Review
Biofilm in Endodontics: In Vitro Cultivation Possibilities, Sonic-, Ultrasonic- and Laser-Assisted Removal Techniques and Evaluation of the Cleaning Efficacy

Uros Josic 1, Claudia Mazzitelli 1, Tatjana Maravic 1, Ales Fidler 2, Lorenzo Breschi 1,* and Annalisa Mazzoni 1

1 Department for Biomedical and Neuromotor Sciences, University of Bologna-Alma Mater Studiorum, 40139 Bologna, Italy; uros.josic2@unibo.it (U.J.); claudia.mazzitelli@unibo.it (C.M.); tatjana.maravic@unibo.it (T.M.); ales.fidler@mf.uni-lj.si (A.M.)
2 Dental Clinic, Faculty of Medicine, University of Ljubljana, 1000 Ljubljana, Slovenia; annalisa.mazzoni@unibo.it
* Correspondence: lorenzo.breschi@unibo.it

Abstract: Incomplete and inadequate removal of endodontic biofilm during root canal treatment often leads to the clinical failure. Over the past decade, biofilm eradication techniques, such as sonication of irrigant solutions, ultrasonic and laser devices have been investigated in laboratory settings. This review aimed to give an overview of endodontic biofilm cultivation methods described in papers which investigated sonic-, ultrasonic- and Er:Yag laser-assisted biofilm removal techniques. Furthermore, the effectiveness of these removal techniques was discussed, as well as methods used for the evaluation of the cleaning efficacy. In general, laser assisted agitation, as well as ultrasonic and sonic activation of the irrigants provide a more efficient biofilm removal compared to conventional irrigation conducted by syringe/needle. The choice of irrigant is an important factor for reducing the bacterial contamination inside the root canal, with water and saline being the least effective. Due to heterogeneity in methods among the reviewed studies, it is difficult to compare sonic-, ultrasonic- and Er:Yag laser-assisted techniques among each other and give recommendations for the most efficient method in biofilm removal. Future studies should standardize the methodology regarding biofilm cultivation and cleaning methods, root canals with complex morphology should be introduced in research, with the aim of simulating the clinical scenario more closely.

Keywords: biofilm; endodontics; irrigation; sonic; ultrasonic; laser

1. Introduction
The elimination of pathogen microorganisms is routinely performed in conventional daily dental practice, being imperative for a predictable endodontic therapy [1–5]. The persistence of intraradicular infection due to incomplete bacteria removal or pathogen’s leakage within the canals is the most common cause of failure of root canal therapy. Enterococcus faecalis (E. faecalis), a Gram-positive, facultative anaerobic microorganism is most frequently detected inside the root canal, with a prevalence of 77% in persistent endodontic infections [6]. The ability of E. faecalis to form endodontic biofilm has been well studied and established [7–9]. Biofilm formation provides E. faecalis with better protection to environmental threats as well as enhanced tolerance to antimicrobials [10]. Literature reports the presence of E. faecalis biofilm in medicated root canals and its survival in conditions of severe alkaline stress (pH = 11.2) [7,11]. In addition, the complexity of the root canal system (isthmus, lateral canal and apical ramification) complicates traditional chemomechanical debridement and makes biofilm removal challenging [3,12,13].

Typically, different irrigant solutions are used between instrumentation during orthograde as well as retrograde endodontic therapy [14], with the goal of removing bacteria in the endodontic lumen, dissolving the smear layer, and disinfecting the canals. Sodium
hypochlorite (NaOCl) used alone or in combination with other disinfectants, such as EDTA, citric acid, chlorhexidine etc., represents the most used endodontic irrigant. Notwithstanding the effective disinfectant potential of NaOCl, it is not able to completely remove the smear layer created during instrumentation and cases of reinfection have been observed over time.

In an attempt to enhance the effect of irrigants, provide a biofilm-free surface and make the endodontic treatment more predictable, sonic techniques (<20,000 Hz) have been introduced [15–17]. It was noteworthy that the sonic technique alone was unsuccessful in biofilm eradication from the root canal surface [16]. Further attempts to remove biofilm completely have been made and include investigation of ultrasonic (>20,000 Hz) effect in combination with various irrigants [18–20]. This decontamination strategy has been shown to remove the intracanal biofilm more efficiently when compared to conventional methods of irrigation [21]. Still, similar to sonic techniques, no complete biofilm removal could be achieved by using the ultrasonic system [22].

In the early 2000s, the application of Er:Yag lasers was suggested as a means of disinfecting root canal space [23]. Since then, studies focused on the investigation of the efficacy of the Er:Yag laser family in biofilm removal. It is considered that the bactericidal potential of Er:Yag laser is related to the evaporation effect of cellular water, which expands quickly during the laser pulse and leads to the disintegration of bacterial cell wall [16,24–26].

Another emerging technique, photon-induced photoacoustic streaming (PIPS), which is provided by Er:Yag lasers, has shown promising results in providing the removal of the biofilm from the root canal surface [15,27]. This technique implies positioning of the PIPS tip inside the pulp chamber with a consequent activation of irrigants inside the root canal through a profound photoacoustic and photomechanical phenomenon. Each impulse created by the PIPS tip is absorbed by the water molecules, further creating a strong “shock wave” that leads to the formation of an effective streaming of fluids inside the canal while avoiding side effects, such as high temperature [28].

The contemporary biofilm removal techniques, such as sonic-, ultrasonic- and Er:Yag laser-assisted techniques, have become increasingly popular over the last years. Consequently, this paper aimed to provide an overview of the published work discussing in vitro endodontic biofilm cultivation methods associated with sonic-, ultrasonic- and laser-assisted removal techniques. Furthermore, the effectiveness of these removal techniques was discussed and an overview of the evaluation of the cleaning methods was provided.

2. Materials and Methods

An extensive literature search of articles was performed by two investigators (U.J. and C.M.) using the electronic databases PubMed and Scopus. The following keywords and strings were used: ultrasonic OR YSGG OR YAG OR ER OR Er,Cr AND biofilm AND root canal OR endodontics. No time restrictions were set, and the only filter applied was articles published in the English language. The last search was conducted in February 2022 and it yielded 122 titles. Abstracts were read and excluded if the reported article did not have any possible applications in endodontic biofilm removal achieved by the sonic, ultrasonic and Er:Yag devices. Finally, 41 articles were fully read and included in the present paper.

3. Results and Discussion

Findings from the included articles were sorted into the following sections:

1. Biofilm cultivation;
2. Biofilm removal techniques;
3. Evaluation of biofilm removal.

3.1. Biofilm Cultivation
3.1.1. Monospecies Biofilm

The endodontic bacteria are usually organized in biofilm communities which are present not only in the main canal, but in the overall root canal system [29]. The extracel-
lular matrix of the biofilm offers bacteria higher survival rates in challenging growth and environmental conditions [11]. In order to mimic conditions that are well established within infected root canals, authors put their efforts into growing *E. faecalis* biofilms since it is considered to be the leading pathogen associated with failed endodontic treatment. Among the microorganisms commonly isolated in the endodontic space, this microorganism represents the leading pathogen largely associated with failed endodontic treatment [7].

According to the reviewed articles, potential double origins, lab-adapted strains [15,16,18–21,24,26,30–46] and clinically isolated *E. faecalis* were noted [25]. Types of *E. faecalis* strains which are most widely used by different authors and bacteria origin are shown in Table 1. *E. faecalis* strain isolated from root canal of pulpless teeth is available, but only two authors reported using it [24,30], while the majority of the studies used strains of *E. faecalis* isolated from different tissues and fluids.

**Table 1.** Details of the biofilm cultivation methods and cleaning evaluation techniques of the reviewed studies. CFU: colony forming unit; SEM: scanning electron microscope; CLSM: confocal laser scanning microscope; PCR: polymerase chain reaction; TEM: transmission electron microscope.

| Author, Year | Microorganism | Period of Incubation (Days) | Substrate | Methodology Assessment |
|--------------|---------------|-----------------------------|-----------|-----------------------|
| Noiri et al. (2008) | *E. faecalis* ATCC 19246 | 21 | HA disc | CFU, SEM |
| Shen et al. (2010) | Subgingival plaque | 21 | HA disc | CLSM |
| Bhuva et al. (2010) | *E. faecalis* OMGS 3202 | 3 | Human dentin | SEM |
| Alves et al. (2011) | *E. faecalis* ATCC 29212 | 30 | Human dentin | CFU, PCR |
| Peters et al. (2011) | Oral bacteria | 6–8 intraorally, 15 in vitro | Human dentin | CFU, histology |
| Grundling et al. (2011) | *E. faecalis* ATCC 29212 | 50 | Animal teeth | SEM |
| Meire et al. (2012) | *E. faecalis* ATCC 10541 | 1 | Human dentin | CFU |
| Case et al. (2012) | *E. faecalis* ATCC 29212 | 12 | Human dentin | CFU |
| Halford et al. (2012) | *E. faecalis* ATCC 29212 | 7 | Human dentin | CFU |
| Cheng et al. (2012) | *E. faecalis* ATCC 4083 | 28 | Human dentin | CFU and SEM |
| Seet et al. (2012) | *E. faecalis* ATCC 700802 | 28 | Human dentin | SEM |
| Bhardway et al. (2014) | *E. faecalis* ATCC 29212 | 3 | Human dentin | SEM |
| Niazi et al. (2014) | *E. faecalis* OMGS 3202, *Propionibacterium acnes*, *Staphylococcus epidermidis*, *Actinomyces radinisdensis*, *Streptococcus mitis* | 14 | Hydroxyapatite discs | CFU, CLSM |
| Ordinola-Zapata et al. (2014) | Oral biofilm | 3 days intraorally, 2 days in vitro | Animal dentin | SEM |
| Macedo et al. (2014) | Biofilm mimicking with hydrogel | / | Solidifying polydimethylsiloxane | High-speed camera |
| Al Shahrani et al. (2014) | *E. faecalis* ATCC 4083 | 21 | Human dentin | CFU, SEM |
| Olivi et al. (2014) | *E. faecalis* vancomycin-resistant | 28 | Human dentin | SEM |
| Nelaakantan et al. (2015) | *E. faecalis* ATCC 29212 | 21 | Human dentin | CFU, CLSM |
| Author, Year     | Microorganism                        | Period of Incubation (Days) | Substrate | Methodology Assessment                      |
|------------------|--------------------------------------|----------------------------|-----------|--------------------------------------------|
| Layton et al. (2015) | E. faecalis ATCC 29212              | 21                        | PEG-modified PDMS | crystal violet assay                       |
| Chirsto et al. (2016) | E. faecalis ATCC 700802              | 28                        | Human dentin     | CFU                                        |
| Joy et al. (2016)   | Biofilm mimicking with collagen      | /                         | Human dentin     | Digital images                             |
| Balic et al. (2016) | E. faecalis ATCC 29212              | 15                        | Human dentin     | PCR, CFU                                   |
| Pladisai et al. (2016) | E. faecalis ATCC 29212            | 21                        | Human dentin     | CFU                                        |
| Mohmmed et al. (2016) | E. faecalis ATCC 19433             | 10                        | Clear liquid photopolymer material | fluorescence microscope with high-resolution CCD camera |
| Cherian et al. (2016) | E. faecalis ATCC 29212          | 7                         | Human dentin     | CFU, SEM                                   |
| De Meyer et al. (2017) | E. faecalis (strain ATCC 10541) Streptococcus mutans (strain LMG 14558) | 2                        | Resin | CFU                                        |
| Toljan et al. (2017) | E. faecalis ATCC 29212              | 1                         | Human dentin     | CFU                                        |
| Bao et al. (2017)   | Mixed biofilm                       | 28                        | Human dentin     | SEM                                        |
| Mohmmed et al. (2017) | E. faecalis ATCC 19433             | 10                        | Clear liquid photopolymer material | SEM, CLSM, TEM |
| Kasic et al. (2017) | E. faecalis Candida albicans        | 7                         | Human dentin     | CFU                                        |
| Cheng et al. (2017) | E. faecalis (clinically isolated)   | 28                        | Human dentin     | SEM                                        |
| Golob et al. (2017) | E. faecalis vancomycin-resistant    | 28                        | Human dentin     | SEM                                        |
| Maden et al. (2017) | E. faecalis ATCC 29212              | 21                        | Human dentin     | CFU                                        |
| Betancourt et al. (2018) | E. faecalis ATCC 29212          | 1                         | Glass            | CFU and atomic force microscope           |
| Sasanakul et al. (2019) | E. faecalis ATCC 29212            | 21                        | Human dentin     | CFU                                        |
| Zhang et al. (2019) | Sub- and supragingival biofilm      | 14                        | Human dentin     | Quantitative real-time PCR                 |
| Hartmann et al. (2019) | E. faecalis ATCC 19433            | 26                        | Human dentin     | CFU                                        |
| Suer et al. (2020)  | E. faecalis ATCC 29212              | 1                         | Human dentin     | SEM                                        |
| Hoedke et al. (2021) | E. faecalis ATCC 29212 and Streptococcus oralis ATCC 35037 | 5                        | Human dentin     | CFU                                        |
| Choi et al. (2021)  | E. faecalis OG1RF Streptococcus mitis ATCC 49456 and Campylobacter rectus ATCC 33238 | 21                        | Human dentin     | CFU, CLSM, TEM                             |
| Afkhami et al. (2021) | E. faecalis ATCC 29212            | 28                        | Human dentin     | CFU                                        |

When choosing the strain of *E. faecalis*, dental researchers should be aware of its genetic heterogeneity which is observed inside a single population, as well as that different strains of *E. faecalis* can be detected in the oral cavity of one individual [47,48].
3.1.2. Multispecies Biofilm

Mixed endodontic infections are more common than infections caused by a single microorganism [11]. Collecting subgingival plaque from dental patients is a method used to grow multispecies biofilm [17,49]. Another approach for multispecies biofilm sampling is an intraoral contamination process by wearing a custom-made appliance for 6–8 days. However, this method is very subjective as the patients voluntarily carry the appliance and follow the diet recommendations [49]. A simplified, lab-adapted, dual-species biofilm model of *E. faecalis* and *Streptococcus mutans* was introduced, as well as a three-species biofilm composed of *E. faecalis*, *Streptococcus mitis* and *Campylobacter rectus* [43,50]. Only one study investigated the removal of dual-species biofilm composed of vancomycin resistant *E. faecalis* and *Candida albicans* [35]. Niazi et al. (2014) used a biofilm consisting of five different species of microorganisms (Table 1) [51].

3.1.3. Biofilm Mimicking

Few studies included in this paper used non-bacterial approaches to test different methods of biofilm removal. Macedo et al. (2014) proposed a hydrogel model to provide visualization of biofilm removal by ultrasonic techniques. As stated by the author, viscoelastic properties of hydrogel can be compared to the one of bacterial biofilm and therefore it may be suitable for replacing bacterial biofilm in in vitro studies [52]. Joy et al. (2015) applied layers of stained collagen to the dentin surface and analyzed digital images of its removal by ultrasonic irrigation [22].

3.1.4. Substrate and Period of Incubation

The majority of research included in this paper uses human [15,16,18,19,21,22,24,25,27,30–35,37,38,40–46,49,53,54] and animal-bovine dentin [20,55] as substrate for biofilm growth and formation (Table 1). A general pattern in preparing samples for bacterial inoculation was observed among the studies which included both human and bovine dentin: after examining the extracted teeth, root canals were enlarged and shaped using endo files, with sodium hypochlorite (NaOCl) serving as an irrigant and EDTA used for smear layer removal. In contrast, Meire’s et al. (2012) presented a different approach since the crowns were firstly cut and dentin slices of standardized thickness were obtained for further testing [38]. Similarly, Bao et al. (2017) used a split tooth model which, after biofilm removal efforts have been made, allows dissembling the tooth and gaining a clear insight into the dentin surface [53]. Another methodology observed in the reviewed studies focuses on use of bovine dentin sections that serve as a substrate for multispecies biofilm cultivation. These sections were incorporated within an orthodontic device and worn by a volunteer allowing oral bacteria to accumulate on the dentin surfaces [55].

Hydroxyapatite (HA) discs are frequently used in dental research and possess the affinity towards bacteria colonization [56,57]. Consequently, both Noiri et al. (2008) and Shen et al. (2010) used hydroxyapatite discs for *E. faecalis* biofilm cultivation [17,26].

Further, six studies included in this review used root canal models as substrate for biofilm formation. In the most recent studies, root canal models were created using CAD technology and 3D printing. The goal of 3D printing is to create a desirable, transparent and anatomically standardized model which would allow an insight into real-time interaction between irrigants and biofilm removal [39,58].

Time plays an important role in biofilm formation, allowing bacteria to aggregate and form a network of polymer strands. Scanning electron microscope (SEM) investigations revealed that after 1 week of incubation, a biofilm-like structure can be observed on dentin surface. After 2, 3 and 4 weeks, biofilm becomes thicker and thus more challenging to remove. Mature biofilm with characteristic honey-comb like structures can be observed after 6 weeks of incubation [9].

The period of incubation used for biofilm cultivation is presented in Table 1. As seen from the table, no consensus in terms of incubation period between different authors was
found. However, Cheng et al. (2012, 2017) and Mohmmed et al. (2016, 2017) were consistent in choosing the same incubation period for their studies over the course of years [24,25,39,58]. The average period of incubation for the reviewed studies was 14 days for multi- and 17.8 days for monospecies biofilm, with major variation between 1 and 50 days.

In studies reviewed in this paper, the authors determined the incubation period based on data available from the literature and their personal preference. However, as pointed out earlier, various incubation periods result in different maturity and thickness of the biofilm, which eventually can lead to unequal effort towards biofilm removal.

3.2. Biofilm Removal Techniques

3.2.1. Sonic Devices

Table 2 shows the sonic devices, settings and irrigant solutions found during the literature search. Among the different sonic devices, the EndoActivator was the most used apparatus. According to its manufacturer, deep cleaning of the root canal system and subsequent biofilm removal could be expected.

Table 2. Details on irrigants, mode and time of agitation, as well as type of sonic device used for biofilm removal.

| Author                  | Sonic Device                              | Irrigant                              | Mode of Agitation     | Time of Agitation |
|-------------------------|-------------------------------------------|---------------------------------------|-----------------------|-------------------|
| Shen et al. (2010)      | Endo Activator                            | 2% chlorhexidine digluconate (CHX), CHX plus | Medium power          | 1–3 min           |
| Halford et al. (2012)   | Endo Activator                            | Sterile water, 5.25% NaOCl, or microbubble emulsion | Full energy           | 20 s              |
| Seet et al. (2012)      | Endo Activator                            | Saline, 4% NaOCl                      | Full energy           | 60 s              |
| Balic et al. (2016)     | Endo Activator                            | 2.5% NaOCl and QMiX solution 10,000 cpm | 10,000 cpm            | 30 s              |
| Mohmmed et al. (2016)   | Endo Activator                            | 2.5% NaOCl                            | High power            | 30 s              |
| Mohmmed et al. (2017)   | Endo Activator                            | 2.5% NaOCl                            | High power            | 30 s              |
| Maden et al. (2017)     | Endo Activator                            | 5.25% NaOCl                           | 167 Hz                | 60 s              |
| Swimberghe et al. (2019)| Eddy (VDW) and EA                         | Water                                 | 6000 Hz               | 60 s              |
| Hoedke et al. (2021)    | SONICflex, (KaVo, Warthausen, Germany)    | Saline, 1% NaOCl                      | Intensity mode 3      | 60 s              |

Sodium hypochlorite (NaOCl) is one of the most commonly used irrigants in endodontic practice. Authors are in agreement that sonic energizing with different concentrations of NaOCl offers greater biofilm disruption than sonic energizing with water or saline [15,33]. Furthermore, sonic energizing with NaOCl was found to be an effective and promising technique in biofilm reduction in many different studies. [15,16,39,58]. Maden et al. (2017) developed a prototype device which using low electric current is able to sonically agitate
the NaOCl solution. This device was able to significantly reduce biofilm in comparison to other sonic devices [37]. Chlorhexidine (CHX) is also a popular irrigant due to its antimicrobial effect [59]. It has been shown that the antimicrobial effect of sonic irrigation with 2% chlorhexidine was superior when compared to sonic saline irrigation. Additionally, it was concluded that longer exposure time to irrigants (up to 3 min) and use of CHX–Plus contributed to higher number of dead bacterial cells [17].

Alternative irrigants used in the reviewed studies were microbubble-emulsion (ME) and QMiX solution. Halford et al. (2012) examined the synergistic effect of ME and sonic agitation. This combination provided bacteria reduction 3 mm from the apical terminus, but left a considerable number of viable bacteria 1 mm from the apical terminus [60]. Interestingly, EndoActivator in combination with QMiX solution provides more favorable antibiofilm efficacy than NaOCl needle irrigation. However, as stated by the authors of the study, this result may also be due to chemical properties of QMIX solution in which the detergent plays an important role in weakening the biofilm structure [15].

3.2.2. Ultrasonic Devices

Passive ultrasonic irrigation (PUI) is a term used in endodontics for describing irrigation of root canal system without additional shaping of the canal wall [61]. With the intention to avoid possible confusion and misunderstanding, PUI will be referred to as “ultrasonic irrigation” in further text. In contrast to previously discussed studies where EndoActivator is the most commonly used sonic device, authors used different units in an attempt to enhance biofilm removal by ultrasonic agitation of irrigants. Table 3 shows reported details of ultrasonic agitation when investigating biofilm removal efficacy. Non-consistent power settings of ultrasonic devices, various shapes, and sizes of ultrasonic tips, different irrigant concentrations and time of irrigation used in research, make comparison of the studies and their findings quite problematic and prone to subjective interpretation. Nevertheless, we aimed to summarize findings from the reviewed studies, based on similarities observed in their methodology.

Table 3. Details on type of ultrasonic devices and instruments (manufacturers), irrigant, mode and time of agitation used in the studies included in the review.

| Author              | Ultrasonic Device | Irrigant                  | Mode                  | Time of Agitation | Instrument         |
|---------------------|-------------------|---------------------------|-----------------------|-------------------|--------------------|
| Shen et al. (2010)  | E7 of Varios 350 LUX (Nakanishi Inc., Kanuma, Japan) | Saline, 2% CHX, CHX-plus | Medium power         | 60–180 s          | Ultrasonic tip     |
| Bhuva et al. (2010) | Piezon Master 400 (Electro Medical Systems SA, Nyon, Switzerland) | 1% NaOCl | 1/2 of maximum power | 40 s              | Size #15 ultrasonic file |
| Alves et al. (2011) | Piezoelectric (Enac-Osada, Tokyo, Japan) | 2.5% NaOCl, 0.2% CHX | Not specified         | 60 s              | Size #15 K-file    |
| Peters et al. (2011)| EMS 600 ultrasonic (Nyon, Switzerland) | 6% NaOCl | 5/10 of maximum power | 30 s              | Non-cutting insert |
| Grundling et al. (2011) | Nac Plus ultrasonics (Adiel, Ribeirao Preto, SP, Brazil) | Distilled water, 2% NaOCl | Scale power 2        | Not specified     | Size #40 K-file    |
| Case et al. (2012)  | Ultrasonic scaler (Perioscan; Sirona, Bensheim, Germany) | Saline | 70 kHz and 200 mW/cm² | 120 s             | Size #15 K-file    |
Table 3. Cont.

| Author                  | Ultrasonic Device                                                                 | Irrigant                                                                 | Mode                              | Time of Agitation | Instrument                                      |
|-------------------------|------------------------------------------------------------------------------------|--------------------------------------------------------------------------|-----------------------------------|-------------------|------------------------------------------------|
| Halford et al. (2012)   | P5 Newton unit (Acteon Group, Norwich, UK)                                        | Sterile water, 5.25% NaOCl, microbubble emulsion                       | Power setting 10                 | 60 s              | Size #10 K-file                                 |
| Bhardway et al. (2014)  | Ultrasonic unit (Satelec, Merignac Cedex, France)                                 | 1% NaOCl                                                                | ¼ of maximum power               | 40 s              | Size #15 ultrasonic file                        |
| Ordinila-Zapata et al. (2014) | Satelec P5 suprasson ultrasonic unit (Suprasson P5; Satelec Acteon group, Acteon, Merignac, France) | 6% NaOCl                                                                | Power setting 4                  | 60 s              | Irrisafe file 20.00 (Acteon, Merignac, France) |
| Niazi et al. (2014)     | Ultrasonic unit (Piezon Master 400; Electro Medical Systems, Nyon, Switzerland)    | Trypsin, Proteinase K, NaOCl, saline, CHX                               | ¼ of the maximum power           | 20 s              | 15 ultrasonic file (Endosonore File, Dentsply Maillfer) |
| Macedo et al. (2014)    | Ultrasonic device (Suprasson P-Max, Acteon Satelec, Acteon, Merignac, France)     | Water and 8.7% NaOCl                                                    | Power setting ‘Yellow 5’         | 20 s              | IrriSafe file (Acteon, Merignac, France)        |
| Layton et al. (2015)    | 1. ultrasonic device (P5 Newton unit; Satelec); 2. PiezoFlow device               | Sterile water                                                           | 1. power setting 10; 2. power setting 5 | 20 s              | 1. non-cutting steel wire, 200 µm; 2. ultrasonic irrigation needle, 500 µm |
| Nelaakantan et al. (2015) | EMS 600 ultrasonic unit (Nyon, Switzerland)                                    | Saline, 6 and 3% NaOCl, 18% etidronic acid, 17% EDTA                   | Not specified                    | 30 s              | Ultrasound file                                 |
| Joy et al. (2015)       | Not specified                                                                      | 2.5% NaOCl                                                              | Not specified                    | Not specified     | Size #15 K-file                                 |
| Pladisi et al. (2016)   | Piezoelectric ultrasonic device (P5; Satelec Acteon, Merignac, France)            | 2.5% NaOCl                                                              | Power setting 4                  | 60 s              | Irrisafe tip K20/21 (Acteon, Merignac, France)  |
| Toljan et al. (2016)    | Ultrasonic device (Piezon Master 400; EMS, Nyon, Switzerland)                     | 3% NaOCl                                                                | Medium power                     | 30 s              | Size #15 K-file                                 |
| Cherian et al. (2016)   | Ultrasonic unit (Varios 750, NSK Nakanishi Inc., Tochigi, Japan.)                  | 2% CHX, 0.1% octenidine dihydrochloride                                  | ¼ of maximum power               | 40 s              | Ultrasonic file size 15                        |
| Mohammed et al. (2016)  | Satelec P5 Newtron piezon unit (Acteon, Merignac, France)                        | 2.5% NaOCl                                                              | Power setting 7                  | 30 s              | Irrisafe instrument 20/02 (Acteon, Merignac, France) |
With the aim of investigating purely mechanical effects of ultrasonic devices, only saline or distilled water was used during biofilm removal (Table 3). Ultrasonic agitation of saline had proven to be more efficient in multispecies biofilm removal than simple irrigation with saline delivered by syringe and needle. This result can be due to pure mechanical effect of the ultrasonic agitation, since no antibacterial agent was used [50]. The results are in agreement with a similar research [32], that reported bacterial reduction using a comparable approach in monospecies biofilm elimination. Similarly, Grundling et al. (2011) and Hartmann et al. (2019) stated that ultrasonic irrigation with distilled water offers significant biofilm reduction when compared to manual agitation of saline with hand files. Furthermore, this study was based on a microscopy evaluations (SEM) method and confirmed a significant difference in apical and middle thirds between manually agitated saline and ultrasonic irrigation with distilled water [20,62].

NaOCl can also be used as an irrigant during ultrasonic agitation. Bhuva et al. (2010) demonstrated that ultrasonic irrigation with NaOCl is superior to saline needle/syringe irrigation in biofilm removal at all three levels of the root canal [19]. Comparatively, other studies noted similar results, although the evaluation method of biofilm removal was different and included plate counting (CFU method) [41,44,45]. In addition, it was shown that ultrasonic NaOCl irrigation offers better bacterial reduction than ultrasonic irrigation with water or saline, which can be explained by the antimicrobial effect of NaOCl [30,33]. Both the ultrasonic device and GentleWave system were effective in reducing the bacteria inside the root canal space [63].
Similarly to NaOCl, CHX can be ultrasonically agitated. Cherian et al. (2016) investigated the effectiveness of ultrasonic agitation of CHX and compared it to CHX syringe irrigation. It was concluded that ultrasonically delivered CHX provides significant bacterial reduction in comparison to syringe CHX irrigation [21]. Furthermore, Shen et al. (2010) compared the antimicrobial efficacy of CHX with CHX-Plus, both ultrasonically agitated, and found a significant difference in the number of cells killed. CHX-Plus was more efficient in biofilm reduction, which can be contributed to the chemistry of the antimicrobial agent itself [17]. Yet, when observing the study, it should be noted that HA discs were used as substrate for multispecies biofilm formation, which is notably different when compared to the morphology of the root canal system. Similarly, when activated ultrasonically, enzymes are more efficient in biofilm removal compared to saline alone [51].

Lastly, ultrasonic effect within simulated biofilm and root canal models was also investigated. For this purpose, Macedo et al. (2014) introduced a transparent root canal model with isthmus and lateral canals which were filled with hydrogel. As a result, the main canals were better cleaned with water used as an irrigant rather than NaOCl. Different from lateral canals, isthmi were equally well rinsed regardless of the agent used for ultrasonic irrigation [52]. Another study used root canal models to investigate fluid dynamics generated by syringe irrigation and both continuous and intermittent ultrasonic technique [36]. Continuous ultrasonic agitation was found to be significantly better in biofilm removal compared to syringe irrigation and intermittent ultrasonic technique. The superior action of continuous ultrasonic agitation can be due to complete oscillating amplitude of the ultrasonic tip inside the root canal which, consequently, generates maximum acoustic microstreaming. Unlike complete oscillating amplitude achieved by the continuous tip, the intermittent ultrasonic tip comes in occasional contact with the canal wall, thus resulting in weakened microstreaming effect [36]. Very recently, Moommed et al. explored the effect of different agitation methods using NaOCl as irrigant within 3D printed root canals [39]. The results indicated an effective biofilm removal with NaOCl ultrasonic agitation especially when compared to sonic and syringe irrigation. Additionally, microscopic images evaluations showed that 1 mm from the apex manual and sonic treatment left the biofilm intact, while complete biofilm removal at the same level was associated with ultrasonic agitation of NaOCl [39,58].

3.2.3. Er:Yag Laser Group
Er:Yag Laser

In one of the pioneer studies which investigated the effect of Er:Yag laser in biofilm removal, Noiri et al. (2008) directly irradiated hydroxyapatite discs that had previously been contaminated with multispecies biofilm. As shown in Table 4, different energy pulses were applied and it was discovered that low laser energy offers anti-biofilm effect [26]. Although the study demonstrated encouraging results in biofilm removal, a notably different scenario in clinical conditions may be found, since the laser tip is not able to reach all parts of the complex root canal anatomy. Meire et al. (2012) used a rather similar laboratory approach, although the authors evaluated the effect of Er:Yag laser in monospecies biofilm removal and used dentin discs as substrate. Additionally, NaOCl was introduced as an irrigant, and it was concluded that the combination of Er:Yag irradiation and NaOCl irrigation can be used as joint techniques during root canal disinfection since it provides better biofilm removal than Er:Yag laser alone [38].

However, similarly to the previous study, a uniform irradiation of dentin discs was possible due to the laboratory setup of the experiment. Complex root canal morphology in clinical conditions represents a greater challenge in biofilm removal, but, nonetheless, the results of the mentioned studies confirm beneficial effect of laser irradiation in attempts to remove biofilms.
A more relevant clinical approach was proposed by Cheng et al. (2012) who compared the results of biofilm removal using different techniques and irrigants. Although conventional 5.25% NaOCl syringe irrigation of canals was effective in eliminating the bacteria from the surface of root canals, CFU counting revealed it was not able to successfully remove *E. faecalis* from deep dentin layers. By applying Er:Yag laser and NaOCl as irrigant, better biofilm reduction was achieved deep inside dentinal tubules, thus suggesting that Er:Yag laser supports penetration of NaOCl. Furthermore, the study emphasized the importance of the synergistic effect of NaOCl Er:Yag laser agitation since it showed better results in comparison to NaOCl syringe irrigation or saline Er:Yag agitation [24].

| Author | Laser Type | Irrigant | Laser Tip | Laser Parameters (Wavelength, Power, Pulse Energy, Pulse Frequency, Pulse Duration) | Time | Position of the Tip |
|--------|------------|----------|-----------|----------------------------------------------------------------------------------|------|--------------------|
| Noiri et al. (2008) | Er:YAG laser (Arwin; MORITA, Osaka, Japan) | No irrigant | Custom made tip, diameter 650 µm | 2940 nm not specified 20, 40, 80 mJ not specified | 10 s | 3 mm from the HA disc |
| Meire et al. (2012) | Er:Yag laser (Fidelis; Fotona, Ljubljana, Slovenia) | 0.25% NaOCl | RO2 handpiece (Fotona) | 2940 nm not specified 50, 100 mJ 15 Hz not specified | 20 s | Directly over the dentin disc |
| Cheng et al. (2012) | 1. Er:Yag laser (Fontona Lasers) 2. Er,Cr:YSGG laser (Biolase, Irvine, CA) | 1. 5.25% NaOCl, saline, distilled water 2. Not specified | 1. Optical fiber, 200 µm diameter 2. Optical fiber, 415 µm diameter | 1. 2940 nm 0.3 W not specified 15 Hz not specified 2. 2.780 nm 1 W not specified 20 Hz not specified | 1. 20 s 2. 60 s | 1. Orifice of the root canal 2. 1 mm from the working length |
| Seet et al. (2012) | Er:Cr:YSGG laser (WaterLase, Biolase Technology, Irvine, CA, USA) | Saline, 4% NaOCl | Radial firing tip (17 mm, 52°) | Not specified 0.25 W not specified 20 Hz not specified | 60 s | 4 mm into the canal, withdraw coronally |
| Christo et al. (2016) | Er:Cr:YSGG laser (Waterlase, Biolase Technology, Irvine, CA, USA) | Saline, 0.5, 1 and 4% NaOCl | RFT 3 (diameter 415 µm, length 17 mm) (Endolase, Biolase Technology) | 2.780 nm 0.5 W 25 mJ 20 Hz 140 µs | 60 s | 5 mm apically from the orifice |
| Kasic et al. (2017) | Er:Cr:YSGG laser (Waterlase, Biolase, Irvine, CA, USA) | Saline | RTF 2 (200 µm) | Not specified 1.25 W not specified 15 Hz 150 µs | Not specified | 5 mm apically from the coronal access |
| Betancourt et al. (2018) | Er:Cr:YSGG laser (Waterlase iPlus BIOLASE Technology, Irvine, CA, USA) | Saline, 0.5% and 5% NaOCl | RFT 2 tip (Endolase, BIOLASE Technology, Inc.; 200 µm in diameter, length 21 mm, calibration factor of >0.55) | 2.780 nm, 1 W, 100 mJ 10 Hz 140 µs | 60 s | Tip placed in the cylindric reservoir |
| Suer et al. (2020) | Er:Cr:YSGG laser | 2.5% NaOCl | Fiber tip | Not specified 2 W/0.75 W Not specified 20 Hz | 40 s | Placed into the canal towards the apex |

Table 4. Details on laser type, irrigant, laser tip design and parameters, time of irradiation and position of the tip used for biofilm removal in the studies included in the review.
Er,Cr:YSGG Laser

The pure effect of Er,Cr: YSGG laser on biofilm removal without the presence of irrigant/dry canal was investigated by Cheng et al. (2012) [24]. Using laser parameters as shown in Table 5, it was concluded that Er,Cr: YSGG laser is less effective than 5.25% NaOCl irrigation. Furthermore, the same study revealed that Er:Yag NaOCl agitation is superior to Er,Cr: YSGG laser irradiation alone.

Table 5. Details on laser type, irrigant, laser tip design and parameters, time of irradiation and position of the tip used during PIPS.

| Author            | Laser Type (Fidelis; Fotona, Ljubljana, Slovenia) | Irrigant | Laser Tip (Pulse Rate, Pulse Energy, Pulse Duration, Power) | Time | Position of the Laser Tip |
|-------------------|-------------------------------------------------|---------|-------------------------------------------------------------|------|---------------------------|
| Peters et al. (2011) | Er,Yag laser Fidelis; (Fotona, Ljubljana, Slovenia) | 3.6% NaOCl | 21-mm-long, 400-µm endodontic fiber | 10 Hz 50 mJ not specified not specified | 30 s | Coronal reservoir |
| Olivi et al. (2014) | Er,Yag laser LightWalker AT, Fotona, Ljubljana, Slovenia | 5% NaOCl followed by 17% EDTA | 9-mm, 600-µm quartz tip | 15 Hz 20 mJ 50 µs 0.3 W | 90 s | Access cavity |
| Ordinola-Zapata et al. (2014) | Er,Yag laser Fidelis; (Fotona, Ljubljana, Slovenia) | 6% NaOCl | 12-mm, 400-µm quartz tip | 15 Hz 20 mJ 50 µs 0.3 W | 60 s | Access cavity |
| Al Shahrani et al. (2014) | Er,Yag laser LightWalker AT, Fotona, Ljubljana, Slovenia | 6% NaOCl, saline | 9-mm, 600-µm quartz tip | 15 Hz 20 mJ 50 µs 0.3 W | 90 s | Access cavity |
| Neelakantan et al. (2015) | Er,Yag laser (Fidelis; Fotona, Ljubljana, Slovenia) | Saline, NaOCl-EDTA-NaOCl, NaOCl-EDTA, NaOCl-editrionic acid | 21 mm long, 400 microns endodontic conical fiber tip | 10 Hz 50 mJ 50 ms not specified | 30 s | Coronal reservoir |
| Balic et al. (2016) | Er,Yag laser LightWalker AT, Fotona, Ljubljana, Slovenia | 2.5% NaOCl, QMiX solution | 600-µm fiber tip | 15 Hz 20 mJ 50 µs not specified | 60 s | Access cavity |
| Kasic et al. (2017) | Er,Yag laser LightWalker AT, Fotona, Ljubljana, Slovenia | Saline | 14-mm, 400 µm tapered tip | 15 Hz 20 mJ 50 µs 0.3 W | 40 s | Access cavity |
Surprisingly, other studies found no difference in biofilm removal between Er,Cr: YSGG 4% NaOCl agitation and 4% NaOCl syringe irrigation [33]. On the other hand, Seet et al. (2012) discovered that Er,Cr: YSGG 4% NaOCl agitation offers better biofilm eradication compared to 4% NaOCl syringe irrigation [16]. Interestingly, both authors used the identical E. faecalis strain, same period of incubation and the same irrigant concentration and time of agitation. Even though Seet et al. (2012), Betancourt et al. (2019) and Suer et al. (2020) used lower laser power settings compared to Chriso et al. (2016), they still found superior results in biofilm removal which were associated with Er,Cr: YSGG laser agitation of the irrigant, rather than conventional syringe irrigation [46,64].

PIPS

The goal of PIPS is to enhance biofilm removal by creating photoacoustic shockwaves that would travel through the root canal system which is filled with an irrigant [65]. When applying the PIPS technique, the laser tip is usually positioned in the access cavity (pulp chamber or canal entrance). Many authors are consistent in their methodologies with an emphasis that, during studies, the position of the tip was limited to the access cavity only, without further insertion towards the root canal [27,30,54,55]. Instead, De Meyer et al. (2017) inserted the PIPS tip into the canal, 6 mm short of the working length, only to discover equal effect of PIPS, regardless of the position of the laser tip [50].

As seen from Table 5, the same laser parameters, i.e., pulse rates from 10 to 20 Hz and pulse energies from 10 to 40 mJ, which were considered to have no thermal or ablative effect on
canal walls, were used during investigation of PIPS effect in biofilm reduction \[15,27,30,35,54,55\]. However, different laser tip designs as well as various irrigant concentrations and activation times are noted in the reviewed articles (Table 5).

In general, the PIPS technique is considered to be superior to conventional syringe/needle irrigation, regardless of the irrigant used \[15,25,30,43,50,54\]. Confocal laser scanning microscopy images taken by Al Shahrami et al. (2014) revealed that conventional NaOCl irrigation leaves viable bacteria deep inside dentinal tubules, while PIPS with NaOCl offers deeper penetration of the irrigant, consequently killing more bacteria \[30\].

When comparing PIPS to sonic agitation, Ordinola-Zapata et al. (2014) demonstrated that PIPS significantly reduces the number of bacteria within bovine root canal models when NaOCl was used as irrigant \[54\]. Contrarily, Balic et al. (2016) and Hage et al. (2019) concluded that both PIPS and sonic irrigation of NaOCl remove biofilm evenly from the root canal \[15,66\].

Up to the present time, it has been confirmed that, compared to ultrasonic techniques, PIPS offers enhanced biofilm removal in the apical part of root canals \[49\]. SEM images from different studies confirm PIPS superiority over ultrasonic methods in biofilm reduction inside root canals \[55\]. Moreover, by evaluating treatment results by CLSM and CFU, Nelaakantan et al. (2015) concluded that PIPS agitation of NaOCl and etidronic acid provides better biofilm removal when compared to conventional and ultrasonic techniques with the same irrigants \[40\]. Furthermore, PIPS was more efficient than sonic devices in removing hydrogel from the isthmus when using only water as irrigant \[67\].

Only one study compared the effect of PIPS to Er,Cr:YSGG laser in dual-species biofilm removal. The study used saline as irrigant and therefore it was possible to estimate solely the physical effect of lasers. Er,Cr:YSGG laser agitation of non-antimicrobial agent performed better at E. faecalis and C. albicans biofilm removal in comparison to PIPS \[35\].

Lastly, Golob et al. (2017) suggested a modified PIPS protocol, which offered promising results in disinfection of root canals \[27\]. Unlike the classic PIPS protocol, the authors introduced PIPS with EDTA, prior to NaOCl irrigation, and removed the mineralized part of the smear layer, opening dentinal tubules, thus enabling deeper penetration of NaOCl. Additionally, in order to increase the safety of the PIPS treatment, laser energy was reduced by 50% and no difference was found in biofilm removal between higher and lower power settings.

### 3.3. Evaluation of Biofilm Removal

The most frequently used methods for evaluating biofilm removal efficacy include counting of colony forming units (CFU) and analysis of scanning electron microscope (SEM) images, while confocal laser scanning microscopy (CLSM), polymerase chain reaction (PCR) and transmission electron microscopy (TEM) are found to be less mentioned in the reviewed studies. Additionally, it was found that some authors used more than one means of evaluation while assessing the success of biofilm removal \[18,21,24,26,30,40,45\] (Table 1).

#### 3.3.1. CFU—Plate Counting

Methods of obtaining samples for further microbial analysis differ among the studies. It is suggested that after treatment protocol, root canals are filled with sterile saline, followed by syringe aspiration, centrifugation and counting of CFU \[42\]. Similarly, paper points leave the integrity of the dentin surface intact and have also been used in collecting samples for bacteriological evaluation \[24,30\]. On the other hand, Hedstrom files \[32\], round dental \[34\], Gates Glidden burs \[21,40\] and Peeso reamer \[41\] allowed researchers to retrieve dentin samples from various depths and use them for later analysis. Regardless of the sampling technique used, the CFU method provides information on the number of viable bacteria found either on the root canal surface or at various dentin depths.

#### 3.3.2. SEM

An innovative proposal introduced by Bhuva et al. (2010) involves SEM image observation and analysis by endodontists with different levels of experience \[19\]. Briefly, a scoring
system was created in relation to percentage of root canal which was covered with biofilm and dentists rated the SEM images according to their personal opinion and observations. A similar approach in SEM analysis was also used a few years later by Bhardway et al. (2013) and Ordinola-Zapata et al. (2014) [31,55]. Eventually, dividing the root canal surface into three areas—coronal, middle and apical—and taking SEM images of the mentioned sections is also widespread in methodologies reviewed by this paper [16,19,20]. The observed level of magnification used varies, ranging from 40 up to 10,000× [19,21,30,39,53]

Overall, SEM allows visualization of morphological structures of biofilms, their amount and distribution on dentin surface, as well as in deeper dentin layers. [20,24] However, it should be noted that sample preparation for SEM analysis might result in changes of the biofilm’s extracellular polymer matrix [11].

3.3.3. CLSM

Based on the reviewed papers, it was noted that the main advantage of using CLSM techniques is the author’s capability to distinguish viable and dead cells within biofilms. When observing the CLSM images taken after the treatment, live cells are usually seen as green, while dead cells are painted red [17,30,39]. Additionally, 3D reconstruction can be achieved and the ratio between live and dead cells can also be determined [40].

3.3.4. Other Methods

TEM: Mohmmed et al. (2017) used TEM as well as CLSM and SEM to evaluate the results of biofilm removal. TEM images enabled an insight into cellular integrity and level of damage caused by different removal techniques [39].

PCR: Only two authors used PCR with the purpose of confirming identification of E. faecalis and to determine the presence of bacteria even in low numbers or stationary phase and therefore avoiding false negative results [15,18]. Quantitative real time PCR analysis was also reported as a valid method for the evaluation of the bacterial removal [63]. However, one should keep in mind that even DNA from dead cells, as well as free extracellular DNA, could be amplified and detected, eventually giving misleading data.

Histology: Brown–Brenn staining technique can be used to determine bacterial penetration into dentinal tubules. Only one study was found to use this technique, with an observational magnification set to 100× and 400× [49].

High speed camera: One study used a high speed camera attached to a microscope to record the hydrogel removal process from transparent root canal models. The recorded films of hydrogel removal were analyzed in MATLAB and later discussed [52]. A similar methodology was used by Mohmmed et al. (2016), although this study investigated the removal of E. faecalis biofilm in contrast to the previously described study that investigated removal of biofilm mimicking model [58].

Colorimetric assay: Layton et al. (2015) used colorimetric assay, a rapid technique for biofilm quantification, while assessing the results of root canal cleaning. Additionally, by using micro PIV system, this research provided valuable findings concerning irrigation and fluid dynamics in simulated root canals [36].

Digital images: A special imaging method that allows estimation of the canal surface which is covered with collagen was introduced by Joy et al. (2015). As stated by the author, the method used in this study allows the three-dimensional irregularities on the root canal surface to become two-dimensional surfaces on the images [22].

It is important to emphasize that the evaluators should always be blinded to the treatment protocol in all techniques that employ image analysis.

4. Future Research

Cultivating a biofilm formation in in vitro conditions may seem easily replicable, low cost and offering researchers control over the period of incubation and maturity of biofilms introduced in studies. However, one should consider that different growth media, different
conditions and time of incubation lead to various viscoelastic behaviors of biofilms, which is ultimately an important feature for the resistance of biofilms.

In the reviewed articles, the models used for establishing biofilm infection were straight, single-rooted and single-canal or simulated canal. In order to provide more relevant clinical implications and draw comparison of the results from the in vitro trials more accessible, the following strategies should be considered:

1. standardize the pathogens’ growth conditions which can lead to more uniform viscoelastic properties of biofilms and their thicknesses;
2. confirm biofilm formation by SEM/CLSM before initiating the treatment protocol;
3. introduce root canals with complex morphology to surveys;
4. align sonic, ultrasonic and laser parameters, respectively, and standardize them;
5. besides CFU, introduce SEM and CLSM, which would allow a more detailed insight into the effectiveness of disinfection methods in the coronal, middle and apical parts of root canal, as well as the distinction between live and dead bacterial cells.

5. Conclusions

Within the limitations of this study, it can be concluded that sonic, ultrasonic and Er:Yag laser agitation, in general, offer better biofilm removal when compared to conventional irrigation methods delivered by syringe and needle. The choice of the right irrigation solution is an important factor for removal of the endodontic biofilm, with water and saline being less effective compared to NaOCl and CHX. However, due to heterogeneity in methodologies, it is difficult to compare adjuvant endodontic techniques with one another and give recommendations for the most efficient method in biofilm removal. Lastly, this review emphasizes the importance of standardizing methodologies in experimental protocols, as well as introducing strategies which would provide more relevant clinical implications.

Author Contributions: Conceptualization, U.J., A.F., L.B. and A.M.; methodology, U.J., C.M., T.M. and A.F.; writing, U.J., L.B., A.M., A.F. and C.M.; supervision, L.B. and A.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Sjogren, U.; Figdor, D.; Persson, S.; Sundqvist, G. Influence of infection at the time of root filling on the outcome of endodontic treatment of teeth with apical periodontitis. *Int. Endod. J.* 1997, 30, 297–306. [CrossRef] [PubMed]
2. Siqueira, J.F., Jr. Aetiology of root canal treatment failure: Why well-treated teeth can fail. *Int. Endod. J.* 2001, 34, 1–10. [CrossRef] [PubMed]
3. Nair, P.N. On the causes of persistent apical periodontitis: A review. *Int. Endod. J.* 2006, 39, 249–281. [CrossRef] [PubMed]
4. Endo, M.S.; Ferraz, C.C.; Zaia, A.A.; Almeida, J.F.; Gomes, B.P. Quantitative and qualitative analysis of microorganisms in root-filled teeth with persistent infection: Monitoring of the endodontic retreatment. *Eur. J. Dent.* 2013, 7, 302–309. [CrossRef] [PubMed]
5. Mazzitelli, C.; Ionescu, A.; Josic, U.; Brambilla, E.; Breschi, L.; Mazzoni, A. Microbial contamination of resin composites inside their dispensers: An increased risk of cross-infection? *J. Dent.* 2022, 116, 103893. [CrossRef]
6. Stuart, C.H.; Schwartz, S.A.; Beeson, T.J.; Owatz, C.B. *Enterococcus faecalis*: Its role in root canal treatment failure and current concepts in retreatment. *J. Endod.* 2006, 32, 93–98. [CrossRef]
7. Distel, J.W.; Hatton, J.F.; Gillespie, M.J. Biofilm formation in medicated root canals. *J. Endod.* 2002, 28, 689–693. [CrossRef]
8. Kishen, A.; George, S.; Kumar, R. *Enterococcus faecalis*-mediated biomineralized biofilm formation on root canal dentine in vitro. *J. Biomed. Mater. Res. Part A* 2006, 77, 406–415. [CrossRef]
9. Duggan, J.M.; Sedgley, C.M. Biofilm formation of oral and endodontic *Enterococcus faecalis*. *J. Endod.* 2007, 33, 815–818. [CrossRef]
10. Neelakantan, P.; Romero, M.; Vera, J.; Daood, U.; Khan, A.U.; Yan, A.; Cheung, G.S.P. Biofilms in Endodontics-Current Status and Future Directions. *Int. J. Mol. Sci.* 2017, 18, 1748. [CrossRef]
Polymers 2022, 14, 1334

11. Ran, S.; He, Z.; Liang, J. Survival of Enterococcus faecalis during alkaline stress: Changes in morphology, ultrastructure, physio-chemical properties of the cell wall and specific gene transcripts. Arch. Oral Biol. 2013, 58, 1667–1676. [CrossRef] [PubMed]

12. Kato, A.; Ziegler, A.; Higuchi, N.; Nakata, K.; Nakamura, H.; Ohno, N. Aetiology, incidence and morphology of the C-shaped root canal system and its impact on clinical endodontics. Int. Endod. J. 2014, 47, 1012–1033. [CrossRef] [PubMed]

13. Nair, P.N.; Henry, S.; Cano, V.; Vera, J. Microbial status of apical root canal system of human mandibular first molars with primary apical periodontitis after “one-visit” endodontic treatment. Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod. 2005, 99, 231–252. [CrossRef] [PubMed]

14. Corsentino, G.; Mazzitelli, C.; Mazzoni, A.; Ambu, E.; Perotto, C.; Franciosi, G.; Grandini, S. Sealing ability of two root-end filling materials at different retro-preparation lengths. J. Oral Sci. 2022, 64, 80–84. [CrossRef]

15. Balic, M.; Lucc, R.; Mehadjizic, K.; Bago, I.; Anic, I.; Jakovljevic, S.; Plecko, V. The efficacy of photon-initiated photoacoustic streaming and sonic-activated irrigation combined with QMiX solution or sodium hypochlorite against intracanal E. faecalis biofilm. Lasers Med. Sci. 2016, 31, 335–342. [CrossRef]

16. Seet, A.N.; Zilm, P.S.; Gully, N.J.; Cathro, P.R. Qualitative comparison of sonic or laser energisation of 4% sodium hypochlorite on an Enterococcus faecalis biofilm grown in vitro. Aust. Endod. J. 2012, 38, 100–106. [CrossRef]

17. Shen, Y.; Stojicic, S.; Qian, W.; Olsen, I.; Haapasalo, M. The synergistic antimicrobial effect by mechanical agitation and two chlorhexidine preparations on biofilm bacteria. J. Endod. 2010, 36, 100–104. [CrossRef]

18. Alves, F.R.; Almeida, B.M.; Neves, M.A.; Moreno, J.O.; Rocas, I.N.; Siqueira, J.F., Jr. Disinfecting oval-shaped root canals: Effectiveness of different supplementary approaches. J. Endod. 2011, 37, 496–501. [CrossRef]

19. Bhuva, B.; Patel, S.; Wilson, R.; Niazi, S.; Beighton, D.; Mannocci, F. The effectiveness of passive ultrasonic irrigation on intraradicular Enterococcus faecalis biofilms in extracted single-rooted human teeth. Int. Endod. J. 2010, 43, 241–250. [CrossRef]

20. Grundling, G.L.; Zechin, J.G.; Jardim, W.M.; de Oliveira, S.D.; de Figueiredo, J.A. Effect of ultrasonics on Enterococcus faecalis biofilm in a bovine tooth model. J. Endod. 2011, 37, 1128–1133. [CrossRef]

21. Cherian, B.; Gehlot, P.M.; Manjunath, M.K. Comparison of the Antimicrobial Efficacy of Octenidine Dihydrochloride and Chlorhexidine with and Without Passive Ultrasonic Irrigation—An Invitro Study. J. Clin. Diagn. Res. JCDR 2016, 10, ZC71–ZC77. [CrossRef] [PubMed]

22. Joy, J.; Mathias, J.; Sagir, V.M.; Babu, B.P.; Chirayath, K.J.; Hameed, H. Bacterial Biofilm Removal Using Static and Passive Ultrasonic Irrigation. J. Int. Oral Health JIOH 2015, 7, 42–47. [PubMed]

23. Schoop, U.; Moritz, A.; Kluger, W.; Patruta, S.; Goharkhay, K.; Sperr, W.; Wernisch, J.; Gattringer, R.; Mrass, P.; Georgopoulos, A. The Er:YAG laser in endodontics: Results of an in vitro study. Lasers Surg. Med. 2002, 30, 360–364. [CrossRef] [PubMed]

24. Cheng, X.; Guan, S.; Lu, H.; Zhao, C.; Chen, X.; Li, N.; Bai, Q.; Tian, Y.; Yu, Q. Evaluation of the bactericidal effect of Nd:YAG, Er:YAG, Er,Cr:YSGG laser radiation, and antimicrobial photodynamic therapy (aPDT) in experimentally infected root canals. Lasers Surg. Med. 2012, 44, 824–831. [CrossRef] [PubMed]

25. Cheng, X.; Xiang, D.; He, W.; Qiu, J.; Han, B.; Yu, Q.; Tian, Y. Bactericidal Effect of Er:YAG Laser-Activated Sodium Hypochlorite Irrigation Against Biofilms of Enterococcus faecalis Isolate from Canal of Root-Filled Teeth with Periapical Lesions. Photomed. Laser Surg. 2017, 35, 386–392. [CrossRef] [PubMed]

26. Noiri, Y.; Katsumoto, T.; Azakami, H.; Ebisu, S. Effects of Er:YAG laser irradiation on biofilm-forming bacteria associated with endodontic pathogens in vitro. Endod. J. 2004, 38, 826–829. [CrossRef]

27. Golob, B.S.; Olivi, G.; Vrabec, M.; El Feghali, R.; Parker, S.; Benedicenti, S. Efficacy of Photon-induced Photoacoustic Streaming in the Reduction of Enterococcus faecalis within the Root Canal: Different Settings and Different Sodium Hypochlorite Concentrations. J. Endod. 2017, 43, 1730–1735. [CrossRef]

28. Olivi, G.; DiVito, E. Photoacoustic endodontics using PIPS™: Experimental background and clinical protocol. J. Laser Health Acad. 2012, 1. [CrossRef]

29. Ricucci, D.; Siqueira, J.F., Jr. Biofilms and apical periodontitis: Study of prevalence and association with clinical and histopathologic findings. J. Endod. 2010, 36, 1277–1288. [CrossRef] [PubMed]

30. Al Shahran, M.; DiVito, E.; Hughes, C.V.; Nathanson, D.; Huang, G.T. Enhanced removal of Enterococcus faecalis biofilms in the root canal using sodium hypochlorite plus photon-induced photoacoustic streaming: An in vitro study. Photomed. Laser Surg. 2014, 32, 260–266. [CrossRef]

31. Bhaward, A.; Velmurugan, N.; Sumitha; Ballal, S. Efficacy of passive ultrasonic irrigation with natural irritants (Morinda citrifolia juice, Aloe Vera and Propolis) in comparison with 1% sodium hypochlorite for removal of E. faecalis biofilm: An in vitro study. Indian J. Dent. Res. Publ. Indian Soc. Dent. Res. 2013, 24, 35–41. [CrossRef] [PubMed]

32. Case, P.D.; Bird, P.S.; Kahler, W.A.; George, R.; Walsh, L.J. Treatment of root canal biofilms of Enterococcus faecalis with ozone gas and passive ultrasonic activation. J. Endod. 2012, 38, 523–526. [CrossRef] [PubMed]

33. Christo, J.E.; Zilm, P.S.; Sullivan, T.; Cathro, P.R. Efficacy of low concentrations of sodium hypochlorite and low-powered Er,Cr:YSGG laser activated irrigation against Enterococcus faecalis biofilm. Int. Endod. J. 2016, 49, 279–286. [CrossRef]

34. Nagahashi, T.; Yahata, Y.; Handa, K.; Nakano, M.; Suzuki, S.; Kakiuchi, Y.; Tanaka, T.; Kanehira, M.; Suresh Venkataiah, V.; Saito, M. Er:YAG laser-induced cavitation can activate irradiation for the removal of intraradicular biofilm. Sci. Rep. 2022, 12, 4897. [CrossRef] [PubMed]
35. Kasic, S.; Knezovic, M.; Beader, N.; Gabric, D.; Malcic, A.I.; Baraba, A. Efficacy of Three Different Lasers on Eradication of Enterococcus faecalis and Candida albicans Biofilms in Root Canal System. *Photomed. Laser Surg.* 2017, 35, 372–377. [CrossRef] [PubMed]

36. Layton, G.; Wu, W.I.; Selvaganapathy, P.R.; Friedman, S.; Kishen, A. Fluid Dynamics and Biofilm Removal Generated by Syringe-delivered and 2 Ultrasonic-assisted Irrigation Methods: A Novel Experimental Approach. *J. Endod.* 2015, 41, 884–889. [CrossRef] [PubMed]

37. Maden, M.; Ertugrul, I.F.; Orhan, E.O.; Erik, C.E.; Yetis, C.C.; Tuncer, Y.; Kahririm, M. Enhancing antibacterial effect of sodium hypochlorite by low electric current-assisted sonic agitation. *PLoS ONE* 2017, 12, e0183895. [CrossRef]

38. Meire, M.A.; Coenye, T.; Nelis, H.J.; De Moor, R.J. Evaluation of Nd:YAG and Er:YAG irradiation, antibacterial photodynamic therapy and sodium hypochlorite treatment on *Enterococcus faecalis* biofilms. *Int. Endod. J.* 2012, 45, 482–491. [CrossRef]

39. Mohmmed, S.A.; Vianna, M.X.; Penny, M.R.; Hilton, S.T.; Mordan, N.; Knowles, J.C. Confocal laser scanning, scanning electron, and transmission electron microscopy investigation of *Enterococcus faecalis* biofilm degradation using passive and active sodium hypochlorite irrigation within a simulated root canal model. *MicrobiologyOpen* 2017, 6, e00455. [CrossRef]

40. Neelakantan, P.; Cheng, C.Q.; Mohanraj, R.; Sirraman, P.; Subbarao, C.; Sharma, S. Antibiofilm activity of three irrigation protocols activated by ultrasonic, diode laser or Er:YAG laser in vitro. *Int. Endod. J.* 2015, 48, 602–610. [CrossRef]

41. Pladisai, P.; Ampornmaramveth, R.S.; Chivatxaranukul, P. Effectiveness of Different Disinfection Protocols on the Reduction of Bacteria in *Enterococcus faecalis* Biofilm in Teeth with Large Root Canals. *J. Endod.* 2016, 42, 460–464. [CrossRef] [PubMed]

42. Toljan, I.; Bago, I.; Juric; Anic, I. Eradication of Intracanal Enterococcus Faecalis Biofilm by Passive Ultrasonic Irrigation and RinsEndo System. *Acta Stomatol. Croat.* 2016, 50, 14–22. [CrossRef] [PubMed]

43. Afkhami, F.; Ahmadi, P.; Chiniforush, N.; Sooratgar, A. Effect of different activations of silver nanoparticle irrigants on the elimination of *Enterococcus faecalis*. *Clin. Oral Investig.* 2021, 25, 6893–6899. [CrossRef] [PubMed]

44. Hoedke, D.; Kaulika, N.; Dominisch, H.; Schlafer, S.; Shemesh, H.; Bitter, K. Reduction of dual-species biofilm after sonic- or ultrasonic-activated irrigation protocols: A laboratory study. *Int. Endod. J.* 2021, 54, 2219–2228. [CrossRef] [PubMed]

45. Choi, M.J.; Kim, M.A.; Choi, Y.; Neelakantan, P.; Yu, M.K.; Min, K.S. A novel three-dimensionally printed model to assess biofilm removal by ultrasonically activated irrigation. *Int. Endod. J.* 2021, 54, 1871–1877. [CrossRef]

46. Suer, K.; Ozkan, L.; Guvenir, M. Antimicrobial effects of sodium hypochlorite and Er, Cr:YSGG laser against *Enterococcus faecalis* biofilm. *Niger. J. Clin. Pract.* 2020, 23, 1188–1193. [CrossRef]

47. Pinheiro, E.T.; Anderson, M.J.; Gomes, B.P.; Drucker, D.B. Phenotypic and genotypic identification of enterococci isolated from canals of root-filled teeth with periapical lesions. *Oral Microbiol. Immunol.* 2006, 21, 137–144. [CrossRef]

48. Tong, Z.; Ling, J.; Lin, Z.; Li, X.; Mu, Y. The effect of MTADN on 10 *Enterococcus faecalis* isolates and biofilm: An in vitro study. *J. Endod.* 2013, 39, 674–678. [CrossRef]

49. Peters, O.A.; Bardsley, S.; Fong, J.; Pandher, G.; Divito, E. Disinfection of root canals with photon-initiated photoacoustic streaming. *Int. Endod. J.* 2011, 44, 1008–1012. [CrossRef]

50. De Meyer, S.; Meire, M.A.; Coenye, T.; De Moor, R.J. Effect of laser-activated irrigation on biofilms in artificial root canals. *Int. Endod. J.* 2017, 50, 472–479. [CrossRef]

51. Niazi, S.; Clark, D.; Do, T.; Gilbert, S.; Foschi, F.; Mannocci, F.; Beighton, D. The effectiveness of enzymatic irrigation in removing a nutrient-stressed endodontic multispecies biofilm. *Int. Endod. J.* 2014, 47, 756–768. [CrossRef]

52. Macedo, R.G.; Robinson, J.P.; Verhaagen, B.; Walmsley, A.D.; Versluis, M.; Cooper, P.R.; van der Sluis, L.W. A novel methodology providing insights into removal of biofilm-mimicking hydrogel from lateral root canals during irrigation procedures. *Int. Endod. J.* 2014, 47, 1040–1051. [CrossRef]

53. Bao, P.; Shen, Y.; Lin, J.; Haapasalo, M. In Vitro Efficacy of XP-endo Finisher with 2 Different Protocols on Biofilm Removal from Apical Root Canals. *J. Endod.* 2017, 43, 321–325. [CrossRef]

54. Olivi, G.; DiVito, E.; Peters, O.; Kaitasas, V.; Angiero, F.; Signore, A.; Benedicenti, S. Disinfection efficacy of photon-induced photoacoustic streaming on root canals infected with *Enterococcus faecalis*: An ex vivo study. *J. Am. Dent. Assoc.* 2014, 145, 843–848. [CrossRef]

55. Ordinola-Zapata, R.; Bramante, C.M.; Aprecio, R.M.; Handsides, R.; Jaramillo, D.E. Biofilm removal by 6% sodium hypochlorite activated by different irrigation techniques. *Int. Endod. J.* 2014, 47, 659–666. [CrossRef] [PubMed]

56. Vacca Smith, A.M.; Bowen, W.H. In situ studies of pellicle formation on hydroxyapatite discs. *Arch. Oral Biol.* 2000, 45, 277–291. [CrossRef]

57. Madhwni, T.; McBain, A.J. Bacteriological effects of a Lactobacillus reuteri probiotic on in vitro oral biofilms. *Arch. Oral Biol.* 2011, 56, 1264–1273. [CrossRef] [PubMed]

58. Mohmmed, S.A.; Vianna, M.E.; Penny, M.R.; Hilton, S.T.; Mordan, N.; Knowles, J.C. A novel experimental approach to investigate the effect of different agitation methods using sodium hypochlorite as an irrigant on the rate of bacterial biofilm removal from the wall of a simulated root canal model. *Dent. Mater. Off. Publ. Acad. Dent. Mater.* 2016, 32, 1289–1300. [CrossRef] [PubMed]

59. Josic, U.; Maravic, T.; Mazzitelli, C.; Del Bianco, F.; Mazzoni, A.; Breschi, L. The effect of chlorhexidine primer application on the clinical performance of composite restorations: A literature review. *J. Esthet. Restor. Dent.* 2021, 33, 69–77. [CrossRef]

60. Halford, A.; Ohl, C.-D.; Azarpazhooh, A.; Basrani, B.; Friedman, S.; Kishen, A. Synergistic effect of microbubble emulsion and sonic or ultrasonic agitation on endodontic biofilm in vitro. *J. Endod.* 2012, 38, 1530–1534. [CrossRef]
61. van der Sluis, L.W.; Versluis, M.; Wu, M.K.; Wesselink, P.R. Passive ultrasonic irrigation of the root canal: A review of the literature. *Int. Endod. J.* 2007, 40, 415–426. [CrossRef] [PubMed]

62. Hartmann, R.C.; Neuvald, L.; Barth, V., Jr.; de Figueiredo, J.A.P.; de Oliveira, S.D.; Scarparo, R.K.; Waltrick, S.B.; Rossi-Fedele, G. Antimicrobial efficacy of 0.5% peracetic acid and EDTA with passive ultrasonic or manual agitation in an *Enterococcus faecalis* biofilm model. *Aust. Endod. J.* 2019, 45, 57–63. [CrossRef] [PubMed]

63. Zhang, D.; Shen, Y.; de la Fuente-Nunez, C.; Haapasalo, M. In vitro evaluation by quantitative real-time PCR and culturing of the effectiveness of disinfection of multispecies biofilms in root canals by two irrigation systems. *Clin. Oral Investig.* 2019, 23, 913–920. [CrossRef] [PubMed]

64. Betancourt, P.; Merlos, A.; Sierra, J.M.; Camps-Font, O.; Arnabat-Dominguez, J.; Vinas, M. Effectiveness of low concentration of sodium hypochlorite activated by Er,Cr:YSGG laser against *Enterococcus faecalis* biofilm. *Lasers Med. Sci.* 2019, 34, 247–254. [CrossRef] [PubMed]

65. DiVito, E.; Lloyd, A. ER:YAG laser for 3-dimensional debridement of canal systems: Use of photon-induced photoacoustic streaming. *Dent. Today* 2012, 31, 122, 124–127. [PubMed]

66. Hage, W.; De Moor, R.J.G.; Hajj, D.; Sfeir, G.; Sarkis, D.K.; Zogheib, C. Impact of Different Irrigant Agitation Methods on Bacterial Elimination from Infected Root Canals. *Dent. J.* 2019, 7, 64. [CrossRef] [PubMed]

67. Swimberghe, R.C.D.; De Clercq, A.; De Moor, R.J.G.; Metre, M.A. Efficacy of sonically, ultrasonically and laser-activated irrigation in removing a biofilm-mimicking hydrogel from an isthmus model. *Int. Endod. J.* 2019, 52, 515–523. [CrossRef]