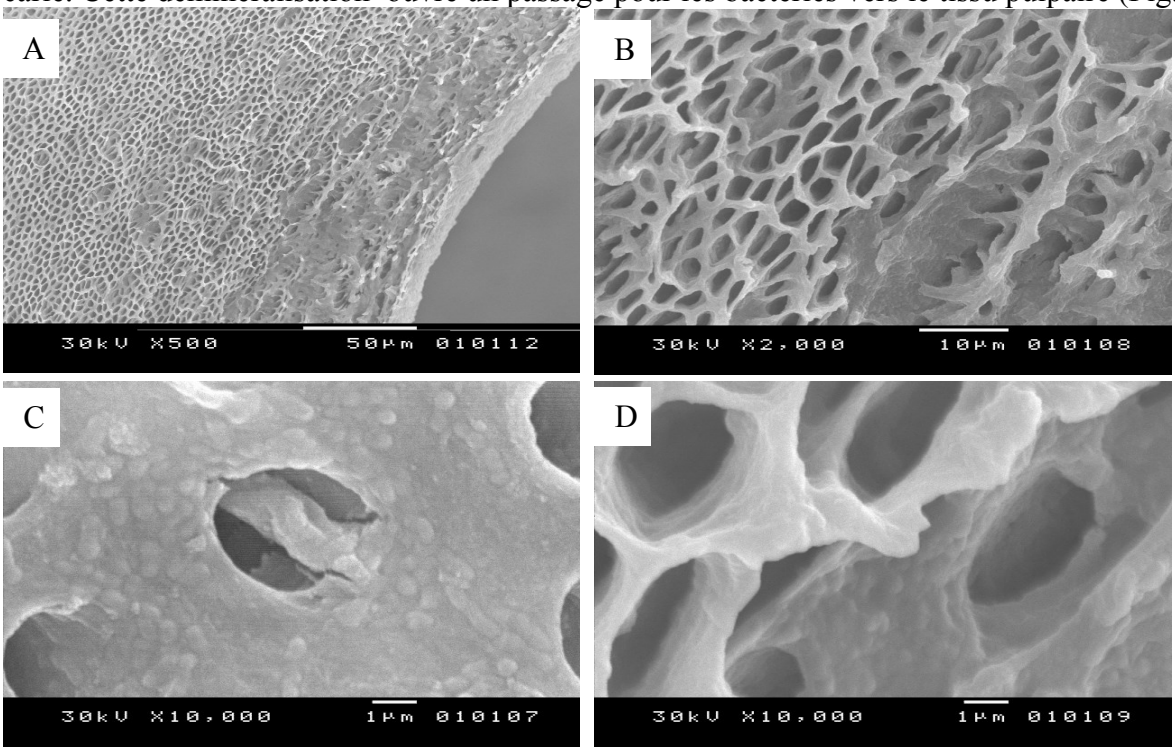

Figure 4. structure de la dentine canalaire, A,B- La dentine artificiellement infectée en différent grandissements, C,D- Plus grands grandissements du même échantillon, Les modifications de la taille et de la forme des ouvertures des tubuli dentinaire avec des bactéries attachée à la surface dentinaire peuvent être notés

Cependant, une inflammation du tissu pulpaire (pulpite) pourrait être observée à la suite d'un traumatisme (33), de malformations congénitales des dents (34), d'une restauration dentaire défectueuse (35) et éventuellement de l'anachorèse (33, 37). Suite à l'intrusion bactérienne dans l'espace endodontique, les tubuli dentinaires seront très vites colonisés par des microorganismes (Fig.6), mais le degré d'invasion bactérienne varie en fonction de la perméabilité des tubules dentinaires dans différentes régions de l'espace endodontique (38). L'attaque persistante bactérienne et le processus inflammatoire continu de la pulpe mènent à la nécrose pulpaire et plus tard aux pathologies périapicales avec pour conséquence principale la perte des dents en l'absence de traitement.

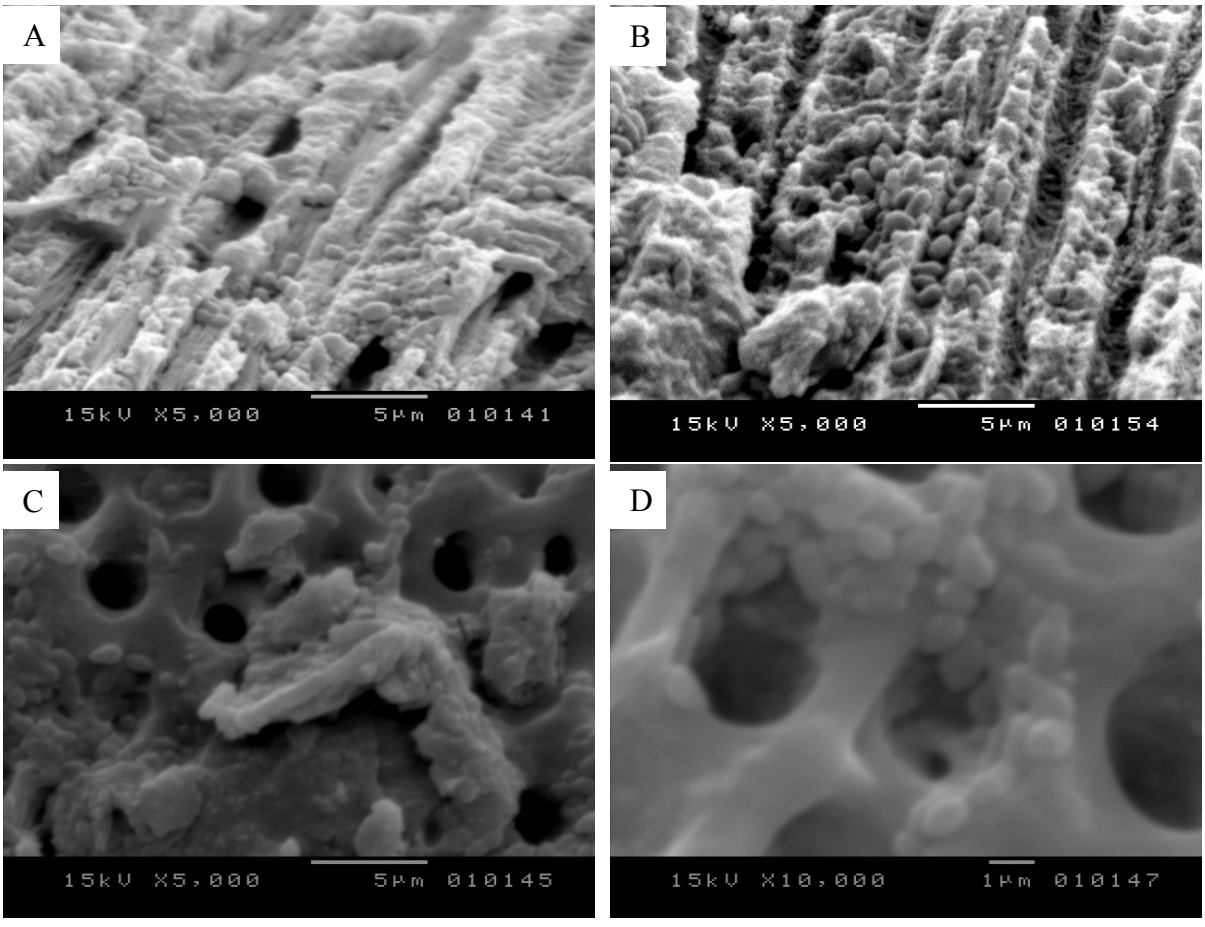

Figure 5. Les images MEB de l'invasion bactérienne des tubuli dentinaires, A,B- Des bactéries peuvent être récupérées seule ou en paquets, C,D- Bactéries peuvent pénétrer dans des tubuli dentinaires grâce à leur petite taille

1.5. La composition du biofilm endodontique

Les bactéries associées aux infections endodontiques causées par des lésions carieuses sont différentes de celles initiées lors des pathologies non carieuses. Les *Streptocoques* et les bactéries du genre *Actinomyces* sont des composants indispensables de la plaque dentaire et des lésions carieuses, mais les microorganismes anaérobies stricts sont les colonisateurs principaux de l'espace pulpaire au cours de l'infection canalaire (Table.1) (38). Les bactéries du biofilm endocanalaire se retrouvent de la chambre pulpaire à l'apex de la dent. Ainsi, il existe des gradients de distribution différents des bactéries à l'intérieur du réseau canalaire. La présence d'oxygène au niveau de la chambre pulpaire se traduit par une zone aérobie en partie coronaire et en direction de l'apex, la quantité d'oxygène diminuant, une zone anaérobie avec un gradient entre les 2 pôles. De la même manière, selon les nutriments (exemple, régime alimentaire de l'hôte et des micros fuites près de la couronne) (19). En outre, comme dans chaque microenvironnement naturel, les capacités d'adaptation des microorganismes augment exponentiellement lorsqu'ils sont organisés en biofilm.

	Aerobic species	Facultative species	Anaerobic species
Gram-positive cocci		Enterococcus faecalis	Peptostreptococcus micros
		Enterococcus faecium	Peptostreptococcus prevotii
		Staphylococcus warneri	Peptostreptococcus magnus
		Staphylococcus lentus	Peptostreptococcus
		Streptococcus anginosus	asaccharolyticus
		Streptococcus constellatus	
		Streptococcus intermedius	
		Streptococcus gordonii	
		Streptococcus mitis	
		Streptococcus mutans	
		Streptococcus oralis	
		Streptococcus salivarius	
		Streptococcus sanguis	
Gram-positive rods		Corynebacterium xerosis	Actinomyces naeslundii
		Lactobacillus acidophilus	Actinomyces israelii
		Lactobacillus catenaforme	Actinomyces meyeri
		Lactobacillus fermentum	Actinomyces odontolyticus
		Lactobacillus salivarius	Actinomyces viscosus
			Atopobium minutum
			Cryptobacterium curtum
			Eubacterium brachy
			Eubacterium lentum
			Eubacterium nodatum
			Mogibacterium timidum
			Propionibacterium acnes
			Propionibacterium
			granulosum
			Propionibacterium propionicus
			Pseudoramibacter alactolyticus
			Slakia exigua
Gram-negative cocci		Neisseria spp.	Veillonella parvula
Gram-negative rods	Pseudomonas aeruginosa	Campylobacter curvus	Dialister pneumosinites
		Campylobacter rectus	Eikenella corrodens
		Campylobacter sputorum	Fusobacterium nucleatum
		Capnocytophaga ochracea	Fusobacterium necrophorum
			Porphyromonas gingivalis
			Porphyromonas endodontalis
			Prevotella oralis
			Prevotella oris
			Prevotella buccae
			P. intermedia
			Prevotella denticola
			Prevotella dentalis
			Prevotella melaninogenica
			Prevotella loescheii
			Selenomonas sputigena

Table1. Diversité des bactéries isolée de l'infection endodontique

Medical Biofilms: Detection, Prevention and Control. Edited by Jana Jass, Susanne Surman and James Walker, 2003 John Wiley & Sons,

Cette approche “écologique” de l’infection canalaire est fondée sur le concept selon lequel le plus dangereux des pathogènes n'est pas représenté par une seule espèce, mais une entité poly-microbienne, qui subit des changements physiologiques et génétiques différents initiés par des changements environnementaux au sein du réseau canalaire. Cependant, des infections monobactériennes peuvent être observées (par exemple les espèces de genre d'*Enterococcus*), en particulier dans les régions apicales et périapicales (6, 7).

La première colonisation au niveau de la surface dentinaire se fait par les *Streptocoques*, car ces bactéries aéro-anaérobie facultatives consomment de l'oxygène et modifient les conditions environnementales en faveur des espèces anaérobies. Les bactéries dans l’infection du réseau canalaire et au niveau dentinaire pourraient pénétrer jusqu’à 500µm à l’intérieur des tubuli dentinaires (3).

1.6. Méthodes de caractérisation des biofilms oraux

Plusieurs méthodes de caractérisation des biofilms oraux ont été mises au point et étudiées dans la littérature. On peut les diviser en méthodes de cultures, méthodes d'identification directe ou méthodes d'observation.

1.6.1. Culture bactérienne

La reproduction *in vitro* d'un biofilm endocanalaire avec les bactéries venue de biofilms endodontiques est difficile. Car de nombreux milieux de culture doivent être utilisés pour permettre l'isolement et la croissance sélective des bactéries prélevées depuis l'espace canalaire. En fait la récupération de toutes les bactéries semble impossible en raison des difficultés à fournir des conditions de croissance appropriées pour les différentes espèces.

1.6.2. Méthodes d'identification

1.6.2.1.PCR

La PCR fréquemment utilisée en biologie moléculaire permet, quant à elle, d'amplifier un petit segment de l'ADN bactérienne produire suffisamment de matériel génétique pour diverses analyses telles que l'identification des microorganismes. Siqueira et coll. (43, 46, 47) a démontré que la technologie PCR facilite la détection des espèces bactériennes qui sont difficiles ou même impossibles à isoler et cultiver. La PCR est rapide, sensible et plus précise que les méthodes de culture traditionnelles. En endodontie, la PCR a contribué à augmenter notre connaissance sur les communautés bactériennes présentes lors d'une infection endodontique.

1.6.3. Méthodes d'observation : la Microscopie

La microscopie optique est appréciée pour différencier les espèces Gram positif des Gram négatif après coloration histologique, et permet la différenciation morphologique des bactéries présentes dans le biofilm (filaments, cocci, bacilles,... etc.) mais les limites sont vite atteintes à cause des faibles grandissements. La microscopie optique au fond noir permet d'autre part d'observer la densité des bactéries, leur morphologie ou surtout la présence de bactéries mobiles (*Spirilles, Spirochètes*) (51).

Le microscope électronique à balayage (MEB) est un équipement qui permet d'observer l'état de surface de biofilm et son substrat, l'arrangement des bactéries entre elles, la présence de divisions bactériennes et de la prédominance ou non de certains morphotypes. (52).

Le MEB ne permet pas d'identifier les bactéries directement et offre un champ d'observation limité car seules les surfaces peuvent être observées (53).

Le Microscope électronique à Transmission (MET) permet d'analyser les structures internes et périphériques des bactéries et permet, entre autre, de distinguer les constituants internes de la cellule, comme les Lipopolysaccharides (LPS) qui se retrouvent à l'extérieur du peptidoglycane et donc de distinguer les bactéries Gram positif des bactéries Gram négatif.

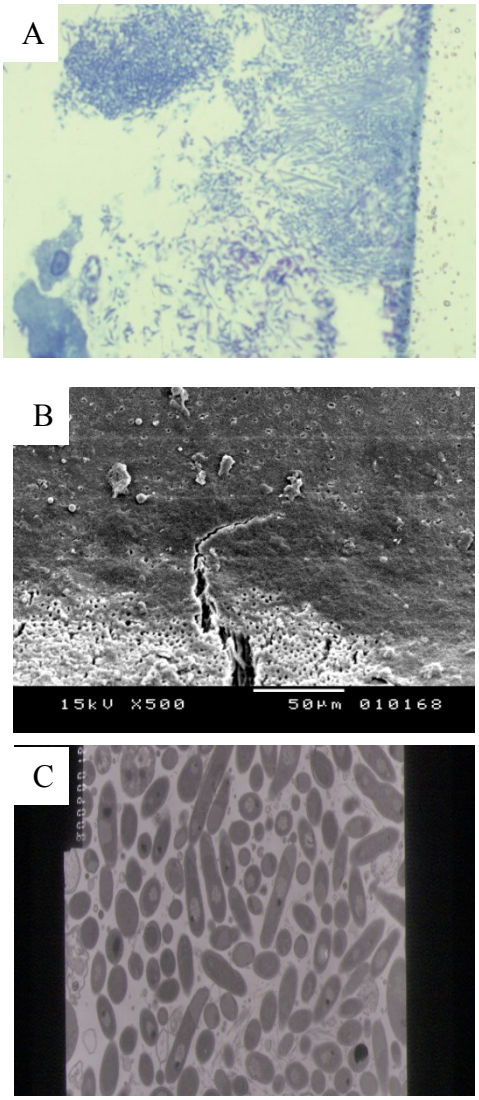

Figure 6. A- Coupe transversale de la plaque dentaire (Prof. JP ROCCA), montrant la présence des différentes forme des bactéries, B- Vue au MEB de la surface dentinaire couverte par le biofilm, C- Image MET de la plaque dentaire, la morphologie de diverses bactéries dans des couches différentes est identifiable

1.6.4. Hybridation *in situ* en fluorescence et microscopie confocale

Afin de suppléer aux limites de la microscopie optique et électronique, l'hybridation *in situ* en fluorescence en combinaison avec la microscopie confocale représente un intérêt réel. Cette technique permet la visualisation des bactéries associées et liées à la matrice extracellulaire même dans des biofilms denses. La FISH combine génétique moléculaire et informations visuelles de la microscopie optique. Le microorganisme peut être repéré et étudié dans son habitat ou dans son tissu hôte. L'hybridation *in situ* a été introduite par Pardue et coll. (60) et John et coll. (61) qui ont introduit des ARN radioactifs dans la cellule afin de l'hybrider avec l'ADN nucléaire. Les hybrides formés ont alors été visualisés par autoradiographie.

Aujourd'hui, on utilise plutôt un marquage fluorescent direct des oligonucléotides. C'est ce qui est généralement utilisé lors de l'hybridation *in situ*. Des marqueurs fluorescents (Fluorochromes) se fixent à l'extrémité 5' de la sonde oligonucléotide.

Les fluorochromes ont différents niveaux d'excitation et d'émission; cela permet l'identification de deux ou plusieurs micro-organismes en même temps. La principale cible moléculaire en microbiologie est l'ARNr 16S grâce à sa stabilité génétique et son nombre élevé de copies (Fig.6) (62). Les séquences de l'ARNr 16S sont identifiées pour la plupart des bactéries cultivables et de nombreuses espèces microbiennes non cultivables. Elles ont été recueillies dans des bases de données et sont publiquement disponibles (63, 64). Il faut noter que les séquences des sondes conçues pour la majorité des ARNr 16S sont stockées dans différents programmes en ligne comme ARB (65) et probeBase (66).

Le protocole FISH comprend typiquement quatre étapes : la fixation et la permeabilisation de l'échantillon, l'hybridation, l'élimination des sondes non attachées par rinçage et la détection des cellules marquées par microscopie en épi fluorescence ou confocale(67).

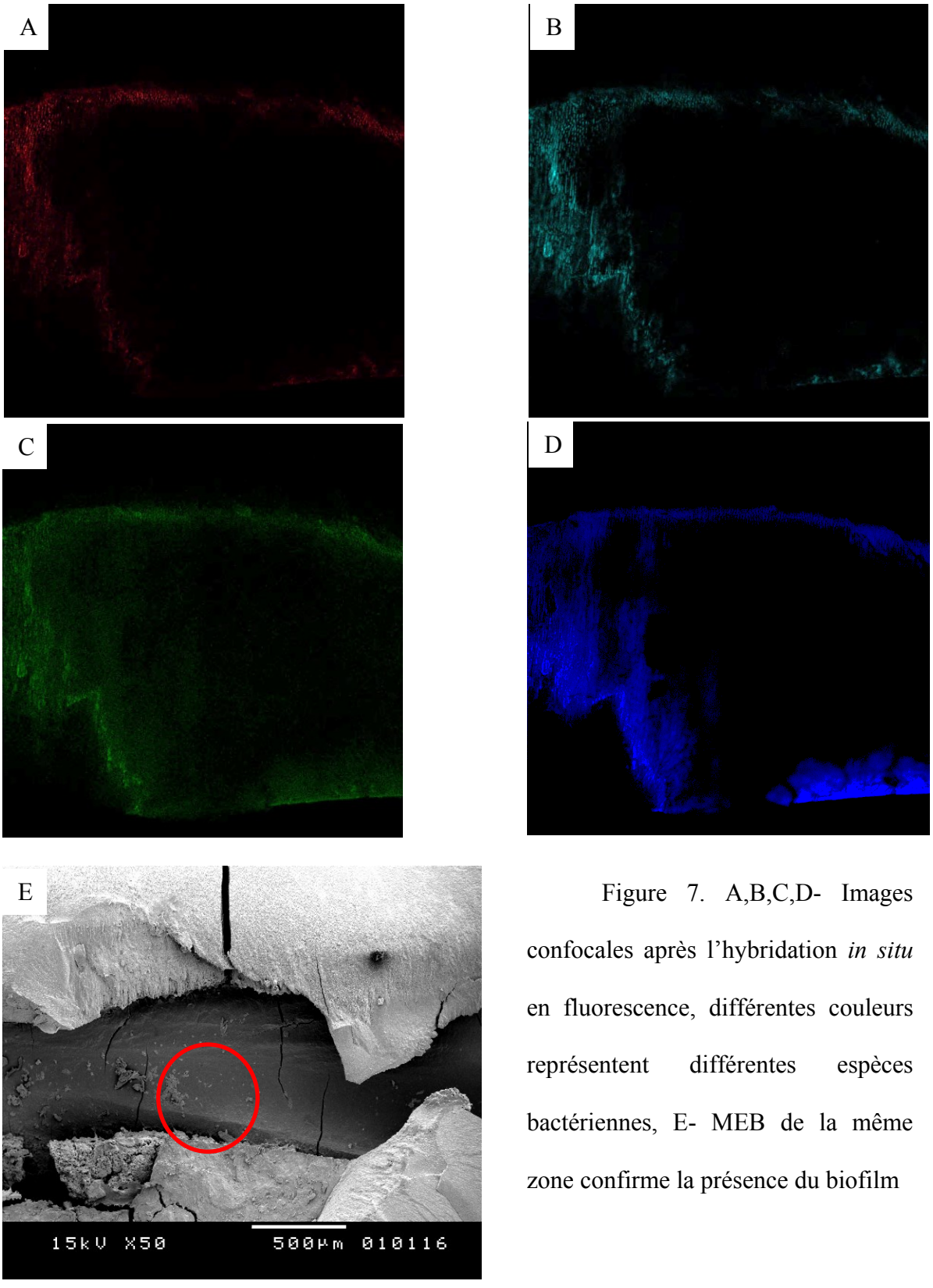

Figure 7. A,B,C,D- Images confocales après l'hybridation *in situ* en fluorescence, différentes couleurs représentent différentes espèces bactériennes, E- MEB de la même zone confirme la présence du biofilm

1.7. Biofilm artificiel

Pour concevoir un protocole efficace de décontamination de l'espace endodontique, il est important de concevoir un modèle de biofilm microbien qui ressemble le plus possible à celui que l'on pourrait trouver dans un canal radiculaire infecté. L'éradication du biofilm bactérien est un défi important lors du traitement endodontique des dents infectées. L'efficacité d'un agent chimique, physique ou mécanique antibactérien seul ou combiné dans un protocole dit de « désinfection canalaire » peut se mesurer par l'élimination efficace du biofilm intra canalaire. Un biofilm artificiel reproductible proche d'un type sauvage de biofilm dans ses principales caractéristiques structurales semble indispensable. La conception d'un modèle in vitro dépend de plusieurs facteurs importants. La diversité des espèces microbiennes composant ce biofilm, l'âge et la qualité des nutriments sont certains de ces facteurs.

L'analyse microscopique a mis en évidence une variation distincte de l'ultrastructure des biofilms formés dans des conditions expérimentales différentes. Il a été par exemple montré que la pénétration des bactéries dans les tubuli dentinaires est en relation directe avec la qualité des nutriments (68). On sait aussi que l'âge et l'état nutritionnel des biofilms peuvent interférer avec les effets d'agents antimicrobiens (69). Dans la littérature, des biofilms monobactériens ont été longtemps utilisés comme modèle d'évaluation des étapes de désinfection endodontique. Les biofilms utilisés dans ces essais sont faciles à construire (70, 71) et relativement faciles à éliminer. Cependant, il est encore plus facile d'éliminer les bactéries planctoniques par divers agents de désinfection : cela ne peut pas refléter pas l'action antibactérienne de ces mêmes agents dans des biofilms mono – bactériens, encore moins dans des biofilms poly-bactériens. Il a été montré que les bactéries au sein de biofilms peuvent être 100 à 1000 fois plus résistantes aux agents antibactériens que leurs homologues planctoniques (72, 73). En raison de cette grande différence,

un nombre croissant d'études se portent désormais sur l'évaluation des agents antibactériens par la destruction du biofilm au lieu de bactéries planctoniques (74). Dans un biofilm endodontique on rencontre le plus souvent différentes formes de microorganismes : coques, bacilles, filaments et plus rarement des spirochètes (74). La microanalyse dispersive en énergie (EDS) a montré une augmentation significative du taux de Calcium (Ca2 +) du biofilm formé dans des conditions anaérobies, lorsque les bactéries sont privées de nutriment. Au contraire, la profondeur de pénétration bactérienne était significativement augmentée en présence de nutriments (68).

1.8. Impact de la présence ou l'absence du biofilm sur le résultat de traitement endodontique

L'élimination des microorganismes dans l'espace radiculaire infecté est l'un des objectifs principaux du traitement endodontique (51, 52). L'infection microbienne joue un rôle important dans l'évolution de la nécrose pulpaire et la formation éventuelle de lésions périapicales (52). Bien que le la mise en forme canalaire instrumentale combinée à l'irrigation à l'aide d'un agent chimique antibactérien semble supprimer la majeure partie des microorganismes dans un réseau canalaire infecté, l'infection du réseau canalaire étant tri dimensionnelle, des bactéries résiduelles sont toujours détectables avant de réaliser l'obturation canalaire (4, 81, 82).

Certains problèmes opératoires comme une instrumentation insuffisante, un canal non traité (cas des pluri radiculées), une irrigation insuffisante, la virulence de certaines espèces bactériennes, ou une obturation canalaire et coronaires non étanches pourraient conduire à un échec post opératoire (4). Un autre obstacle est représenté par la variation anatomique de la ou des canaux, qui rend le débridement chimio mécanique inefficace dans l'éradication des bactéries (83).

Cela comprend par exemple les canaux latéraux ou pulpo-parodontaux qui ne peuvent être désinfectés en raison de leur localisation ou de leurs petits diamètres (84).

Le taux de réussite des traitements endodontiques est plus élevé lorsque le canal est stérile (cultures négatives) avant l'obturation (85). Les succès du traitement peuvent atteindre 94% de succès en l'absence de bactéries pour diminuer à environ 68% en présence de la bactérie (86).

1.9. Différentes méthodologies de désinfection endodontique

1.9.1. Irrigation ultrasonore passive (PUI)

La littérature a démontré que l'irrigation canalaire associée à l'instrumentation de mise en forme permettait d'obtenir un espace radiculaire "plus propre" que celui obtenu seulement avec une instrumentation mécanique (87, 88). Une irrigation idéale devrait avoir les caractéristiques suivantes :

- Un effet antibactérien sur tous les types de bactéries de type planctonique ou organisées en biofilms
- être organolytique;
- Inactiver les endotoxines;
- éliminer les boues dentinaires produites lors de l'instrumentation
- Être biocompatible (66);

Il n'existe pas de solution d'irrigation unique qui possède toutes ces qualités. Aujourd'hui, la plupart des auteurs préconisent l'association du NaOCl et de l'EDTA) pour répondre à ce cahier des charges. Ce protocole a été établi afin de palier le défaut de chaque solution d'irrigation prise séparément (90, 91). L'utilisation d'unités ultrasonores permettant d'agiter ces solutions a été par ailleurs proposée pour améliorer désinfection du réseau canalaire (93, 94). Deux types d'activation

par ultrasons ont été proposés. Le premier concerne l'instrumentation ultrasonique et l'irrigation simultanément. Le deuxième est l'activation passive. L'agitation ultrasonore étant faite une fois que l'instrumentation est terminée (88). Seul le deuxième type est quant à lui utilisé car la lime ultrasonore est susceptible de recréer de la smear layer des lors qu'elle est en contact avec les parois canalaire. La diffusion acoustique est le phénomène qui se produit lorsque la lime ultrasonique est activée dans le canal rempli d'irrigant. Cela améliore la capacité des irrigants à dissoudre les tissus résiduels ou la smear layer (93).

Techniques photoniques

Depuis le premier laser développé par Maiman en 1960 (100), cet outil a été utilisé dans divers domaines de la dentisterie (101-108). Parmi les premières applications endodontiques du laser, la fermeture de l'apex a été tentée à l'aide d'un laser CO_2 avec une puissance élevée (109). Les risques de carbonisation des surfaces a conduit à son abandon en endodontie.

D'autres tentatives ont été réalisées avec un laser Nd: YAG sur les traitements de chirurgie apicale et d'obturation a retro : (110) abandonné pour des risques thermiques au niveau du tissu osseux. C'est l'effet antibactérien des lasers qui a ensuite attiré l'attention des cliniciens et chercheurs pour tenter de trouver une méthode et des longueurs d'ondes adaptées à la situation (décontamination endodontique) (111-118).

Les lasers hauts puissances semblent présenter des avantages et désavantages dans le domaine de la désinfection canalaire.

L'effet bactéricide des différents lasers a été décrit dans littérature (119-120). Ces lasers de haut niveau d'énergie ont des inconvénients parfois dangereux. Ils peuvent engendrer une augmentation de la température tel durant l'irradiation qu'il peut excéder le niveau physiologique

acceptable d'élévation thermique (plus de 5,6 ° C). Ce changement de température peut provoquer la mort des cellules périapicales (95, 96). Ces lasers infrarouges peuvent créer des zones de carbonisation, fondre la dentine, créer des fissurations et former des boues dentinaires sur les parois canalaires (120-122). À ces complications s'ajoutent aussi des propriétés d'usage et de réglages complexes et un coût élevé (102).

Grâce à l'évolution constante de la technologie laser et des expériences scientifiques, il a pu être établi que des Lasers à énergies plus basses pourraient atteindre les objectifs visés.

Le laser peut intervenir dans les protocoles de décontamination endodontique comme un assistant à l'irrigation canalaire ou comme un activateur des solutions d'irrigation. Les lasers Erbium par la notion de vaporisation explosive et les lasers diode avec leurs fréquences de 100Hz jusqu'à 20000-50000Hz pourraient produire un effet de cavitation à l'intérieur d'un canal rempli de solution d'irrigation. Quelques études ont montré des parois canalaire propre même dans la région apicale. Dans cet usage, le Laser n'a pas besoin d'énergie élevée et les effets secondaires indésirables thermiques et physiques sur la dentine ou autres tissus environnants sont très limités.

Il est connu que des solutions d'irrigation canalaire pourraient absorber la lumière de différentes longueurs d'onde de 513 nm pour la chlorhexidine à 2200 nm pour l'acide citrique. Ces solutions d'irrigation ont aussi un taux d'absorption élevé pour des longueurs d'onde supérieures à 2500 nm. Ces caractéristiques optiques permettent l'usage de tous les types de solutions d'irrigation pour (LAI) (123).

Thérapie photodynamique

La thérapie photodynamique (PDT) est un traitement médical qui utilise la lumière pour activer un agent appelé l'agent photosensible en présence d'oxygène. Il y a beaucoup d'agents

photosensibles et chimiques comme le bleu de Toluidine (148, 149), le bleu de méthylène (150, 151) et des photosensibilisateurs naturels comme la curcumine (152). L'effet de la PDT dans différents protocoles avec les différentes lumières d'activation a été testé. Toutefois, il semble que le fait de changer la source lumineuse n'améliore pas nécessairement les résultats obtenus (109).Le premier, Wilson, a observé les effets bactéricides de la PDT dans les maladies bucco-dentaires (154, 155). Par la suite, le rôle potentiel de la PDT dans le traitement de l'infection canalaire a fait l'objet de nombreuses publications (157-160). Pour s'en convaincre il suffit d'observer le nombre croissant de publications sur ce sujet.

Le mécanisme de la thérapie photodynamique est très différent de l'interaction physique de LAI qui repose sur la production d'ondes dans les irrigants ou de la décontamination par lasers proche de l'Infra-rouge, qui fonctionne par élévation thermique. La PDT est une réaction chimique pure et il n'est pas possible d'éliminer totalement le biofilm microbien de l'espace canalaire, lorsque utilisée seule. Il s'agit en fait d'un complément et non d'un substitut au débridement chimio-mécanique conventionnel. Mais l'intérêt repose sur le fait qu'il n'y a ni résistance ni sélectivité : la PDT pourrait donc servir à éliminer les bactéries de l'espace endodontique sans risques collatéraux.

2

Matériaux et Méthodes

Trente-trois dents mono-radiculées ont été préparées jusqu'à la lime K ISO 40 puis stérilisées et conservées à 4°C. Les échantillons ont été incubés pendant 7 jours avec un mélange de Porphyromonas gingivalis (ATCC 33277), Streptococcus salivarius (ATCC 7073), une souche sauvage de Enterococcus faecalis et une souche sauvage de Prevotella intermedia, qui ont été fournis par le laboratoire de bactériologie de l'Hôpital Archet 2 CHU Nice – France.

Parmi les dents du groupe contrôle, deux échantillons ont été coupés longitudinalement en 2 parties et ces dernières ont été recouvertes d'une fine couche d'or à l'aide de la machine de pulvérisation cathodique (JOEL JFC-11F LTD, Japon). Cette étape nous a permis de visualiser les biofilms artificiels formés sur les parois dentinaires au Microscope électronique à balayage (JSM-5310LW total vide, JEOL LTD, Japon) en vide totale. (Fig. 2).

Ensuite, les autres dents ont été divisées en trois groupes de test.

Le groupe 1 a été traité avec la PDT. La source lumineuse utilisée est une LED rouge (635nm) et un dérivé commercial de bleu de Toluidine a été utilisé comme photosensibilisant (Aseptim™, Leutkirch im Allögo, Allemagne). Le photosensibilisant a été injecté dans le canal à

l'aide d'une aiguille G26. La procédure s'est poursuivie en agitant le produit chimique à l'intérieur du canal pendant une minute. Puis la solution a été activée pendant 120 secondes par la lumière LED.

Le groupe 2 a été traité avec un protocole de PDT composé d'un laser diode de longueur d'onde 650 nm et de bleu de Toluidine concentré à 15 µg/ml comme agent photosensible. Le photosensibilisant a été injecté dans le canal à l'aide d'une aiguille G26. La procédure s'est poursuivie en agitant le produit chimique à l'intérieur du canal pendant une minute. La solution a été ensuite activée pendant 120 secondes par le laser diode à l'aide d'une fibre optique.

Le groupe 3 a été traité selon un protocole d'irrigation ultrasonore. Le canal a été traité pendant 1 minute avec 2,6 % NaOCl et pendant 1 minute avec de l'EDTA à 17 %. Pour agiter les solutions d'irrigation une lime ultrasonique endodontique a été utilisé (IRRISAFE® ACTEON, Mérignac, France).

Échantillonnage : Une fois les procédures de traitement cliniques accomplies, l'échantillonnage microbiologique des canaux radiculaires a été faite avec une lime K ISO 10. Les échantillons ont été cultivés sur une gélose de sang de mouton à 5 %. La technique de culture a été inspirée des publications scientifiques de Bonsor et coll. (119, 120).

Dix dents extraites ont été coupées en tranches de 2 mm à l'aide d'une scie de précision. Les canaux radiculaires ont été préparés comme lors de la Phase 1.

Design du Biofilm artificiel : Le biofilm est composé d'une combinaison de Streptococcus salivarius ATCC 13419, Enterococcus faecalis ATCC 29212, Fusobacterium nucleatum ATCC 25586 et Porphyromonas gingivalis ATCC 33277. Pour les disques, le biofilm a été formé sur la surface dentinaire dans des plaques de culture à 6 puits en ajoutant 2,5 ml de suspension cellulaire

standardisé. Au cours de la période d'incubation, 30 % de milieu de culture a été remplacé deux fois par semaine afin d'approvisionner les nutriments nécessaires pour la croissance des bactéries. Un suivi périodique de l'état de croissance de biofilm (à l'aide de microscope électronique à balayage et la culture bactérienne) a été effectué après 3, 7, 10, 13, 17, 20, 23,27 et 31 jours d'incubation.

Le biofilm a été développé à l'intérieur de l'espace canalaire des échantillons en injectant la suspension cellulaire à l'aide d'une seringue. Puis, ces échantillons ont été mis dans un récipient en plastique stérile avec 24 ml du mélange bactérien. Le conteneur a été incubé en anaérobiose à 37°C sur un agitateur orbital (150 tr/min). Après 24 heures, 8 ml de bouillon Schaedler a été ajouté au conteneur. La période d'incubation a duré 21 jours. De la même manière qu'au niveau des disques dentinaires, 30 % du milieu de culture a été remplacé avec des produits frais, deux fois par semaine, afin de fournir les nutriments nécessaires pour la croissance des bactéries.

Une fois que les procédures d'incubation ont été réalisées, l'échantillonnage microbiologique des disques dentinaires a été fait. Une lime K ISO 10 a été utilisée pour parer la surface dentinaire afin de recueillir des bactéries viables. L'échantillon a été alors cultivé sur de la gélose de sang de mouton à 5 %.

Microscopie :

FISH : Des sondes oligonucléotides ADN ont été conçus avec différents colorants fluorescents à l'extrémité 5', comme décrit dans le tableau. 4 (Biomers.net®, Ulm, Allemagne). Pour tester l'efficacité des sondes, une première hybridation a été effectuée sur une culture pure de chaque espèce bactérienne. Le tampon d'hybridation a été formulé pour une utilisation sur des cultures bactériennes pures. Le tampon a été préparé en mélangeant 0,01 % (p/v) de dodécylsulfate de Sodium (SDS), 0,9 M de NaCl, 20 mM de Tris/HCl. Le tampon d'hybridation a été ajusté en ajoutant 20 % (v/v) de Formamide. Enfin, la concentration des sondes ajoutées au tampon d'hybridation a été calculée pour obtenir une concentration finale de 30 ng/µl. L'hybridation a été effectuée pendant 90 minutes à 46° C.

Une fois l'hybridation accomplie, l'étape de rinçage a été réalisée. Un tampon de rinçage composé de 0,01 % (p/v) de dodécylsulfate de Sodium (SDS), 88 mM de NaCl, 20 mM de Tris/HCl a été préparé. Les échantillons ont été rincés deux fois par le tampon de rinçage chaque fois pendant 10 min à 46° C. Un rinçage final avec de l'eau a été réalisé pour enlever toutes les sondes non attachées.

En ce qui concerne le biofilm formé dans l'espace canalaire, le protocole d'hybridation a été adaptée des publications de Böckelmann et coll. (131) et de Schaudinn et coll. (38). Le tampon d'hybridation a été ajusté avec les proportions suivantes : 0,01 % (p/v) de dodécylsulfate de Sodium (SDS), 0,9 M de NaCl, 20 mM de Tris/HCl. Puis un tampon d'hybridation a été dilué par addition de 35 % (v/v) de Formamide. Des sondes ont été ajoutées au tampon d'hybridation pour obtenir une concentration finale de 5 ng/µl. Après le processus d'hybridation, s'est déroulée l'étape de rinçage. Le tampon de rinçage est composé de 0,01 % (p/v) de dodécylsulfate de Sodium (SDS),

88 mM de NaCl et 20 mM de Tris/HCl. Les échantillons ont été rincés deux fois 10 minutes à 46° C.

L'observation des échantillons sous microscope confocal a été réalisée en mettant les échantillons dans des chambres LabTek™™ (Nunc, ThermoFisher Scientific, International). Les LabTek® étaient remplis avec de l'eau stérile. La microscopie a été faite dans la plateforme Prism, « Plateforme Prism – IBV-CNRS UMR 7277-INSERM U1091-UNS ».

Ensuite, les échantillons, y compris les disques dentinaires, ont été contrôlés à l'aide du Microscope électronique à balayage. Les images ont été utilisées afin d'évaluer la formation de biofilm sur les disques et les parois dentinaires et afin d'évaluer l'état de la surface dentinaire et du biofilm après le traitement clinique.

Test des groupes :

Groupe 1: Les canaux radiculaires ont été traités par le système de préparation unique OneShape® (Micro-Mega, Besançon, France). L'irrigation finale s'est faite en injectant 1 ml de solution Salvizol® EDTA à 8 % (ACTEON, Merignac, France) puis 2 ml de NaOCl 2,6 % dans les canaux radiculaires. Les solutions d'irrigation ont été agitées dans le canal pendant une minute en utilisant une lime endodontique ultrasonore (IRRISAFE® ACTEON, Mérignac, France).

Groupe 2: Les espaces canalaires ont été instrumentés avec le système de préparation unique OneShape® (Micro-Mega, Besançon, France). Dans ce groupe, l'irrigation a été réalisée uniquement avec H2O pour exclure les effets antimicrobiens et chélatants des solutions d'irrigation classiques.

Groupe 3: Les espaces canalaires ont été instrumentés avec le système de préparation unique OneShape® (Micro-Mega, Besançon, France) et uniquement H2O comme solution d'irrigation. Enfin pour finaliser le protocole de PDT, on a utilisé une LED rouge et le bleu de toluidine 15µg/ml. L'agent photosensibilisant a été activé pendant 2 minutes avec la lumière rouge.

Groupe 4: L'espace canalaire a été traités avec NaOCl 2,6 % qui a été agité avec le laser Er :YAG (2940nm) transmis via un embout saphir de 800 µm de diamètre. Le processus a été conclu en 4 X 5 secondes. Entre chaque cycle d'irradiation la solution d'irrigation a été renouvelée.

Groupe 5: L'espace canalaire a été traités avec NaOCl 2,6 % qui a été agité avec le laser Er :YAG (2940nm) transmis via une fibre endodontique de 300 µm de diamètre. Le processus a été conclu en 4 X 5 secondes. Entre chaque cycle d'irradiation la solution d'irrigation a été renouvelée.

Groupe 6: L'espace canalaire a été traités avec NaOCl 2,6 % qui a été agité avec un laser diode (combinaison des longueurs d'onde 915nm et 1064 nm) transmis via une fibre endodontique de 300 µm de diamètre. Le processus a été conclu en 4 X 5 secondes. Entre chaque cycle d'irradiation la solution d'irrigation a été renouvelée.

Évaluation :

Le système scoring a été utilisé, 3 évaluateurs étrangers du projet ont participé à l'évaluation.

Tests statistiques :

Les tests statistiques Kruskal et Wallis, Mann et Whitney et le test T de Student ont été utilisés pour évaluer la signifiance et comparer les protocoles de création du biofilm et des différents groupes de traitement.

3

Résultats

L'analyse statistique a montré une différence significative entre les trois groupes cultivés en condition aérobie ($p < 0,0001$ dans toutes les observations à 24, 48 et 72 heures ; (Fig. 35). Nous n'avons observé aucune différence significative entre les résultats obtenus par la thérapie photodynamique avec le laser diode et la thérapie photodynamique avec le protocole d'Aseptim™ ($p < 0.6267$). L'irrigation par ultrasons s'est démontré être le protocole le plus fiable pour désorganiser les biofilms microbiens ($p < 0,0001$).

Les mêmes procédures d'analyses statistiques ont été réalisées pour les cultures dans des conditions anaérobies. Une différence significative a été observée entre les trois groupes ($P < 0,0001$). L'irrigation par ultrasons a obtenu le meilleur résultat sur la réduction de la charge bactérienne ($P < 0,0001$). Cependant, le protocole Aseptim™ a eu des effets statistiquement plus favorables que la thérapie photodynamique avec un Laser diode en ce qui regarde la réduction de la charge bactérienne ($P < 0,0043$).

Étude Principale :

Les observations périodiques au MEB des disques dentinaires ont permis de mettre en évidence le processus de structuration du biofilm. Nous avons observé que le biofilm se forme de

manière progressive. Les premiers signes de structuration sont observés après 3 jours d'incubation. A 3 semaines, le biofilm atteint une forme mature. Une phase de grand détachement s'observe à partir du 24 jours et dès 27 jours, le biofilm a perdu sa forme habituelle d'amas. Après 31 jours d'incubation le biofilm recommence la construction de sa structure.

FISH :

Nous avons utilisé un microscope confocal afin de récupérer les signaux fluorescents des sondes ARNr 16S, cela nous a permis de confirmer la présence de toutes les espèces bactériennes présentes dans notre biofilm artificiel. Les marqueurs DY-405, ATTO488, Cy3 et Cy5 ont servi à colorer respectivement P. gingivalis, E. faecalis, F. nucleatum et S. salivarius (Fig. 49). Le test statistique a montré que les bactéries incorporées sont présentes de manière équivalente dans le biofilm couvrant la surface dentinaire ($p > 0,05$).

Toutefois, la colonisation bactérienne à l'intérieur des tubulis n'est pas identique à celle observée au niveau de la surface dentinaire ($p = 0,01$; Fig. 50). Le test Mann-Whitney a révélé que la P. gingivalis est l'espèce bactérienne dominante par rapport aux autres bactéries ($p<0.05$). De la même manière, S. salivarius est statistiquement moins présente que les autres bactéries.

SEM:

Les échantillons observés au MEB montrent que le canal principal est recouvert par le biofilm. De la matrice extra cellulaire et des bactéries sont mis en évidence au niveau des tubulis dentinaires également. Des agrandissements à 1000 fois et 2000 fois permettent d'identifier la morphologie des bactéries dans la couche superficielle du biofilm. (Fig. 51). Des cocci (S. salivarius ou E. faecalis), des bacilles (P. gingivalis) et des bactéries filamenteuses (F. nucleatum)

sont facilement différenciées. Des bactéries présentant une forme bipolaire suggèrent la présence de E. faecalis.

Des bactéries ont été détectées à l'intérieur des tubulis dentinaires lorsque les échantillons ont été visualisés à forts grandissements (2000 X et plus). Des micro-organismes ont été découverts dans les paquets localisés près des orifices des tubulis dentinaires ou individuellement à plus de 500 μm à l'intérieur des tubuli et loin de la surface dentinaire.

Traitement clinique:

Les tests statistiques concernant les résultats de la FISH ($p = 2,55 \times 10^{-9}$) et de la culture bactérienne ($p = 0,002$) ont montré que les protocoles de décontamination ont efficacement éliminé ou réduit la charge bactérienne de l'espace canalaire.

Ces résultats suggèrent que PUI, PDT, les lasers Er:YAG et diode ont montré statistiquement de meilleurs résultats en termes de contrôle de l'infection ($p < 0,05$). Toutefois, les groupes traités avec PUI et Er:YAG ont présenté une meilleure réduction de la charge bactérienne que les groupes PDT et diode laser ($p < 0,05$; Fig. 67).

Les tests statistiques concernant les résultats obtenus avec le MEB ont montré une efficacité des différents protocoles dans le retrait des débris (organiques et inorganiques) de la surface radiculaire ($p = 2.37 \times 10^{-18}$).

Statistiquement il n'y avait aucune différence significative dans l'élimination de l'enduit pariétal avec PUI, les lasers diode et Er:YAG transmis par une fibre endodontique ($p > 0,05$).

Mais l'instrumentation sans les solutions d'irrigation, la PDT et le laser Er:YAG transmis par un embout saphir n'ont pas pu nettoyer les surface dentinaire des débris ($p < 0,05$).

4

Discussion

Les microorganismes ont un rôle prédominant dans le développement des nécroses pulpaires et leurs complications. Le résultat du traitement endodontique est totalement lié à l'élimination de la flore bactérienne infectieuse qui se présente le plus souvent sous forme d'un biofilm organisée dans l'espace canalaire. Les taux de succès du traitement endodontique sont plus élevés lorsque le réseau canalaire est totalement débarrassé des bactéries avant le remplissage canalaire. Mais cliniquement le protocole chimio mécanique conventionnel de débridement ne pourrait pas atteindre complètement cet objectif. Des centaines de protocoles et techniques ont été introduites avec pour objectif d'obtenir un espace radiculaire totalement décontaminé : il n'est pas possible aujourd'hui, in vivo, de stériliser à proprement parler le réseau canalaire

Pour vérifier leur efficacité, chaque procédure de désinfection devrait être testée dans des environnements in vitro avant son application dans la clinique. Les biofilms artificiels pourraient représenter un modèle cible le plus approprié pour évaluer la capacité désinfectante de ces techniques. Une revue de littérature nous a permis de montrer que de nombreux modèles d'infection canalaire artificielle ont été introduits dans le domaine de l'endodontie. Parmi ces modèles, certains sont composés de bactéries planctoniques et d'autres sont formés de bactéries

organisées dans un biofilm. De plus, certains biofilms ne sont composés que d'une seule espèce bactérienne.

Il a été bien établi que le résultat obtenu pour l'éradication des bactéries planctoniques par divers agents de désinfection ne reflète pas l'effet des mêmes agents antibactériens dans des conditions in vivo où les bactéries sont très souvent organisées dans les biofilms complexes. Plusieurs publications existent concernant les biofilms microbiens à partir d'une seule espèce associée à des études de décontamination de l'espace canalaire. Les biofilms utilisés dans ces tests sont faciles à développer (176, 179). Il a été démontré par exemple que les bactéries dans un biofilm peuvent être 100 à 1000 fois plus résistantes aux agents antibactériens que leurs homologues planctoniques (72). C'est pourquoi nous avons conçu un modèle d'étude qui tendrait à certifier ces conclusions.

Dans notre modèle d'étude, nous avons réussi à développer sur toute la surface dentinaire radiculaire un biofilm mature qui s'est fermement attaché à son substrat. Les biofilms de dents avec nécrose pulpaire avec ou sans complication périapicale sont composés de plus de 400 espèces bactériennes dont certaines non encore identifiées. Selon ce concept écologique, la pathogénicité n'est pas liée exclusivement à une seule espèce bactérienne, c'est bien la composition polymicrobienne qui est à l'origine des altérations physiologiques et modifications génétiques, initiées par des changements au sein de l'espace endodontique et qui conduit à l'état pathologique (6, 7). La plus grande partie des organismes présents dans le biofilm est une collection de cocci, bacilles, filaments et plus rarement de spirochètes (52). Cette diversité des bactéries constituées en biofilm en fait sa reproduction difficile dans des conditions de laboratoire (20).

Le modèle de biofilm idéal devrait ressembler à la structure naturelle da la communauté infectieuse naturelle en termes de morphologie et de distribution des bactéries. Nous avons donc sélectionné pour notre étude une bactérie colonisatrice de chaque groupe au sein du biofilm oral pour former un biofilm polybacterien simple mais mature qui ressemble morphologiquement au biofilm de type sauvage. Dans notre étude, nous avons cherché également à former un biofilm artificiel qui pourrait adhérer à la surface dentinaire et pénétrer dans les tubuli dentinaires comme cela arrive lors de l'infection endodontique clinique. Les premiers essais sur des disques de dentine ont permis d'étudier la croissance d'un biofilm artificiel dans son aspect morphologique et chronologique. Les toutes premières images en microscopie électronique à balayage, ont démontré une maturation progressive de la structure du biofilm. Nous avons constaté qu'il fallait attendre 20 jours après incubation pour obtenir la forme la plus mature de ce biofilm. Après 24 jours, il a été observé une réduction de la taille du biofilm probablement à cause de la phase de détachement des bactéries pour revenir à l'état planctonique et recoloniser d'autres zones. Cette pseudo-altération du comportement du biofilm lui permet résister aux changements environnementaux.

Il a été mis en évidence par Shen et al, (69) que les biofilms âgés 3 semaines ou plus résistent mieux aux techniques de désinfection que les biofilms jeunes. Ainsi, une période de 21 jours semble suffisant afin d'obtenir ce biofilm mature artificiel. C'est ainsi que des canaux dentaires stérilisés de dents ont été infectées artificiellement par des bactéries pendant 21 jours puis traitées avec différents protocoles de désinfection. Pour mesurer l'efficacité des protocoles différents de désinfection et pour être certain que le biofilm artificiel a été supprimé du réseau canalaire, il était nécessaire de caractériser les composants de notre biofilm avant traitement.

Dans notre étude, nous avons décidé de caractériser le biofilm artificiel à l'aide de techniques d'hybridation in situ basée sur l'émission fluorescente de sonde ARNr 16S spécifiques

des bactéries composant notre biofilm. Les signaux de fluorescence sont recueillis en microscopie confocale à balayage laser (MCBL) (analyse en 3 dimensions possible). Les échantillons n'étant pas détruits par cette méthode, et afin d'éliminer les faux positifs ou les faux négatifs, les mêmes échantillons ont été observés en microscopie électronique à balayage.

La FISH nous a permis d'obtenir des résultats semi quantitatifs sur la présence et la répartition des espèces bactériennes dans l'espace canalaire avant et après traitement. En outre, la spécificité des sondes fluorescentes ARNr 16S pour chaque espèce conduit à l'identification des bactéries à l'intérieur de la structure des biofilms. Il semble possible de confirmer la présence de chaque micro-organisme et de les localiser in situ dans l'espace endodontique canalaire. Les techniques PCR ne permettent pas de révéler la localisation du biofilm à l'intérieur de l'espace radiculaire. À l'aide de la MCBL nous pouvions récupérer les informations des couches profondes de la structure de biofilm. En mesurant l'intensité de la fluorescence des différents signaux fournis par les sondes ARN 16s au moment de l'hybridation il était possible d'évaluer indirectement l'efficacité des différents protocoles de désinfection sur l'ensemble des bactéries. Par exemple en cas d'absence de signal il semble péremptoire d'affirmer que le protocole de traitement a effectivement éliminé le biofilm. Les images de MEB fournissent des informations sur la présence / absence de biofilm, sa morphologie et donc sur l'efficacité de nettoyage des procédures testées.

L'interprétation des données obtenues à partir des images confocales nous permet de confirmer la présence de toutes les bactéries composantes à l'intérieur du biofilm artificiel mature que nous avons conçu. Les bactéries étaient distribuées uniformément à l'intérieur du biofilm ($p > 0,05$) confirmant que les bactéries utilisées pouvaient coexister et former un biofilm bien développé. Toutefois, la présence de P. gingivalis était plus significative à l'intérieur des tubuli dentinaires que les autres espèces bactériennes. L'hypothèse la plus probable est liée à

l'augmentation de la prévalence des bactéries anaérobies dans le biofilm lors de sa maturation (181).

Les images de MEB démontrent que le biofilm artificiel couvre la surface dentinaire comme un tapis de microorganismes compactés. À des grandissements plus élevés (de 7000 à 10000 fois), la différenciation morphologique de chaque espèce devient possible.

En balayant les régions marquées par des sondes fluorescentes, nous avons pu identifier les bactéries à l'intérieur des tubuli dentinaires jusqu'à 500µm de profondeur. Même si Ma et al. (188) ont montré que la centrifugation de l'inoculum bactérien en contact avec le substrat permet une meilleure pénétration de microorganismes dans les tubuli dentinaires, dans notre étude, nous avons obtenu le même résultat naturellement, par l'injection simple d'inoculum à l'intérieur de l'espace radiculaire à l'aide d'une seringue. Cette technique est moins compliquée et permet d'atteindre le même objectif. Nous avons obtenu des résultats hautement reproductibles de la procédure de développement de nos biofilms (proche de 100%). La reproductibilité est essentielle pour un modèle d'étude in vitro et pour évaluer le résultat des différentes méthodes de décontamination.

La technique FISH-CLSM a été utilisée dans nombreuses expérimentations scientifiques ciblant l'infection dentaire et plus précisément celle de l'espace canalaire. La microscopie confocale et l'hybridation en fluorescence sont des techniques non invasives grâce auxquelles la comparaison in situ des effets des différentes méthodes de désinfection devient possible (188). Toutefois, sauf certaines études pionnières (52-171), l'utilisation de ces techniques est limitée à un simple test "Live/Dead" (189-192). Ce test a pour but de montrer la présence d'un biofilm viable avant traitement et permet de mesurer le rapport entre les cellules mortes et vivantes après

application de la procédure de traitement. En outre, comme déjà dit, les résultats de ces modèles comprenant généralement des biofilms monobactériens ne reflètent pas ce qui se produit lors d'infections poly-bactériennes naturelles. Donc, lorsqu'on utilise un biofilm poly-bactérien, les résultats des tests "Live/Dead" peuvent illustrer la présence et la répartition des bactéries sur son substrat mais ne peut pas servir à démontrer la sensibilité des différentes bactéries face à un traitement spécifique. Nous rappellerons également qu'il y a des limitations comme dans tout travail scientifique in vitro. Dans notre étude, le bruit de fond est présent même dans les témoins négatifs. Au cours du processus d'hybridation, en raison de la structure anatomo-histologique du canal radiculaire et pour éviter tout risque de distorsion du biofilm, l'étape de lavage était compliquée. En outre, la mise au point du microscope n'était pas possible dans la même observation pour les parois latérales et le fond du canal, ce qui nous a obligé à segmenter chaque image. Enfin il faut ajouter la possibilité de certains flous mineurs d'observations dues aux conditions dans lesquelles les sections d'échantillons ont été observées (présence d'eau dans la chambre du LabTek ®).

Une fois que le biofilm a été standardisé, nous avons conçu nos groupes de test. L'effet de l'irrigation ultrasonore sur l'infection canalaire et son aspect de débridement sont bien établis dans la littérature.

Il n'existe pas à l'heure actuelle de solution d'irrigation permettant d'agir à la fois sur le substrat organique et sur le substrat minéral des contenus canalaires instrumentés ou mis en forme mécaniquement. L'hypochlorite de sodium (ClONa) par son effet organolytique, dissout les tissus organiques comme le biofilm bactérien et l'éthylène diamine TétrAcétique (EDTA) par son effet chélatant, dissous les débris minéraux donc nettoie la surface dentinaire des matériaux

inorganiques résiduels (88, 93, 94) et ce que nous avions déjà effectué dans notre étude pilote (137).

Les techniques d'irrigation permettant d'activer les solutions de façon passive par les Ultra-sons (PUI) se trouve être plébiscité par la littérature comme le "gold standard" (138) et constituera naturellement notre groupe témoin-référence, en matière d'efficacité de la procédure testée. Le deuxième groupe a été traité uniquement avec l'instrumentation rotative et de l'eau stérile pour évaluer l'effet du débridement mécanique sur la réduction de la charge bactérienne. Le troisième groupe a été géré par l'instrumentation mécanique puis par la thérapie photodynamique de l'espace radiculaire. Nous avons utilisé le laser Er :YAG pour activer les solutions d'irrigation dans les quatrième et cinquième groupes pour lesquels le faisceau laser a été utilisé respectivement avec un embout saphir et une fibre optique de 400µm. Le dernier groupe a été traité avec le laser diode à l'aide d'une fibre optique endodontique. En termes de réduction de la charge bactérienne, nous avons pu comparer l'intensité des signaux récupérés des sondes fluorescentes par la microscopie confocale. À l'exception du groupe 2 traité par instruments rotatifs et qui ne montrait aucune différence avec le groupe témoin (biofilm seul), dans tous les autres groupes la charge bactérienne a été réduite de manière significative. Cependant, comme illustré dans la figure 63, PUI montre de meilleurs résultats que les groupes PDT et diode.

Nous n'avons pas pu recueillir de signaux de fluorescence émis par les sondes ARNr 16S dans les groupes 5 et 6. comme dans le groupe PUI. Cela confirmerait que le laser Er :YAG dans les deux méthodes de d'irradiation du faisceau laser (le saphir et la fibre) pourraient réduire l'infection bactérienne de façon aussi efficace que la technique PUI. Mehl et al. (93) ont obtenu les mêmes résultats avec un laser Er :YAG.

L’irrigation activée par laser (LAI) a l'avantage d'exciter les solutions d’irrigation dans l’espace canalaire à l'aide de très faible puissance du laser : on protège ainsi les tissus dentaires de tout effet iatrogène, y compris des dommages thermiques liés au mécanisme de vaporisation explosive. Nous avons utilisé des paramètres proches de ceux décrits par De Moor et al. (195, 196). Ces études ont montré que les laser Er :YAG et Er,Cr:YSGG ont efficacement éliminé les boues dentinaires du canal radiculaire avec une énergie de 75mJ et un temps d'irradiation total de 20 s (4 x 5 s). Nous avons utilisé 80mJ pendant le même temps d’application avec la fibre optique et le saphir. Comme mentionné auparavant, les images confocales obtenues des deux groupes (saphir et fibre) ont montré l'éradication bactérienne maximale comme pour le groupe PUI ; néanmoins, certaines observations au MEB se sont révélées surprenantes. Dans le groupe traité par le Laser et saphir les tubuli dentinaires étaient entièrement recouverts de débris. En comparaison avec les différents groupes (figure 65), il se pourrait que l’association laser Er :YAG / fibre endodontique se soit montrée aussi efficace que la PUI tant pour l’élimination des boues dentinaires que pour l’élimination du biofilm artificiel.

Si nous analysons la fluence utilisée, il y a une énorme différence entre saphir et fibre optique. L'énergie fournie par la fibre endodontique était de 116J/cm2 et de 15,92 J/cm2 pour le saphir. En outre, le saphir a été maintenu stable à l’entrée canalaire sans contact avec les parois du canal alors que la fibre endodontique a été déplacée dans un mouvement hélicoïdal le long du canal radiculaire et à proximité de la surface dentinaire.. Cependant, il n’y a pas de différence statistiquement significative entre ces 2 groupes pour l'état de surface dentinaire dans la région apicale. Cela pourrait être dû au fait que la fibre reste plus longtemps au niveau apical. Nous n’avons utilisé que la solution de ClONa, sans agent chélateur pour enlever les boues dentinaires. Le débridement ainsi obtenu semblerait donc être du uniquement à l’agitation « mécanique » et à

la production de microbulles explosives par le laser. Au cours de ce travail, nous avons montré que l'irradiation du canal radiculaire rempli avec ClONa en 4 cycles de 5 secondes est suffisante pour éradiquer la population bactérienne (biofilm et bactéries dans les tubuli) de l'espace radiculaire. Il conviendra de rappeler cependant que le microscope confocal a montré certains signaux fluorescents faibles et statistiquement négligeables dans de très petits débris dentinaires restés sur la surface radiculaire. L'explication la plus probable est qu'un petit nombre de bactéries viables pourraient encore être présentes dans les débris dentinaires. Les photons du laser Er :YAG peuvent être absorbés dans presque tous les type de solution d'irrigation utilisées en endodontie. Ainsi, le débridement chimique de l'espace radiculaire pourrait aussi être conduit en activant de l'EDTA avec le laser Er :YAG. On pourrait alors dissoudre les débris minéraux et donc exposer les bactéries "cachées" à l'action du ClONa et ainsi les éliminer. Les résultats obtenus avec le laser Er :YAG nous amènent à préconiser l'usage d' énergies plus basses et à utiliser exclusivement les fibres endodontiques pour décontaminer l'espace canalaire. Enfin, l'utilisation de l'EDTA est indispensable pour avoir une surface dentinaire propre. Nous pourrions ainsi proposer une séquence d'irrigation superposable à la PUI, utilisant successivement les solutions de ClONa et l'EDTA agitées à l'aide d'un Laser Er :YAG muni d'une fibre optique . D'autres expérimentations et particulièrement des évaluations cliniques de guérison de LIPOE in vivo seraient souhaitables.

Pour la première fois en 1997, Moritz a examiné la capacité d'un laser diode d'une longueur d'onde de 810nm pour éliminer les bactéries radiculaires dans des études in vitro et in vivo (199, 200). En outre, il a été vérifié que cette longueur d'onde est capable de décontaminer les couches profondes de la dentine radiculaire, donc que cette décontamination avait une envergure tridimensionnelle (144). Nous pourrions expliquer cela par le fait que le laser diode n'étant ni absorbé dans l'eau ni dans les matières minérales, la dispersion du faisceau laser pourrait se faire

dans les couches les plus profondes de la dentine (119). Des résultats identiques ont été obtenus avec les longueurs d'ondes 830nm (202), 940nm (203) 980nm (204). Ces différentes études montrent aussi que les lasers diodes de différentes longueurs d'onde sont toutes efficaces en termes de réduction de la charge bactérienne. Dans notre étude, nous avons utilisé un laser à double longueur d'onde qui est une combinaison 915nm/1064nm. Les images confocales obtenues ont confirmé une réduction significative de la charge bactérienne dans l'espace radiculaire. Ces résultats ont été aussi efficaces que ceux obtenus avec le laser Er :YAG mais les signaux fluorescents obtenus ont été plus importants que ceux récupérés après la PUI.

Les cultures bactériennes des prélèvements des échantillons traités avec le laser diode n'a pas permis de mettre en évidence une charge bactérienne résiduelle après traitement. Les observations des images en MEB confirment l'absence de microorganismes sur la surface dentinaire. Toutefois, cela pourrais être aux différents états de surface des parois dentinaires laissées par procédures d'agitations : les observations au MEB ont montré des surfaces plus ou moins rugueuses et plus ou moins anfractueuses. Cela aurait pu entrainer des difficultés d'élimination de certaines sondes fluorescentes après la procédure de rinçage.

Il ne faut cependant pas négliger le risque de dommages thermiques lors de l'usage des lasers diode. Selon da Costa Ribeiro les dommages thermiques provoqués par un laser diode sur les parois dentinaires sont négligeables lorsque des paramètres ''raisonnables'' sont utilisés (205). L'élévation thermique peut atteindre 8,6 ° C en mode continu, alors que ce ne sera que de 1,2 à 3,3 ° C en mode discontinu avec période de repos. Un repos de 20 seconds est crucial pour éviter tout dommage au niveau des tissus péridentaires. Les changements morphologiques provoqués par les lasers diode sur la dentine radiculaire sont dépendants de la puissance. Les lasers diodes enlèvent les boues dentinaires à une puissance affichée de 1.5W. L'augmentation de la puissance

conduit à des modifications de la structure dentinaire comme sa fonte en surface (206-208). Néanmoins, malgré cette altération morphologique, les lasers à diode n'ont aucun effet néfaste sur la caractéristique de composition de matrice minérale du canal radiculaire (208). Pour éviter tout effet thermique cumulatif, il est indispensable de tenir compte des temps de repos pendant les intervalles entre chaque irradiation. Pour cette raison, nous avons utilisé une puissance affichée de 2 W avec une fréquence de 100 Hz et nous avons irradié chaque canal pendant 5sec et en prenant soin de renouveler à chaque fois la solution de ClONa. Selon Gutknecht et al. et Alfredo et al. (209, 210) en respectant les temps de repos nécessaires (10ms d'irradiation avec ensuite 10ms temps de repos) les lasers diodes peuvent être considérés comme un dispositif sans danger qui nous permet d'augmenter la puissance affichée jusqu'à 3W. Pour éviter la surchauffe et la fonte de la dentine et des dommages thermiques aux tissus environnants, la fibre endodontique n'était jamais immobile comme décrit dans la littérature (209-211). L'observation au MEB montre que l'élimination des boues dentinaires peut être réalisée à l'aide d'un laser diode en conjonction avec des solutions d'irrigation (207). Selon Alfredo et al. à nouveau l'activation une solution d'EDTA 17% par un laser diode pourrait aider à l'élimination des boues dentinaires alors que l'activation de ClONa à 1 % avec la même longueur d'onde entraine la production de boues dentinaires (132). Dans ce travail, nous avons testé l'effet de l'activation d 'une solution de ClONa à 2,6% activée au laser diode à une puissance affichée de 2W et une fréquence de 100Hz sur notre modèle de biofilm et sur la propreté de surface dentinaire. Nous n'avons donc pas utilisé d'EDTA pour exclure son effet chélateur. Contrairement aux résultats d'Alfredo et al. nous avons noté que l'agitation d'une solution de ClONa à 2,6% avec un laser diode couplé des deux longueur d'ondes (915nm et 1064nm) (DeltaCube3™, Erma-Électronique, La Teste de Buch, France) conduit à une élimination satisfaisante de la charge bactérienne sans provoquer la création de débris

supplémentaires sur la surface dentinaire. Les résultats de l'analyse d'images MEB à nouveau sont superposables à ceux de groupe PUI en matière d'élimination de l'enduit pariétal apical. Les images obtenues ont montré que ce laser DeltaCube® combinant plusieurs longueurs d'ondes a efficacement paré les parois du canal radiculaire de toute bactérie dans la zone apicale que coronaires et médianes en laissant les tubuli dentinaires ouverts. Les lasers diode pourraient contribuer à l'activation des solutions d'irrigation grâce à leur haute fréquence qui atteint 20 à 50 KHz. Cette propriété pourrait promouvoir l'effet de cavitation ce qui aboutirait à un meilleur débridement (140). Neelakantan a démontré que le laser diode était aussi efficace que l'Er :YAG pour activer les solutions d'irrigation dans le canal radiculaire et détruire le biofilm microbien (140). Toutefois, selon George et al. il y a quelques différences entre Er :YAG et la diode pour ce qui regarde les qualités de cavitation au sein de l'irrigant. La formation de bulles avec le laser diode arrive avec un retard d'environ 5 secondes après la première irradiation. En raison du mouvement lent des fluides lors de l'irradiation par diode, la possibilité d'extrusion des irrigants au-delà d'apex est inférieure à celle avec le Er :YAG (142).

Cependant, selon George et al., pour le laser diode il y a une relation proportionnelle entre le volume d'irrigant dans l'espace radiculaire et la puissance nécessaire pour l'activer. La cavitation se produit toujours à un niveau de puissance plus que 2W mais pour éviter les effets thermiques indésirables il faut toujours respecter le temps de repos. Pour assurer ce temps de repos le laser diode doit être utilisé en mode discontinu et un protocole d'irrigation avec renouvellement d'irrigant. La forme de la fibre peut améliorer le résultat.

La thérapie photodynamique (PDT) est un traitement médical qui utilise la lumière pour activer un agent dit photosensible en présence d'oxygène. Les résultats des études concernant cette méthode de désinfection de l'espace endodontique sont toutefois controversés. Il a été démontré

que la désinfection photo-activée conventionnelle (PAD) ne peut pas perturber un biofilm poly-bactérien, mais qu'elle peut réduire un biofilm monobactérien d'*Enterococcus faecalis* (214). Yao et al. ont suggéré que la PDT était plus efficace sur les formes planctoniques des bactéries que sur le biofilm dans les canaux radiculaires (215). Ainsi, pour être efficace, il faudrait d'abord perturber mécaniquement le biofilm intracanalaire avant d'appliquer la PDT (150). Les essais cliniques de Jurič et al. ont montré que l'application de la PDT après débridement conventionnel (instrumentation canalaire sous irrigation ClONa) de l'espace radiculaire permet d'obtenir un canal libre de bactéries (216), mais sur un biofilm monobactérien. Dans notre étude, nous avons testé l'effet du PDT sur un biofilm mécaniquement perturbé par une instrumentation endodontiques. Dans notre étude pilote (217), nous savions déjà que la PDT ne pouvait pas éradiquer le biofilm dans un temps de travail cliniquement acceptable. De même nous avions montré que le laser diode avec son émission unidirectionnelle cohérente et que la LED avec sa lumière diffusée ne permettaient pas plus (pas de différence statistiquement significative) la décontamination d'un espace radiculaire envahi par un biofilm poly-bactérien mature. Certes, notre contrôle de décontamination n'était basé que sur une culture bactérienne. Cependant la PDT réalisée avec LED sur une solution de bleu de toluidine spécifique (AseptimTM Plus, Leutkirch im Allögo, Germany) peut avoir un effet de réduction de la charge bactérienne sans toutefois éliminer le biofilm(137). C'est pourquoi, nous avons évalué l'effet de la PDT sur un biofilm mécaniquement perturbé à l'aide d'instruments mécanisés. Selon Soukos et al. (158) et Bonsor et al. (218) les émetteurs ou un embout diffuseur de lumière peuvent propager la lumière unidirectionnelle du laser diode dans l'espace canalaire pour assurer le maximum contact entre la lumière et l'agent photosensible même dans les zones apicales du canal radiculaire. Les LED sont aujourd'hui décrites comme une excellente source lumineuse pour activation les agents photosensibles (219). Nous avons montré

avec des canaux simulés endodontiques que la lumière de la LED irradie facilement dans toutes les directions sans qu'il soit nécessaire de déplacer l'embout. Cela peut conduire à de meilleurs résultats en termes de réduction de la charge bactérienne par comparaison avec la PDT utilisant un laser diode unidirectionnel (217). Récemment, l'étude de Sabino a montré que lorsque des microorganismes sont irradiés par le laser diode muni d'une fibre qui diffuse le faisceau unidirectionnel du laser, la réduction de la charge bactérienne est 100 fois plus élevée que lorsque on l'utilise une fibre optique normale (220). C'est pourquoi nous avions traité nos échantillons avec la LED du système Aseptim™ avec une puissance de 900mW pour activer le bleu de Toluidine dont la capacité antimicrobienne est bien démontrée dans la littérature (148, 149).

Les résultats obtenus par Souza et al. (221) comme les nôtres (217), ont montré que différents protocoles de PDT ne peuvent pas éliminer le biofilm sans sa perturbation mécanique préalable avec des méthodologies de débridement de routine (instrumentation et irrigation). Des résultats prometteurs ont été obtenus avec des temps d'irradiation de l'agent photosensible beaucoup plus longs (158, 222). Une étude in vitro de Komine et Tsujimoto confirme que la plus grande quantité d'oxygène singlet produite par la PDT ne peut être atteinte qu'en allongeant le temps d'irradiation (151). Ainsi, ces conditions temporelles requises semblent difficiles à appliquer dans les procédures cliniques quotidiennes en particulier lors du traitement des dents pluri radiculées. Par exemple selon ces protocoles pour traiter un canal il faut incuber pendant 5 minutes l'agent photosensible dans l'espace canalaire et puis l'activer pendant 5 minutes, en total 10 minutes. Si on tient en compte une molaire avec 3 ou 4 canaux la procédure prendra au moins 40 minutes. Certains auteurs proposent de réaliser la PDT en 2 visites pour gérer ce problème de temps d'irradiation (223, 224). Les médications temporaires intra-canalaires ainsi que les obturations coronaires temporaires, bref la désinfection en en 2 ou plusieurs rendez-vous restent

controversées en matière du maintien de la décontamination obtenue à la première séance par risque de recolonisation par micro-infiltration. D'autres controverses sont apparues sur la durée d'irradiation : Yildirim et al. montre dans son étude qu'il n'y a pas de différence entre 1 minute et 4minutes d'irradiation de l'agent photosensible (225). Gardant à l'esprit ces controverses, nous avons constaté qu'une maximum d'irradiation du produit photosensible dans un temps cliniquement acceptable n'est observé qu'en utilisant une LED. La lumière LED est diffusée dans toutes les directions de l'espace canalaire et elle est donc en contact avec l'agent photosensible tout au long de l'irradiation sans nécessité de la déplacer la source. Ce phénomène ne pourrait pas arriver avec une lumière laser en raison de ses propriétés physiques. La lumière laser est en effet unidirectionnelle et pour activer la solution chimique à l'intérieur de l'espace canalaire, nous devons passer la fibre dans tous les sens (mouvement hélicoïdal). Cela pourrait être la raison permettant d'obtenir de meilleurs résultats avec une LED par comparaison avec une source laser même si nous avons montré par la technique FISH-confocale que PDT est efficace pour tuer les communautés infectieuses mécaniquement perturbées

Les images confocales ont révélé que dans les biofilms intacts (canaux non instrumentés) les bactéries sont toujours détectables dans les couches profondes. Il convient donc de perturber efficacement des biofilms endodontiques avant application de la PDT. Les images en MEB ont démontré que la PDT en raison de sa nature chimique de l'action ne peut pas être un substitut pour le débridement chimio-mécanique de l'espace radiculaire. Les débris organiques et inorganiques doivent être parés selon les normes de ‘’gold standard’’. La PDT n'a aucune action sélective et la résistance à ce traitement est rare (223). Il semble donc logique de considérer que la PDT peut aider et représenter une opportunité pour neutraliser les bactéries survivantes envahissant l'espace

endodontique en trois dimensions. Nous pouvons affirmer que la PDT joue un rôle contributeur pour renforcer la décontamination endodontique.

5

Conclusion

La gestion de l'infection endocanalaire est la clé du succès d'un traitement endodontique. Au laboratoire MICORALIS (EA 7354), nous nous sommes intéressés à l'élaboration d'un modèle qui ressemble structurellement au biofilm endodontique sauvage. Après la conception et la réalisation de notre biofilm artificiel, nous avons caractérisé ce biofilm avant et après différents protocoles de décontamination endodontique. Dans ce but nous avons utilisé la technique FISH-Confocal (en utilisant des sondes spécifiques ARNr 16S) ainsi que les techniques d'imagerie en microscopie électronique à balayage. À notre connaissance, un tel protocole n'a jamais été utilisé jusqu'à présent pour caractériser *in situ* un biofilm endodontique, protocole qui peut ouvrir la voie à de nouvelles pistes pour des études concernant la désinfection endodontique.

Dans les conditions de cette étude, les résultats obtenus nous permettent de conclure que :

- le choix des 4 espèces bactériennes utilisées dans ce projet (*S. salivarius, E. faecalis, F. nucleatum and P. gingivalis*) peut coexister et former un biofilm polybactérien *in vitro* sur les surfaces dentinaires.

- l'âge de 21 jours semble suffisant pour avoir un biofilm mature et structuré qui peut résister aux protocoles antibactériens. Ce biofilm pourra donner une image plus fidèle de ces protocoles dans les conditions cliniques *in vivo* contre le biofilm endodontique sauvage.

- la technique FISH-Confocal avec des sondes spécifiques ARNr 16S pourra aider à illustrer *in situ* la présence et la distribution des bactéries situées en profondeur du biofilm au-dessus des surfaces dentinaires et dans les tubuli dentinaires. Cette observation confirme l'aspect tri- dimensionnel de l'infection endodontique.

- la combinaison des deux technique FISH-Confocal et MEB fournit l'imagerie la plus complète à ce jour du biofilm bactérien à l'intérieur du réseau endocanalaire.

- ayant la qualité de '' gold standard'', l'irrigation ultrasonore (PUI) a montré les meilleurs résultats en termes de l'éradication du biofilm de l'espace canalaire avec des surfaces dentinaires les plus propres possible.

- regardant l'effet antimicrobien, les lasers Er:YAG et diode transmis avec une fibre endodontique ont démontré des résultats statistiquement comparables avec ceux obtenu avec PUI.

- le laser Er:YAG transmis avec une fibre endodontique doit être préféré à l'utilisation d'un embout saphir afin de prévenir de possibles dommages et d'obtenir des parois dentinaires libres de débris organiques et inorganiques.

- la PDT est active en ce qui regarde la réduction de la charge bactérienne lorsque le canal endodontique est préalablement instrumenté et la structure du biofilm bactérien est mécaniquement détruite.

- la lumière LED doit être favorisée par rapport au faisceau unidirectionnel des lasers afin d'activer l'agent photosensible. Les LED assurent un contact plus long avec le photosensibilisateur pendant le temps d'irradiation. Ces lumières peuvent parcourir tout au long de l'espace endodontique sans besoin recourir à des mouvements spéciaux (cas des fibres endodontiques).

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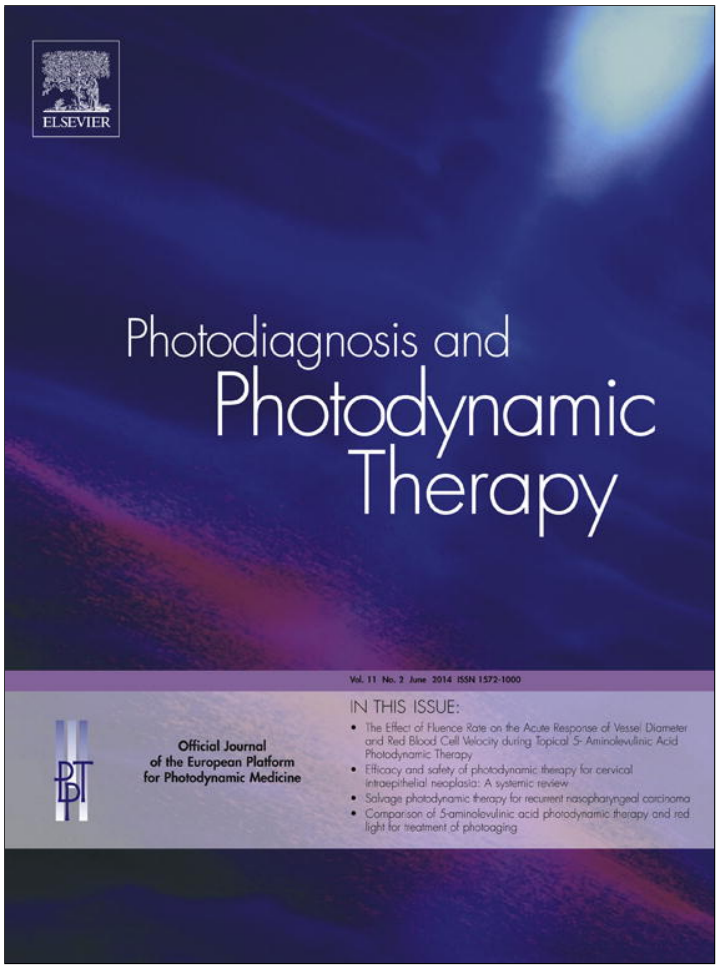

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Photodynamic therapy versus ultrasonic irrigation: Interaction with endodontic microbial biofilm, an ex vivo study

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KEYWORDS
Root canal disinfection;
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Passive ultrasonic irrigation

Summary
Introduction: Photodynamic therapy was introduced as an adjuvant to conventional chemo-mechanical debridement during endodontic treatment to overcome the persistence of biofilms. The aim of this study was to evaluate the ability of photodynamic therapy (PDT) to disrupt an experimental microbial biofilm inside the root canal in a clinically applicable working time.
Materials and methods: Thirty extracted teeth were prepared and then divided in three groups. All samples were infected with an artificially formed biofilm made of *Enterococcus faecalis*, *Streptococcus salivarius*, *Porphyromonas gingivalis* and *Prevotella intermedia* bacteria. First group was treated with Aseptim Plus® photo-activated (LED) disinfection system, second group by a 650 nm Diode Laser and Toluidine blue as photosensitizer, and the third group, as control group, by ultrasonic irrigation (PUI) using EDTA 17% and NaOCl 2.6% solutions. The working time for all three groups was fixed at 3 min. Presence or absence of biofilm was assessed by aerobic and anaerobic cultures.
Results: There was no statistically significant difference between results obtained from groups treated by Aseptim Plus® and Diode Laser ($P < 0.6267$). In cultures of both groups there was a maximal bacterial growth. The group that was treated by ultrasonic irrigation and NaOCl and EDTA solutions had the best results ($P < 0.0001$): there was a statistically significant reduction of bacterial load and destruction of microbial biofilm.

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Conclusion: Under the condition of this study, Photodynamic therapy could not disrupt endodontic artificial microbial biofilm and could not inhibit bacterial growth in a clinically favorable working time.

Introduction and background

Clinical management of microorganisms and their elimination from root canal space is the main goal of endodontic treatment [1,2]. Most failures occur when treatment procedures have not reached an acceptable measure for control and elimination of infection [3—5]. The success rate of endodontic treatments is higher (94%) when the canal is bacteria-free (verified by bacteriological methodologies used just before filling [10]). On the presence of bacteria, this rate is diminished by about 68% [4]. It is apparent that the mechanical debridement combined with chemical irrigation removes the bulk of the infecting microorganisms. But because the infection of root canal system is 3-dimensional, the residual bacteria are still detectable in an important area of the teeth just before filling the root canal [6—8], particularly in small accessory and lateral canals [9]. The antimicrobial susceptibility or resistance to the polymorphous micro flora, which includes anaerobic, facultative anaerobic and aerobic bacteria, may determine the outcome [9]. The important role of *Enterococcus faecalis* in root canal treatment failures and persistent periapical infection is well established in literature [7,10,11,12]. Although *E. faecalis* possesses several virulence factors, its ability to cause periapical disease originates from its ability to persist as a pathogen in the root canals and dentinal tubules of infected teeth [13—15]. Against the traditional views that suggest that the most robust group of organisms are the survivors of root canal treatment, the application of ecological parameters indicates the most important factor is the ability of bacterium to adapt itself to new limiting factors in its corresponding niches. Furthermore, as in every natural microenvironment, the adaptive capabilities of individual organisms are exponentially augmented when growing in biofilm communities. These microorganisms are enclosed in an extracellular polysaccharide matrix. The base of this ecological approach to root canal infections is founded on this concept that the most dangerous pathogen is not an individual species, in a planktonic form, but a polymicrobial entity that undergoes different physiological and genetic changes initiated by changes in root canal environments [16,17]. As an essential part of debridement of root canal space, irrigation makes it possible to achieve a cleaner root canal space more than of that which can be obtained only with mechanical instrumentation [18,19].

Hence, the need for an efficient root canal disinfection method drives researchers toward looking for other more effective technologies in Endodontics. There is no one unique irrigant that can possess all required criteria for the best root canal disinfection, so dual irrigant protocols using NaOCl and EDTA solutions are mainly used in Endodontics [20—22]. The use of ultrasonic devices during irrigation has been proposed to confront the problems observed during cleaning and disinfection of the root canal system [22] and the results are significant in reducing survival bacteria [23,24]. Passive ultrasonic irrigation (PUI) seems to enhance theses results [19], due to an acoustic streaming into the root canal [23].

Since the first laser device was developed by Maiman in 1960 [24], this machine has been used in various fields of dentistry [25—32]. Antimicrobial effects of lasers have driven researchers to use this technology for the purpose of root canal disinfection [33—37]. Er:YAG laser ($\lambda = 2940$ nm) [40], Nd:YAG laser ($\lambda = 1064$ nm) [41], KTP ($\lambda = 532$ nm) [42] and Diode Lasers ($\lambda = 630$ nm, 810 nm [38] to 980 nm [39]), were tested and showed an effective and significant elimination of the bacterial contamination, but most of the studies were performed on mono-bacterial (*E. faecalis*) infected root canals in planktonic form [43,44]. On the other hand, the use of these wavelengths at high power, have considerable disadvantages that make them hazardous for antibacterial purposes [45]. Melted dentin, cracking on the surface, and slight debris formation are other disadvantages of some lasers with high levels of energy [45—49] and, in addition, the complexity and high cost of these devices [50]. The antimicrobial effect of Photodynamic therapy (PDT) has been shown in several publications [51—54] and applied in different fields of dentistry [53,54]. Photodynamic therapy (PDT), sometimes called photo-chemotherapy, is a form of phototherapy using nontoxic light-sensitive compounds (photosensitive agents) that are exposed selectively to a defined wavelength light, whereupon they become toxic to target malignant and other diseased cells. PDT has proven ability to kill microbial cells, including bacteria, fungi and viruses. PDT is popularly used in treating acne. It is used clinically to treat a wide range of medical conditions, including wet age-related macular degeneration and malignant cancers [53], and is recognized as a treatment strategy, which is both minimally invasive and minimally toxic. The physical phenomenon is based on the role of complementary colors [54]. Oskar Raab and Hermann Von Tappeiner used for the first time, in 1900—1904, the term ''Photodynamic action'' to describe the toxic effect of Acridine orange (a dye) on *Paramecia*, when exposed to the sunlight. These researchers told about the importance of the combination of light and atmospheric oxygen. Many years later, in 1975, the first medical treatment using PDT was proposed by Kelly et al. [55] to treat bladder cancer without adverse effects on sound cells. Then PDT, since 90s, was really developed as a an antimicrobial therapy for infectious diseases and the terms of Photo-Activated Antimicrobial Chemo-Therapy and Photo-Activated Disinfection were used [56,57]. This therapy was describe as minimally invasive, to be used several times in the same place, and in combination with surgery, radiotherapy and chemotherapy particularly to treat cancers. In dentistry, Wilson [58], published the first application of PDT in this field, to remove dental plaque. Then, in 2000s, antimicrobial effects on oral biofilms of PDT and its use

to diagnose (PDD) and treat oral cancers were significantly developed [59].

Bacteria could not develop resistance to oxygen derived cytotoxic reactive species such as free radicals and singlet oxygen [60]. Bacteria that grow in biofilms, explored in diseases like cystic fibrosis or periodontitis, are also sensitive to PDT [61,62]. In addition to direct effect on extracellular molecules, singlet oxygen has a photo-damage effect on polysaccharides of the extracellular matrix of polymers within the bacterial biofilm [50]. Light wavelength, intensity and energy, the amount of absorbance of photosensitizer by cells, and exposure time are also other important factors which may influence the results [63]. The upper layers (1—5 mm) of most tissues are penetrated by light at wavelengths of ~630 nm; deeper penetration is achieved at 700—800 nm. Diode Laser systems are used predominantly. Recently, non-laser light sources, such as light-emitting diodes (LED), have also been applied in PDT [64—67]. Different methods, different light sources and many photosensitizers have been examined, all in vitro, ex vivo and in vivo [9,63,68—72].

The aim of this study was to evaluate the capability of two different PDT protocols in disrupting microbial biofilm growth in root canal space, compare their efficiency to PUI using EDTA 17% and NaOCl 2.6% solutions.

Materials and methods

Selection and preparation of teeth

Thirty-four roots obtained from 50 extracted human single and multi-rooted teeth were selected. The presence of just one canal was confirmed by digital radiography. The teeth were stored in saline solution until starting the experiments. All roots were reduced from original height to 14 mm. Afterward, all the samples were placed in an Ultrasonic heated bath (Fisher Scientific Inc., Schwerte, Germany) in order to clean off the dust and dirt accumulated during cutting. All apices were closed with Photac™ Fil Quick Applicap™ Glass-ionomer cement (3M ESPE AG, Seefeld, Germany), to avoid leak loss of irrigants during the root canal preparation.

Root canal shaping

The same operator performed all of the preparation steps. A # 10 K-File (MicroMega, Besançon, France) was introduced into the canal to determine the working length, and confirm the absence of any obstacles in the canal. Root canal shaping was implemented using the Protaper® rotary system (Dentsply-Maillefer, Ballalgues, Switzerland) according to the manufacturer instructions. The final file used was the F2 file (# 25, .09 taper).

Between the use of each instrument, the canals were flushed with 1 ml of NaOCl 2.6% solution using a syringe and a 26G needle (PentaFerte S.p.A, Campli, Italy). A # 10 K-File was used to verify the glide path of the root canal. A final rinsing, aiming to remove smear layer and debris was performed using 1 ml EDTA 17% solution (Root canal enlarger Edeta, Produit Dentaire S.A, Vevey, Switzerland). The solution was advanced into the canals for 1 min using an endodontic ultrasonic smooth file, IRRISAFE® (ACTEON, Merignac, France) mounted on an ultrasonic unit (Piezon® Master 400 EMS Electro Medical Systems SA, Nyon, Switzerland). Then, 2 ml of NaOCl 2.6% were injected in the canal and agitated for 1 min with the same ultrasonic file as mentioned before. To finish the rinsing, 2 ml of NaOCl 2.6% solution was injected into the canals to clean up any remnants without ultrasonic agitation. All prepared teeth were kept in saline solution until ex vivo infection procedures were conducted. All tooth samples were rinsed with water several times to remove any possible remaining debris and disinfecting solutions from the root canal walls. The teeth were then sterilized with an autoclave (130 °C during 1 h).

Biofilm ex vivo creation

The biofilm created in the laboratory for this study was composed of 4 different bacterial species, *Porphyromonas gingivalis* (ATCC 33277), *Streptococcus salivarius* (ATCC 7073), a wild-type strain of *E. faecalis*, and a wild-type strain of *Prevotella intermedia* that were obtained from the laboratory of bacteriology (Hôpital Archet 2 — Nice — France). *E. faecalis* and *S. salivarius* were grown aerobically overnight and at 37 °C on Mueller-Hinton agar plates or on 5% sheep blood agar plates (BioMérieux, Marcy l'Etoile, France), respectively. *P. gingivalis* and *P. intermedia* were cultivated anaerobically on 5% sheep blood agar plates at 37 °C for 3 and 5 days, respectively. For the four strains a standardized suspension containing 10^6 cells ml^{-1} in Schaedler broth (Bio-Rad, Marnes la Coquette, France) was prepared respecting the following proportion: 5% *S. salivarius*, 21% *E. faecalis*, 37% *P. intermedia*, and 37% *P. gingivalis* .

The biofilm was applied onto the teeth by dispensing 2.2 ml of standardized cell suspension within 24-well cell culture plates (Corning Inc., Union city, CA, USA). Cell culture plates were incubated anaerobically at 37 °C on an orbital shaker (150 r.p.m). After 24 h, 0.5 ml of Schaedler broth was added into every well. Seven days after the inoculation, teeth were removed and washed with 0.1 M phosphate buffer saline twice, for 3 min each time. Then, the teeth were randomly divided into 5 groups: 2 groups of 2 teeth (as negative and positive control group), and 3 groups of ten teeth to be treated with different methods to disrupt the biofilm.

Disinfection procedures

Control groups

Cultures were achieved from the 2 teeth of the negative control group (no inoculation) in order to verify that the canal is bacteria-free and a SEM examination is performed under low vacuum to verify the absence of smear layer (Fig. 1). Cultures were also realized from the 2 teeth of the positive control group in order to verify that canal is infected by the biofilm and a SEM examination is performed under low vacuum to verify the presence of biofilm (Fig. 2A and B).

Group A, LED

The ten teeth from group A were treated with PDT protocol using an LED with a wavelength of 635 nm as a source of light (Aseptim Plus®, Leutkirch im Allögo, Germany). Light was delivered using a disposable conical soft plastic tip. The

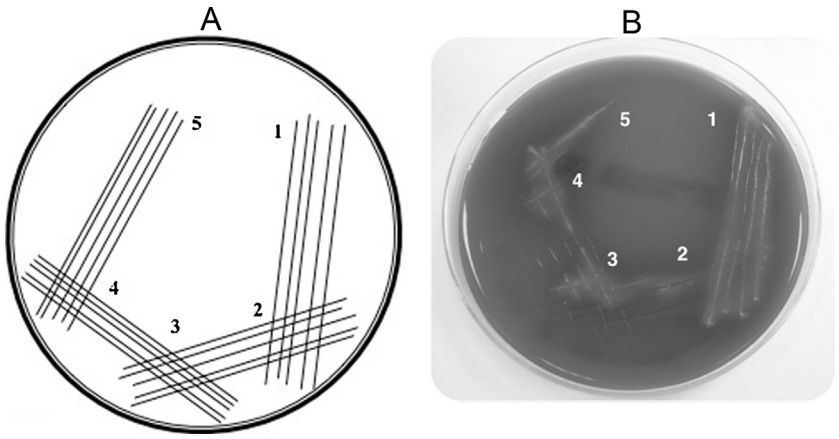

Figure 1 (A) Schematic view of culturing and scoring according to Bonsor et al. [73,74]. (B) Transfer on an agar plate with 5 scores design streaking. Score of 5 is equivalent to heavy bacterial load, score of 2 equivalent to approximately 1.5×10^8 bacteria, score of 0 equivalent to no culturable bacteria.

Photosensitizer was a solution of dilute, pharmaceutical grade Toluidine blue, which was supplied in 0.8 ml syringes. The solution was introduced into the root canal by mean a G26 needle.

For an easy performance of the treatment protocol, teeth were mounted on a base made from Aquasil™, a vinyl polysiloxane impression material (Dentsply DeTrey GmbH, Konstanz, Germany). The work desk was cleaned by alcohol 70% to avoid contamination. Then all procedures were carried out under sterile conditions next to the flame. The excess of the product was collected during injection using a sterile pipette tip and a suction device. The solution was rubbed around inside the root canal one minute using a # 10 K-file to respect the manufacturer's instructions continued the procedure. The sterile, specially designed flexible tip attached to the LED device was inserted into the canal space until a tug-back sensation was felt. Activation of the photosensitizer was commenced for 120 s according to the manufacturer's instructions. Once the procedure was completed, the canal was rinsed with 2 ml of sterile water to remove the photosensitizer from the canal. Afterward, the canal was dried with a sterile paper point.

Group B, Laser

A so-called DeltaCube™ soft laser with a wavelength of 650 nm (Laser 3 S, Pessac, France) with a maximum energy of 60 mW was used. The light was delivered into the canals using a 300 μm optic fiber in a continuous manner for 120 s. The best duration of activation of a photosensitizer (a 15 μg/ml solution of Toluidine blue O; Sigma—Aldrich, Schnelldorf, Germany) by a laser was measured through a pilot study. Before activation, the photosensitizer was agitated for 1 min by means of a # 10 K-file. The photosensitizer was exposed to a red laser light, and after 120 s, the photosensitizer lost its original blue color to pink, in which the light absorbance may change. Because the Laser beam could not diffuse in all directions, the fiber was moved all along of the canals repeatedly to activate the photosensitizer in all regions of the canal space (WL minus 2 mm). After treatment, the canal was rinsed with 2 ml of sterile water and dried by sterile paper points.

Group C, PUI

The ultrasonic irrigation was performed by injecting 1 ml EDTA 17% solution (Produit Dentaire S.A, Vevey, Switzerland) in the root canals. The solution was agitated in the canals for 1 min by means of an endodontic ultrasonic soft file (Irrisafe®, ACTEON, Merignac, France) that was mounted on an ultrasonic unit (Pmax®, ACTEON, Merignac, France) as described for final irrigation before infection protocol. Two ml of NaOCl 2.6% solution was injected in the canal and was agitated for 1 min with the same device. Another 2 ml of NaOCl 2.6% was injected into the canals. Finally, the canal was rinsed with 2 ml sterile water and dried with sterile paper points.

Bacterial load control: microbiological sampling and culturing

Once the clinical procedures were accomplished, microbiological samples were taken from the canals. A # 10 K-file was used to rub the canal walls to collect any possible viable bacteria. Then the samples were cultivated in 5% sheep blood agar. The technique of assessment of culturing was inspired by the protocol of Bonsor et al. [71,72]. A design of 5 parallel lines was created on the agar plate and this was repeated 3 times more, that gave a 5 growing areas to the culture pattern (Fig. 1A). If growth occurred in the well area, a score of one was allocated. If the growth occurred in both the well and the first five streaked lines, this was scored two and so on up to a maximum score of five (Fig. 1B).

Aerobic cultures were performed on all the teeth. Then 3 teeth from each group that were randomly selected underwent anaerobic culturing. For all of the teeth an aerobic culture was performed and for 3 teeth of each group that were randomly selected, an anaerobic culture was done. The plates were incubated at 37 °C. The bacterial growth was observed and the scores recorded all 24, 48 and 72 h after culturing.

SEM observation of biofilm inside the root canal before treatment (Fig. 3A and B), and after LED, Diode and PUI treatments (Fig. 4A and B) from all samples were then taken.

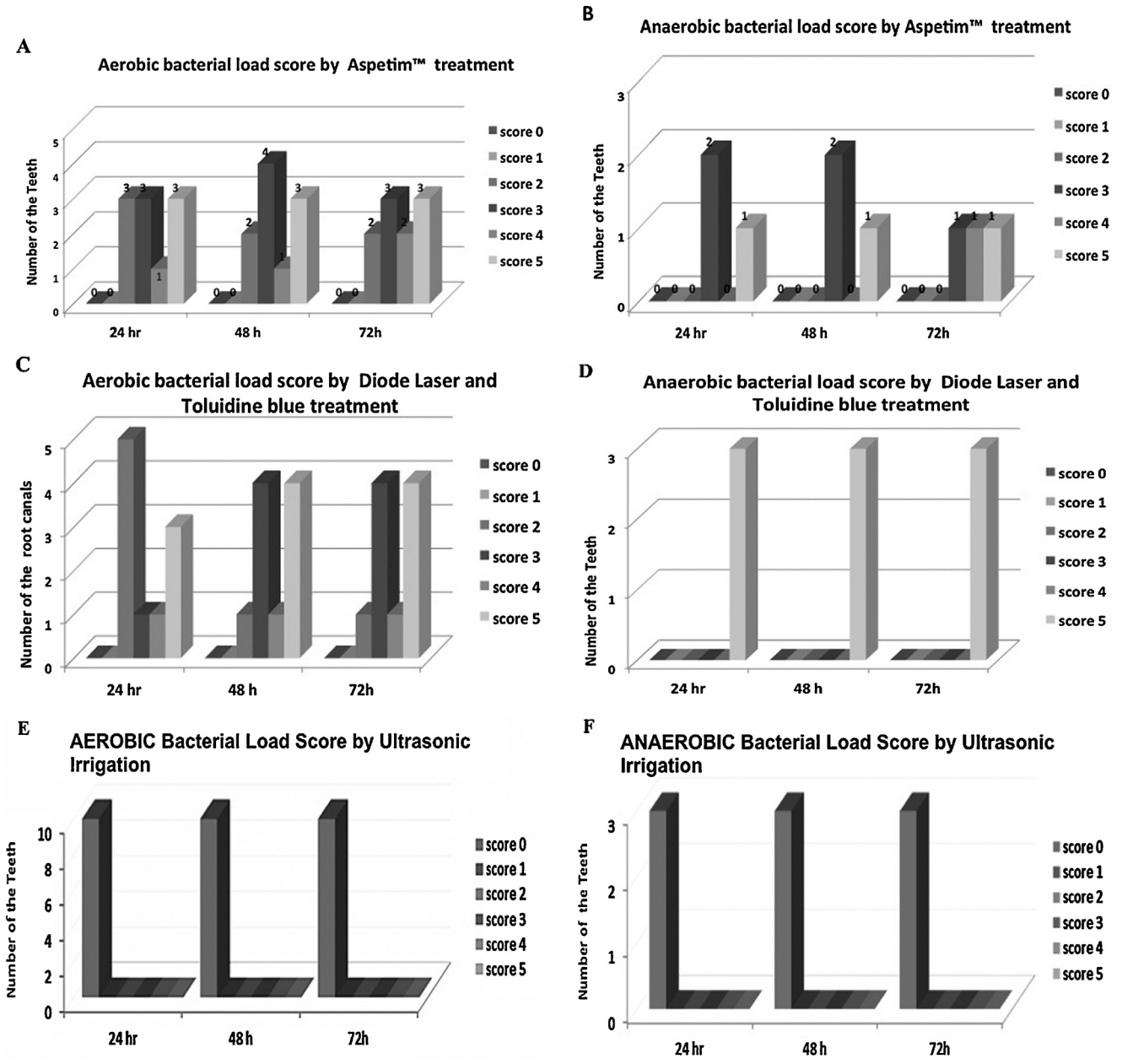

Figure 2 Bacterial load scores per root canals treated: A and B treated by Diode Laser and Toluidine blue, C and D by Aseptim and E and F by PUI. Bar chart shows the numbers of the root canals with aerobic (A, C, E (10 specimens each)) and anaerobic (B, D, F (3 specimens each)) bacterial loads at 24, 48 and 72 h after the treatment.

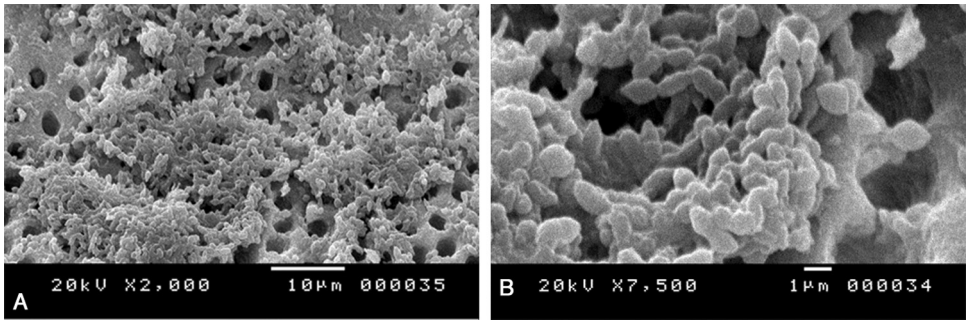

Figure 3 (A) Biofilm in root canal 72 h after inoculation — SEM ×2000. (B) Another view SEM ×7500.

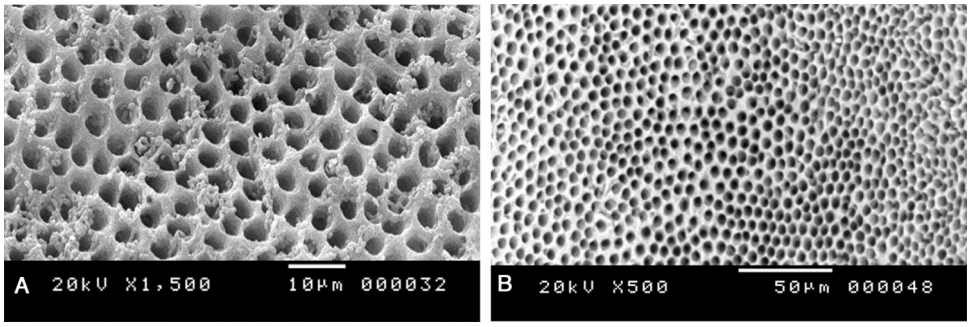

Figure 4 (A) Root canal surface after LED treatment (72 h): some isolated groups of bacteria are still present on the root canal surface and into the dentinal tubules. The biofilm is partially disrupted. (B) Root canal surface after PUI treatment (72 h) (NaOCl and EDTA): Root canal walls are clean. There was no more biofilm or microorganism after using EDTA and NaOCl irrigation solutions, and ultrasonic activation.

Statistical analysis

ANOVA and post hoc tests statistical analysis tests were achieved; all groups were compared by multiple two by two sample tests by Fisher's PLSD and were confirmed by the Student Newman—Keuls test.

Results

Group A, LED

Based on the scoring method previously described, none of the canal cultures had scores of 0 or 1 in the group treated by LED Aseptim® system, meaning that in all canals there was a bacterial load after treatment. Under aerobic conditions, 24 h after treatment, cultures taken from 3 canals showed a score of 2 that was reduced to 2 canals after 48 h and remained unchanged during final observation at 72 h. During the first observation, there were 3 canals with a bacterial load score of 3 that was augmented to 4 canals two days after inoculation and then returned to 3 canals at the end of the experiment. A score of 4 was registered in only 1 culture after 24 and after 48 h of treatment, while after 72 h, 2 cultures showed the same score. Of the 10 root canals evaluated in this group, only three showed maximum bacterial infection with a score of 5 during the observation (from 24 to 72 h after the treatment). The distribution of aerobic bacterial load scores for the culture taken at the different times of bacterial growth is shown in Fig. 2A.

The cultures taken from the root canals did not present anaerobic bacterial load scores of 0, 1 or 2. At the time points of 24 and 48 h after treatment, two root canals had anaerobic bacterial load scores of 3 (Fig. 4A) and one went up to a score of 4 at 72 h. Only one root canal showed a complete anaerobic bacterial infection with a load score of 5 during the entire cultivation experiment. There was no significant difference between the bacterial load scores obtained from anaerobic and aerobic cultivations. Anaerobic bacterial load scores of root canals treated through the Aseptim® system is shown in Fig. 2B. Remnant biofilm inside the dentinal tubules can be seen in Fig. 4A (SEM).

Group B, Diode Laser and Toluidine blue

Teeth presented both aerobic and anaerobic bacterial load. Under aerobic conditions after 24 h, cultures taken from 5, 1, 1 and 3 root canals had bacterial load scores of 2, 3, 4 and 5, respectively. There were no root canals presenting load scores of 0 or 1. Forty-eight hours after treatment, there was a dramatic decrease in the number of root canals with scores of 2 from 5 to 1 and respective increases in number of root canals with scores of 3 from 1 to 4. One root canal with a score of 4 and four root canals with scores of 5 were recorded in this step of observation. The bacterial load scores remained unchanged after 72 h of incubation (Fig. 2C and D).

Interestingly, all 3 root canals evaluated under the anaerobic condition presented a high level of bacterial load score at 5, only after 72 h of incubation (Fig. 2C and D).

Group C, PUI, NaOCl and EDTA

No bacterial load for all samples, under aerobic and anaerobic conditions. PUI using EDTA and NaOCl solutions ultrasonically agitated is effective at disrupt the biofilm and at disinfecting the root canal (Fig. 2E and F). Cleaned surfaces can be seen in Fig. 4B (SEM).

Statistical analysis showed that there is a statistical significant difference between the three groups when culturing under aerobic conditions ($P < 0.0001$ in all observations at 24, 48 and 72 h).

There was no statistically significant difference between cultures obtained from PDT by diode laser group and from PDT by Aseptim® protocol ($P < 0.6267$ for the final observation at 72 h), all of which were confirmed by the Student Newman—Keuls test.

Ultrasonic irrigation showed the best results to disrupt the microbial biofilms ($P < 0.0001$). All statistical plots and tables of cultures taken under aerobic conditions for all observations are shown in Fig. 2.

The same statistical analyses procedures were performed for the cultures incubated under anaerobic conditions. A significant difference was observed in 3 groups and for all observations at 24, 48 and 72 h ($P < 0.0001$). The ultrasonic irrigation had the best effects on reducing bacterial load in anaerobic conditions ($P < 0.0001$). However, Aseptim® had

statistically better effects than photodynamic therapy by Diode Laser to reduce bacterial load in anaerobic conditions ($P < 0.0043$ for final observation at 72 h). All statistical plots and tables of statistical analyses of cultures taken under anaerobic conditions for all observations are shown in Fig. 2.

Discussion

Hundreds of protocols have been introduced in literature aiming to obtain a 100% bacterial free root canal space. PDT was introduced as a measure to attain this goal. PDT was shown to have ability to kill the planktonic bacteria in in vitro study essays.

In the majority of publications concerning PDT and Endodontics, this protocol has played a great role in the reduction of bacterial colonies. However the effect of PDT on microbial biofilm may be related to that of NaOCl used during instrumentation [72]. Subsequently it was suggested always as an adjuvant to conventional root canal treatment procedures and not as a substitute. PDT was performed at the point of chemo-mechanical debridement completion, when the microbial biofilm present in the canal might be logically stressed and disturbed by root canal instrumentation and irrigation. Because intact biofilm remnants attached to canal walls may still be detectable after chemo-mechanical debridement, we investigated the interaction of an intact biofilm with Photodynamic therapy and ultrasonic irrigation in an ex vivo trial. In this study we used an artificially formed biofilm in which there was *E. faecalis* whose role in treatment failures was previously described. We used two different methods of PDT using a 650 nm Diode Laser and LED and we compared the outcome of both of them with passive ultrasonic irrigation.

Garcez et al. [69,70] used PDT as a supplement to chemo-mechanical debridement and placed the photosensitizer in contact with the microbial biofilm resistant to antibiotherapy. A further 4 min were considered for its activation by light, resulting in a total of a 6 min working time. He suggested even a two-visit treatment protocol to obtain bacteria-free root canals.

Soukos et al. [71] examined the effects of PDT on *E. faecalis* in a planktonic state, but he submerged the specimens in photosensitizer solution for 5 min and spent 5 min for photo activation. The reduction of EF in planktonic form was only 77.5%.

Souza et al. [76] irrigated the specimens with two different solutions, one NaOCl 2.5% and other NaCl 0.85%. Then they incubated the canals for 2 min with Toluidine blue and Methylene blue as the photosensitizing agents and for 4 min the Laser irradiated the canal. Ten minutes for a single canal [71] and two visits, becomes 60 min for a multi-rooted tooth in the case of lack of anatomical variation, a problem that is found in other protocols.

Because in the protocols mentioned above and in many of similar publications [63,75—78] the procedures are quite time consuming and not feasible in everyday dental practice, we tried to find and standardize a suitable amount of time for treatment that would be clinically accepted.

The other factor was the photosensitizer itself. A large variety of concentrations from too low (10 μg/ml) to too high (100 μg/ml) of Toluidine blue were tested [79—81]. Because of our objective of feasibility and safety in the clinic (to prevent any possible toxicity and colorization of hard and soft tissues from the photosensitizing agent) we used Toluidine blue with a concentration of 15 μg/ml. The concentration and exact composition of photosensitizer of Aseptim Plus® system was protected by a patent (Scican, States Patent 5,601,430, United States Patent and Trademark Office, sold by Micro-Mega in France).

According to the manufacturer instructions, a 3-minute total procedure time was established for all procedures. In this protocol 1 min was dedicated to agitating the photosensitizer in the root canal with a hand # 10 K file and 2 min were committed to activating the photosensitizing agent with light. The duration of photo activation was tested also for PDT by diode laser through a pilot study. The color modification observed was quite/very much the same with the Aseptim® solution after 120 s of irradiation by LED. As the photosensitizing agent must act as a chromophore corresponding to the exact complementary color of the activator light, in this study we observed that the color of the photosensitizer changed totally from blue (which is a complementary color of the 650 nm red light of Diode Laser and 630 nm of LED) to pink after 30 s of irradiation. The energy absorbed by the photosensitizer could not be the same during all of the irradiation. During another 90 s of activation time, there were different absorption coefficients of the photosensitizer regarding its continuous color changes. Maximum energy absorption by photosensitizer occurred approximately during the first 30 s of the experiment.

However, the period of 180 s was respected in the ultrasonic irrigation group too. We used 2 different solutions, EDTA 17% and NaOCl 2.6%. One ml of each one was injected using an up and down movement for 30 s at WL minus 2 mm and then was agitated for 60 s.

As explained above, all solutions used in all three groups were in a passive state during activation and we did not add photosensitizer, NaOCl, or EDTA.

Standardization of the working time in all three groups aims to measure the capability of those methodologies to act on microbial biofilm.

Diode Laser light was delivered via a 300-μm optic fiber but the LED light was transmitted into the canal by a conical plastic tip that allowed for a better distribution of photons in all directions. Diode Laser light has the collimated characteristic of any other Laser: so to active the entire photosensitizer we had to move the fiber tip up and down (Working Length minus 2 mm) in a helicoidally manner. However there was a slight refraction of the Laser light, so the light might miss some regions in the canal. Moderately better results were achieved by Aseptim Plus® device system which may be due to its non-collimated light produced by LED and/or by the composition and concentration of the photosensitizer.

Ultrasonic irrigation promoted best results in our study. It was efficient time-wise and the most economical. Ultrasonic irrigation enhances the effects of NaOCl and EDTA by acoustic streaming. The solution can reach all parts of the canal even in non-instrumented parts of the root canal where we may find a portion of intact biofilm. But, our results may have to be limited by the carry over effect of antibacterial solutions. Carry-over effect means that the medicament, in active form, follows along with the sample

into the dilution series and even to the culture plate (or liquid culture), where surviving microbes are calculated. A high enough concentration of the disinfectant, in such a situation, can cause a false negative result: the microbes are not killed, but residual medicament in the culture media prevents their growth by a bacteriostatic effect. Thus, carry-over, if undetected, gives a too positive picture of the antibacterial effectiveness of the medicament. Various inactivating agents are used to prevent the effects of carry-over. Citric acid has been used in the root canal to neutralize $Ca(OH)_2$, sodium thiosulfate neutralizes NaOCl, and a mixture of Tween 80 and alpha-lecithin inactivates chlorhexidine (CHX). However, in the literature, this point is controversial because in numerous studies, carry over effect of NaOCl solution is considered as negligible at concentration of 3%. In the study of Rossi-Federer et al. [85], the experiment to determine the effect of carry-over of NaOCl resulted in similar colony counts for the samples from teeth irrigated with NaOCl and water indicating that carry over of NaOCl had no noticeable effect in his experiment.

According to Haapasalo et al. in 2007 [86], this effect is observed in vitro with more powerful disinfectant solutions such as the MTAD. On the other hand, most of the studies showing this effect are made on models (tooth discs, extracted teeth) infected by planktonic bacteria or mono-bacterial biofilm. In the case of multiple bacterial biofilms, this effect seems to be minimized. An other effect, described by Haapasalo [84] is the inhibitory effect of dentin on the antibacterial medicaments: Hydroxyapatite, which is the main inorganic component of dentin, showed a similar effect on calcium hydroxide as dentin, preventing the killing of *E. faecalis* [82,87]. Although the result does not exclude the role of the organic part of the dentin in calcium hydroxide inactivation, it emphasizes the importance of the inorganic dentin components. The strong effect of dentin on the antibacterial action of a saturated calcium hydroxide solution can probably be attributed to the buffering action of dentin against alkali [87]. Studies with dentin powder have shown that dentin has an inhibitory effect on the antibacterial effectiveness of 1% sodium hypochlorite. Dentin powder (18%, w/v) greatly delayed the killing process of *E. faecalis*, which was used as a test organism [87]. When hypochlorite was preincubated with dentin in a closed test tube for 24 h before adding the bacteria, killing all of the bacteria required 24-hour incubation with hypochlorite, whereas after 1-hour incubation all of the bacterial cells were still viable [85—88].

In a pilot study carried out prior to this study to assess our biofilm growth and concentrations of each strain, we used tooth discs on which our biofilms grew. We treated them with the same techniques to test removal of our biofilm. We did not notice any carry over effect following the NaOCl treatment. The SEM observations of samples clearly confirmed the absence of bacteria or trace of biofilm in NaOCl treated root canals (Fig. 4A), while in those treated with the Diode Laser and the Aseptim system, there were still either bacteria present in the tubuli, or traces of biofilm (Fig. 4B).

Also found in this study was the abundant presence of *E. faecalis* and some chains of *S. salivarius* that were observed using the Gram staining test (after the photodynamic therapy of both PDT methods).

This proves the previously described resistance of *E. faecalis* to treatments and the risks of treatment failures due to the presence of this bacterium [8,14—21]. But the fact that all other bacteria were not cultivable does not mean necessarily that they were killed by Photodynamic therapy or even by Ultrasonic irrigation methodology. This could be related to non-viable bacterial cells already there before treatment. This cannot be assessed precisely in the present experimental conditions. Biomolecular techniques as real time PCR [86] or FISH (rRNA 16s) [83] methods have to be used to determine what species are persistent.

Conclusion

Under the conditions of this study, PUI using EDTA and NaOCl solutions was found to be effective in order to eliminate an in vitro-created seven days-old biofilm. PDT is not effective in this purpose. Photodynamic therapy using a non-collimated light and an optimized concentration of photosensitizing agent may have a role in disrupting the biofilm and decreasing the bacterial load of intact microbial biofilm; these effects are statistically inferior to those obtained by passive ultrasonic irrigation.

The present study suggests further in vitro and in vivo studies as:

- Understanding the real interaction of root canal instrumentation on bacterial biofilm.
- The establishment of protocols using a continuous injection of photosensitizer and simultaneous activation by light to overcome the energy absorbance modification related to the photosensitizer's color changes during activation.
- The interest of using Er:YAG Laser before Photodynamic therapy to remove the smear layer and increase wettability of root canal walls.

Conflict of interest

The authors declare to do not have any conflict of interest.

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ORIGINAL ARTICLES

Evolution of the role of phototherapy during endodontic decontamination

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A microbe free root canal space before obturation leads to higher success rate and conventional chemo-mechanical debridement might not achieve this goal completely.
First trials of laser in dentistry started from surgical intervention on caries and bones of oral cavity and extended to prepare cavities and even shaping root canals. Afterward lasers were implicated soon into direct debridement of root canal space.
Anyhow failure of laser to remove debris totally from root canal space is demonstrated recently, additionally it might lead to damages to surrounding tissues or inorganic material of root canal if be used without precaution. Nowadays the theory of light assisted protocols became another start point for laser in endodontics. Laser has been introduced as an adjuvant to conventional debridement of root canals. We used Medline search engine to collect scientific publications to edit this review article in purpose of revealing the evolution of laser position from an ultimate cleaning methodology to an adjuvant to conventional root canal disinfection protocols.

Key words: Decontamination · Diode · Endodontics · Er:YAG · LASER, PDT

Introduction

The conventional endodontic treatment has high success rate. [1)] Nevertheless, this treatment may fail. A great part of failures occur when treatment procedures have not reached an acceptable measure for the control and elimination of infection. [2-4)] It is well-established that the mechanical debridement followed by chemical irrigation removes the bulk of the infecting microorganisms, but because the infection of root canal system is three dimensional, the residual bacteria are still detectable in an important area of the teeth just before filling the root canal. [5-7)] Certain operative problems such as insufficient instrumentation, missed canals or inadequate coronal restoration might lead to periapical pathologies. [7)] The complexity of root canal anatomy is another obstacle to obtain an ultimate bacterial free root canal system, which makes chemo-mechanical debridement ineffective. [8)] This complexity incorporates small accessory canals, isthmuses and dentinal tubules that do not allow direct access during the biomechanical preparation because of their location and/ or their small diameters. [9)]

The success rate of endodontic treatments is higher when the canal is bacterial free at the time of obturation. [10)] This was reported to reach 68 – 85% when so-called rigorous radiographic standards were used. The success rates were approximately 66%, 75%, 77%, and 85% for interventions carried out by general dental practitioners, undergraduate students, graduate students, and specialists, respectively. [11)] Previous studies have shown that in a microorganism free root canal environment just before filling- treatment may achieve a success rate up to 94%. However, because the presence of the bacteria, this rate can be diminished to 68%. [4)] The antimicrobial sensitivity or resistance of the polymorphous micro flora, which includes anaerobic, facultative anaerobic and aerobic bacteria, may deter-

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mine the outcome. [9]

A decade after laser invention by Maiman, [12] Weichman introduced laser, for the first time, for endodontic therapy using a 10.6 μm CO_2 laser and then a 1064 nm Nd:YAG laser; [13] although the results were not encouraging but the original idea brought the bravery for future studies.

After one decade of academic silence, during 1980s, Melcer, using laser in endodontics contributed lots about laser knowledge to dental sciences. [14-17] These studies were continued by other researchers like Miserandino *et al.* [18] Likewise, other wavelengths such as 308 nm, [19] 488 nm, [20] 1064 nm [21] adopted in endodontical treatment studies.

This review throws a glance at evolution of application of different kind of lasers used with different wavelengths in the field of endodontic disinfection and aims to answer whether laser is just a "dreaming star wars" in dentistry or it is real future of the field. [22]

Scientific publications concerning "Endodontics" and "Laser assisted disinfection" in PubMed database could be found from 1971. Using (Endodontics OR Root canal) AND Laser MeSH terms, 1047 articles in English language were found until end of 2014. This search could be narrowed down for defined period of time by using publication date filter of search engine **(Table 1)**.

A huge wave of scientific reports raised in last 5 years. In addition, this search could be restrained for articles that targeted root canal disinfection. The search should be formulated as (Endodontics OR Root Canal) AND Laser AND (Disinfection OR decontamination OR antimicrobial OR Bactericidal), which narrows down the results to 306 published articles in the selected timespan **(Table 1)**. The antimicrobial characteristic of laser was known for many years, but in last 10 years the attention of researchers was specially brought to utilize this device to eliminate root canal bacteria **(Fig. 1)**.

Diode lasers comes to the top of the list of the lasers when their possible ability to decontaminate root canal system is evaluated by publications indexed in PubMed. Anyhow, by a manual revision in suggested articles we found out the search results was included

Table1: Number of publications related to Endodontics and Laser and disinfection from 01.01.1971 to 31.12.2014.

	Total	1971-1981	1982-1992	1993-2003	2004-2009	2010-2014
All Articles about Endodontics and Laser	1047	4	55	314	285	403
Articles about Endodontic disinfection and Laser	306	0	4	44	89	174

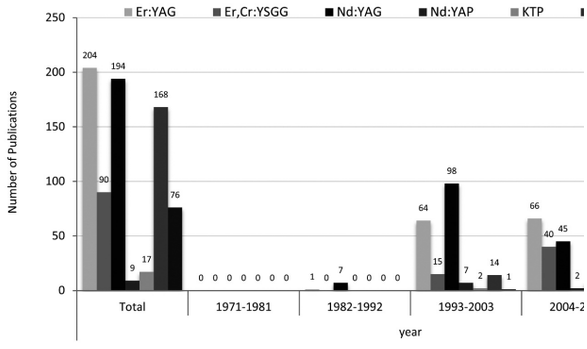

Figure 1: Distribution of scientific publications about "Endodontics" and "Laser" according to specific wavelength and PDT from 01.01.1971 to 31.12.2014.

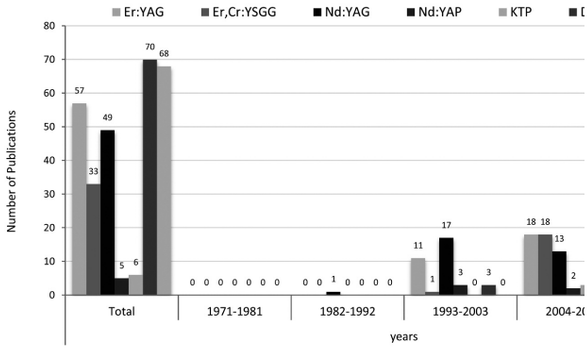

Figure 2: Distribution of scientific publications about "Endodontic Disinfection" and "Laser" according to specific wavelength and PDT from 01.01.1971 to 31.12.2014.

many articles for photodynamic therapy in which diode laser is used as activator of photosensitizer **(Fig. 2)**. It is important to note that the lack of homogeneity in keywords and MeSH terms used in articles may result in many unrelated articles among PubMed search results. Therefore, a manual review of results is compulsory. However, photodynamic therapy came along to be an interesting subject in terms of root canal decontamination in the last 5 years **(Fig. 2)**.

Nd:YAG laser is still hired as machine of choice in certain endodontic protocols, however, the golden period of this laser was in 1990s and beginning of 2000s when the majority of published scientific reports in this field contributed to this wavelength. KTP laser is used anyhow to clean root canal space, but as the first application of 532 nm laser is treatment of vascular lesions, the capability of this device to decontaminate root canal pathogens was ignored **(Fig. 2)**.

Erbium doped lasers are wavelengths which brought attention of researchers from their emergence till now. Er:YAG was the first laser system cleared by FDA in 1997 to treat dental decay and subsequently implicated to endodontic disinfection, thanks to its physical properties specially its affinity to H_2O molecules and superficially limited activity.

Er:YAG

Erbium doped lasers are machines with wide range of application; the laser beam arrives easily in most distal region of oral cavity using sapphire tips. Erbium doped yttrium aluminum garnet (Er:YAG) laser hand pieces are equipped with specially designed fiber to bring the efficiency of this laser light inside root canal space. [23,24] Er:YAG laser has been used more than any other wavelengths to study the dentin-laser interaction in the field of endodontics **(Fig. 1)**. Topçuoğlu using inductively coupled plasma-atomic emission spectrometry (ICP-AES), demonstrated that there is no change in mineral content of dentin inside the root canal after irradiation with Er:YAG laser. [25] Application of Er:YAG laser for intra radicular disinfection, presents optimal results in microbial infection reduction from endodontic space which has been confirmed by Mehl *et al.* [24] These properties are dose-dependent and not selective to any bacterial species. Er:YAG with a wavelength of 2940 nm has highest absorption rate in water and hydroxyapatite. [26] Thus, Er:YAG is able to disrupt organized biofilms and explode the bacterial cells through a well-established mechanism of action by production of explosive vapor.

Er:YAG laser could reduce bacterial load from infected root canal [27] and it is an efficient tool for removing the smear layer. [28] This laser could clean root canal dentin from smear layer and leaves the dentinal tubules open without any harm to the inorganic structure of root canal wall and/ or surrounding periodontal tissue. Although, this result needs a direct contact of optic fiber with root canal walls; it might be a source of thermal damage if it is not controlled. [29] When root canal irradiation is coupled with a chemical antimicrobial agent like NaOCl, it leads to a total eradication of microbes from root canal space. Such an activation with an output power of 0.3 W, 15 Hz for 3 period of 20 seconds could ensure an ideal result in terms of endodontic decontamination. [30] Erbium doped lasers initiate by their explosive nature of action, a cavitation effect inside irrigation solutions passing the root canal; [31] this is the principle of laser activated irrigation (LAI). Matsumoto suggested that a successful root canal treatment, especially in narrow curved root canals, might be achieved when laser activated irrigation is used for disinfection. [32] Likewise, considerable reduction of output power of laser to activate irrigants is another important advantage of LAI. Nowadays, researchers are looking for getting benefits from sub-ablative energy of Er:YAG laser to activate other irrigation solutions inside the root canal. For example erbium doped laser might increase the efficiency of EDTA to remove smear layer. [33, 34] Interestingly it is reported in the literature that ultrasonic irrigation could not encourage chelating character of EDTA. [35]

De Moor evidenced that LAI using an erbium doped laser with low energies (75 mJ) and an intermittent flushing technique (4 times 5 seconds) is as efficient as passive ultrasonic irrigation (PUI) to remove debris from root canal space. [36] Recent innovation in this field is PIPS or photon induced photoacoustic streaming. The mechanism of action is nearly the same than in passive ultrasonic irrigation. The laser produces acoustic waves by low energy pulses and could help irrigants to distribute inside root canal space. Furthermore, DiVito demonstrated that a stationary positioning of the fiber at the canal orifice during PIPS is enough to excite the irrigant even in apical region. [37-39] Recently, eradicative ability of PIPS against mono-species bacterial contamination was confirmed, [40] and this would be a promising technique for future application in the clinical trials.

Er,Cr:YSGG

Erbium, chromium yttrium scandium gallium garnet

(Er,Cr:YSGG) is another member of the erbium doped lasers family. Er,Cr:YSGG with a wavelength of 2780 nm is better absorbed in hydroxyapatite and like Er:YAG, it has a great affinity for H_2O molecules. [26] Identical to Er:YAG, Er,Cr:YSGG laser does not cause thermal damages to dentin that makes this device suitable for root canal smear layer and debris removal. [41]

Eldeniz demonstrated the bactericidal effects of Er,Cr:YSGG laser. [42] This is a time and dose dependent procedure. For instance, 60 seconds irradiation of canal with a 2 W power is as effective as 5% NaOCl irrigation in terms of bacterial load reduction. [43] An ultimate bacterial free canal is not achievable using Er,Cr:YSGG alone, hence it brings the attention of the researchers to test its ability to enhance the effect of other cleansing agents. De Moor *et al.* showed the Er,Cr:YSGG laser is as effective as Er:YAG laser or passive ultrasonic irrigation to activate NaOCl. [36, 44] They demonstrated how a 2780 nm laser is efficient to remove debris from root canal space when the irrigant is triggered with energy of 75 mJ and for 20 seconds (4x5 seconds). Likewise, Bago Jurič showed this activation of NaOCl is efficient in terms of total bacterial charge reduction. [45] They demonstrated the LAI granted more bacterial free sample at the end of treatment than any other test groups. A clinical trial by Martins revealed that Er,Cr:YSGG assisted irrigation of teeth with periapical periodontitis is as effective as conventional irrigation protocols. [46] A follow up of 12 months showed a considerable reduction in peri-apical index (PAI) scores.

Nd:YAG

Nd:YAG is a near infrared laser with a wavelength of 1064 nm and widely used in soft tissue surgery. It is highly absorbed by hemoglobin and dark-colored tissue. [26] Antimicrobial efficacy of Nd:YAG was investigated first by Levy, Rooney and Hardee. [47-49] They all demonstrated a direct relation between dose of irradiation and the bacterial load reduction. Later on, Moshonov and Rahimi demonstrated that Nd:YAG laser could partially clean smear layer and remove bacterial colonies. [50, 51] They proposed a totally cleaned root canal space from bacterial infection could be achieved when Nd:YAG laser and NaOCl are used in synergy.

Hence, Nd:YAG laser is a thermic laser and all bacteria present in root canal microbial biofilm are not pigmented; and Nd:YAG laser is able to inactivate bacteria by local rise of temperature leading to denaturation of enzymes and boiling the liquid presented in canal. [52] Confirming Mehl's study, [53] Meire evaluated the efficiency of 1064 nm laser in terms of bacterial decontamination. They used low parameters (total 80 J) to kill *Enterococcus faecalis*. [54, 55] As the microorganism is not pigmented so the light will pass through the bacterial cytoplasm, therefore the possible mechanism of action is photo-thermic effect of laser on the environment around bacteria. However, the desired effect was not obtained because of inefficiency of laser parameter. Only by increasing the power of Nd:YAG laser beyond clinically allowed dosage, bacterial reduction was observed. Nevertheless, Pirnat demonstrated the near infra-red lasers like Nd:YAG and high power diode lasers could destroy pigmented bacteria presented in the endodontic biofilm. [56]

It is well established in literature that thermal elevation produced by Nd:YAG laser is more than 5.5°C which is physiologically acceptable [57] and could cause unrecoverable response by surrounding tissue. [49, 58, 59]

In addition, Cox and Türkmen [60, 61] showed uncontrolled Nd:YAG laser irradiation may cause many undesirable effects on root canal walls. Smear layer could be formed and occlusion of some dentinal tubules linked with structural modification of dentin (recrystallization, melting and carbonization) may also be observed. Another study indicated that a direct application of Nd:YAG laser could alter structure of dentin even with lower energies (25-50 J/cm^2). [62]

Nd:YAP

Another neodymium doped laser is neodymium doped yttrium aluminum perovskite (Nd:YAP) with a wavelength of 1340 nm. Blum was the first to propose that Nd:YAP inhibits growth of *Streptococcus mitis* with a frequency of 30 Hz and an energy of 300 mJ. [63] Same authors in a further study suggested that the combination of subsonic irrigation and Nd:YAP laser irradiation give better results. [64] Moreover, Moshonov showed Nd:YAP laser could be implicated in root canal cleansing protocol. [65] They observed a cleaner root canal when Nd:YAP laser was involved as an adjuvant to conventional endodontic preparation methods without changing molecular composition of dentin.

Rather than a better absorption in water, Nd:YAP laser has the same photo-thermic mechanism of action when compared with Nd:YAG laser. The temperature elevation during Nd:YAP laser irradiation may become considerable and lead to damages identical to Nd:YAG laser, [66] as use of cooling water and an adapted protocol inside the root canal is required.

KTP

The effect of potassium titanyl phosphate or KTP laser on dentin structure has been studied from its very first emergence in market. Tewfik evidenced that KTP laser is able to make changes on dentin inside the root canal. The parameters were adjusted to 1 W for 1 second or 0.5 W for 0.5 second, because thermal elevation is about 5°C and could not damage periradicular tissues. [67] However, KTP laser resulted in crack formation in dentinal tubules even when using a safe range of energy. In contrast, Machida reported KTP laser is able to remove smear layer and debris from apical dentin using safe parameters (1 W × 6 s, 5 Hz, repeated 5 times and at 2 W x 3 s, 5 Hz, repeated 5 times). [68] This clarified that power and working time influence the outcome of laser treatment on root canal walls. In addition, it is important to consider resting time to avoid any cumulative thermal damage. [69]

KTP is capable to reduce bacterial load, but its efficiency is inferior to the results from NaOCl alone. [70] Since we know that total eradication of bacteria from root canal space is not possible, the ideal protocol seems to be the conjunction of both chemomechanical debridement and laser irradiation. [71]

Diodes

Another group of lasers are semiconductor or diode lasers. Diodes rapidly found their way into laser-assisted dentistry thanks to their small size, ease of use and affordable price. Diode lasers with a big range of different wavelengths from visible to infrared contributed a lot to the field of endodontics especially endodontic disinfection.

For the first time in 1997, Moritz examined an 810 nm diode laser ability to kill root canal bacteria in *in vitro* and *in vivo* studies. [72, 73] Furthermore it was verified that this wavelength is able to decontaminate deep layers of radicular dentin which is an important point to overcome the 3-dimensional aspect of root canal space. [74] It could be explained by the phenomenon that diode laser is not absorbed in water or inorganic material which leads to scattering of laser beam into deeper layer of dentin. [75] Same results were obtained by 830 nm, [76] 940 nm [77] and 980 nm [78] diode lasers. These findings demonstrated different wavelengths of diode laser are all effective in terms of reduction of bacterial load.

da Costa Ribeiro showed photo-thermic damage of diode laser is negligible when reasonable parameters are used. [79] They showed thermal elevation caused by this laser is up to 8.6°C in continuous mode and between 1.2 to 3.3°C in pulsed mode which is crucial to prevent any harm to periodontal tissue. However, still this little thermal change could result in closure of dentinal tubules. To prevent any cumulative thermal effect, it is mandatory to consider recovery time during diode laser irradiation interval. Respecting this rule, diode laser could be counted as a safe laser with different power level [80] which could raise up to 3 W if it has been used in pulsed mode. [81] Stationary contact of fiber tip with dentin leads to overheating and melting the dentin and further thermal damage to surrounding tissues. For this reason constant moving of fiber during irradiation is fundamental. [80-82]

The morphological changes caused by diode lasers in root canal dentin are power dependent. Diode lasers removes smear layer at 1.5 W, but increasing power leads to extreme changes in dentin like melting of surface dentin. [83-85] Nevertheless, despite any morphological alteration initiated, diode lasers have no adverse effect on structural characteristic of mineral matrix of root canal. [85] Smear layer elimination could be achieved using diode laser in conjunction with some irrigation solutions. However, the kind of irrigation solutions determine the outcome of treatment. [84] For example, synergy of diode laser and NaOCl produces smear layer, but combination of diode laser and EDTA results in smear layer elimination. [86] In another study, activation of EDTAC® by a 940 nm diode laser seems to be an ideal protocol to remove smear layer, but irradiation of hydrogen peroxide with same parameters develops no significant effects on smear layer. [87]

Diode lasers might contribute to activation of irrigation solutions thanks to their high frequency that reaches to 20-50 KHz. This property of diode laser could promote cavitation effect inside root canal irrigants and subsequent better debridement. [88] Neelakantan demonstrated the diode laser is as efficient as Er:YAG laser to activate irrigants in the root canal and disturb microbial biofilm. [34] However, in another study it has been indicated that there are some differences in quality of explosive vapor initiated by diode and Er:YAG lasers. The peak of cavitation and bubbles formation with diode laser happens with a delay after irradiation starts. Due to slower fluid movement during irradiation by diode laser, the possibility of irrigants extrusion beyond dental apex is less than that of Er:YAG laser. [89] However, there is a proportional relationship between irrigant volume in root canal spaces and the power needed to activate it. The form of the fiber may enhance the outcome. George introduced a modified

light delivery fiber called honeycomb tip to enhance the agitation and cavitation properties of diode laser. Using this fiber, diode laser makes more bubbles toward root canal walls and less in apical direction. As well the time needed to achieve peak cavitation is inferior to that of plain fiber. However, the factor of power is playing an important role. The cavitation occurs always in a power level more than 2 W. [89]

PDT

Photodynamic therapy (PDT) is a medical treatment which utilizes light to activate an agent called photosensitizer in presence of oxygen. Many Photosensitizers and different from chemical photosensitizing agents like toluidine blue, [90, 91] methylene blue [92, 93] to natural photosensitizers like curcumin [94] have been examined. The outcome of PDT in different protocol with different activating lights has been tested. However, using different types of laser does not improve the outshot of PDT. [95]

As a pioneer, Wilson mentioned bactericidal effects of PDT in dental diseases. [96, 97] Afterward, the potential role of PDT in total eradication of root canal infection was outlined in many researches. [98-101] The number of scientific publication on this topic is increasing in recent years **(Fig. 2)**. A high degree of safety of PDT could be a reason for such significant progress. [102]

The results of studies are controversial. It is demonstrated conventional photo-activated disinfection (PAD) could not disrupt polybacterial plaque but it might reduce a mono-species biofilm made of *Enterococcus faecalis*. [103] Anyhow, Yao suggested that PDT is more effective on planktonic form of bacteria than their biofilm state inside root canals. [104] Thus, the conventional disruption of intracanal biofilm before PDT is critical for success of treatment. [92] Clinical trials of Jurič very well demonstrated application of PDT after conventional debridement of root canal space to obtain a bacterial free canal. [105]

Concerning biofilm – PDT interaction, it is evidenced that different PDT protocols could not be effective without pretreatment of biofilm with conventional decontaminating agents like NaOCl. [106, 107] Anyhow, promising results are reported especially when more incubation time with Photosensitizer and longer irradiation is applied. [99, 108] An *in vitro* study by Komine and Tsujimoto exhibited that the highest amount of singlet oxygen generated through photodynamic therapy could be achieved after longest time of irradiation. [93] Regarding working time, these conditions seem to be difficult to apply in daily clinical procedures particularly when treating multi rooted teeth. To overcome this problem, PDT in 2 visits could be beneficial. [109, 110] However, there are controversies about irradiation time; interestingly, Yildirim reported that there is no difference between 1 minute and 4 minutes irradiation of photosensitizers. [111]

The quality of activating light may also influence the outcome. For example, emitters or light diffuser optic fiber could scatter unidirectional light of diode laser to ensure maximum reach even in the most distal zones of root canal. [99, 112] Light emitting diodes (LED) showed to be promising in terms of activating the photosensitizers. [113] LED light travels easily in all direction with no need to move the optic fiber. This may lead to better results in terms of bacterial load reduction rather than PDT utilizing a unidirectional diode laser **(Fig. 3)**. [107]

Recently, another study by Sabino demonstrated different effect of a same light source on a bioluminescent species of *Candida albicans*. [114] When microorganisms are irradiated with laser using a light diffuser fiber, the reduction in bacterial load is 100 times more than using a normal optic fiber.

CO_2

Zakariasen was the first to propose an exposure of 1 second to a 10 W CO_2 laser beam leads to bacterial death, [115] but later on its effects on root canal dentin were studied. [116] Takeda demonstrated that by placing a conical tip inside the root canal and using 3 W, CO_2 laser could remove smear layer partially. [28] However, in some regions some undesired effects like burning, melting, recrystallization and glazing of dentine were observed too and confirmed by other studies. [61] Likewise, an *in vitro* studies proposed that irradiation of root canal space by CO_2 laser using a hollow fiber at 30 J energy in either pulsed or super-pulsed mode may lead to damage to tissues beyond the apex. [117] Additionally, CO_2 laser light could not be transferred via an optic fiber [118] and trials to deliver the energy of a defocused laser beam inside root canal space also failed. [119] Regarding difficulties in irradiation of CO_2 laser and subsequent undesirable effects, logically and at the present time, there is no more interest to deploy such wavelength in terms of root canal disinfection.

Conclusion

If we look to the milestone of laser in endodontics, the beginning of the way is marked with issues regarding

energy leading to undesirable effects on dentin and periradicular environment. Thanks to constant evolution of laser technology and progressive scientific experiments, nowadays it is well documented that lower energies could provoke the intended objectives.

Laser assisted endodontic decontamination in conjugation with conventional chemical solutions activated by laser should be favored over direct use of laser to remove bacterial biofilm from root canal space. Both erbium doped lasers through explosive vapor and diode lasers with short pulses and high frequencies could produce cavitation effect inside irrigation solutions and result in a 3-dimensionally clean root canal walls, free from smear layer and debris even in apical region. This does not need high energies and could prevent unwanted thermal and physical side effects to root canal dentin or other surrounding tissues.

It is known that common root canal irrigation solutions could absorb light in different wavelengths from 513 nm for chlorhexidine (CHX) to 2200 nm for citric acid. In addition, different irrigants have high absorption rate for wavelengths higher than 2500 nm. These optical properties make all tested irrigants qualified for LAI. [120] The important point is to match the right irrigant with available wavelength or vice versa.

Mechanism of photodynamic therapy is quite different from physical interaction of LAI which is based on wave production in irrigants or direct root canal decontamination by near infra-red lasers which work by thermal elevation. PDT is a pure chemical reaction and it could not clean totally the root canal space from microbial biofilm, alone. It should be kept in mind that photodynamic therapy is an adjuvant and not a substitute to conventional chemo-mechanical debridement. Thus, there is no resistance or selectivity toward PDT, it might be helpful removing bacteria from root canal space especially microbial flora of chronic endodontic disease or/ and those resistant to antibiotics.

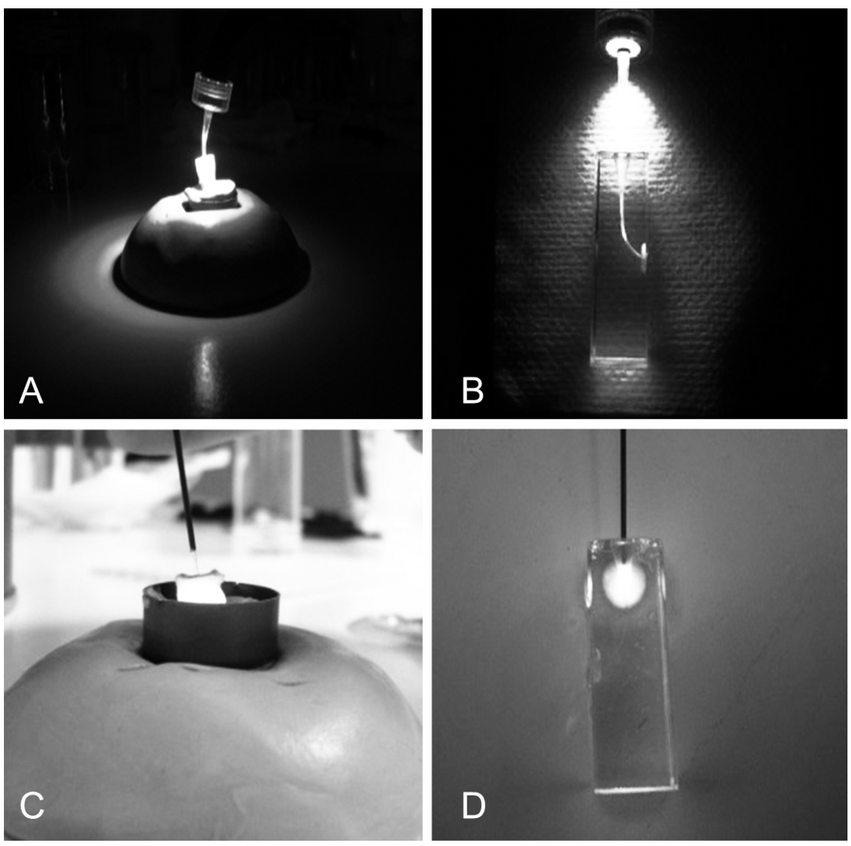

Figure 3: Quality of light may be effective on the outcome of PDT. A, B: Non-collimated, intense LED light could travel in all direction and does not need to be moved to excite photosensitive agent. C, D: Collimated 650 nm diode laser light needs to be moved through the canal to activate photosensitizer. [107]

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