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Article

Activity of two antimicrobial peptides against *Enterococcus faecalis* in a model of biofilm-mediated endodontic infection

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Abstract: *Enterococcus faecalis* is a common cause of biofilm-associated opportunistic infections, which are often difficult to treat. The formation of *E. faecalis* biofilm on the dentinal walls of the root canal is frequently cause of endodontic treatment failure and secondary apical periodontitis. In a preliminary work, two recognized antifungal peptides, KP and L18R, showed antibacterial activity against planktonic *E. faecalis* cells at micromolar concentrations. Moreover, L18R proved to reduce the biomass in early stage of *E. faecalis* biofilm development onto polystyrene plates, while a qualitative biofilm inhibition was demonstrated on hydroxyapatite disks by confocal laser scanning microscopy (CLSM). The aim of this study was to better characterize the effect of both peptides on *E. faecalis* biofilm. A reduction in metabolic activity after peptide treatment was detected by Alamar Blue assay, while a remarkable impairment in the architecture of *E. faecalis* biofilms on hydroxyapatite disks, along with a significant reduction of viable bacteria, was caused mostly by L18R, as assessed by CLSM and scanning electron microscopy. The lack of cytotoxicity of the investigated peptides against L929 murine fibroblasts was also determined. Obtained results suggest L18R as a promising candidate for the development of new strategies for endodontic infection control.

Keywords: antimicrobial peptides; biofilm; confocal microscopy; endodontics; *Enterococcus faecalis*; scanning electron microscopy

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1. Introduction

Apical periodontitis (AP) is a dental pathology that involves an inflammatory lesion of the periradicular tissues caused by microbial infection of the dental pulp and biofilm formation on the dentinal walls of the root canal system [1]. Current AP treatment implies the chemo-mechanical disinfection of the root canals followed by three-dimensional obturation to prevent reinfection [2]. The most common antiseptics employed during canal irrigation are sodium hypochlorite and chlorhexidine [3]. In clinical conditions, the efficacy of these substances is limited by the complexity of the root canal anatomy, which may limit the volume of irrigants that can reach the microorganisms [4]. Another major problem is the higher resistance of bacteria within biofilm communities to antimicrobial agents [5,6]. In particular, antimicrobial failure has been associated with reduced penetration through the biofilm matrix, biofilm-specific expression of efflux pumps and protection against oxidative stress [5,7].

Root canal disinfection could be enhanced by placing an inter-appointment intracanal medication in order to extend the time of exposure [8-11]. The most used endodontic dressing is calcium hydroxide, $Ca(OH)_2$, that inhibits the growth of many pathogens by release of hydroxyl ions and induction of strongly alkaline conditions [12,13]. However, in the radicular environment, the activity of $Ca(OH)_2$ is limited by the inherent

buffer effect of dentine and some endodontic pathogens may survive, leading to persistent infections and secondary AP [14,15].

Primary root canal infections are polymicrobial, dominated by anaerobic Gram-negative bacteria and composed of 10-30 species per canal [16]. In secondary, post-treatment infections, 1-5 species are detected, mostly Gram-positive facultative anaerobes [16-18]. In particular, *Enterococcus faecalis* is the species most frequently associated with cases of endodontic failure and persistent AP [17]. This microorganism normally inhabits human gastrointestinal tract. However, it is a frequent cause of biofilm-associated opportunistic infections of urinary tract and wounds, and can cause endocarditis, which are often healthcare-associated and difficult to treat due to the intrinsic resistance of *E. faecalis* to β-lactam antibiotics and the marked ability to acquire antimicrobial-resistance genes [19].

In the root canal environment, the inherent ability of *E. faecalis* to invade dentinal tubules, survive under unfavorable environmental conditions, such as starvation and alkaline pH, and form biofilms may contribute to its persistence after currently used treatments [20,21]. Due to these features, *E. faecalis* biofilms can be used as in vitro models to preliminarily assess the effect of novel antibiofilm agents for the development of alternative and more effective root canal disinfection strategies [22].

In the last years, there has been a growing interest in the possible use of antimicrobial peptides (AMPs) in endodontic decontamination [23]. AMPs are short, low-molecular-weight peptides of various origins, with a wide spectrum of antimicrobial activities [24-27]. The main reported mechanisms of action of AMPs involve microbial cell membrane permeabilization, but more complex interactions with diverse intracellular targets have been proposed [28].

In a preliminary work, we reported the activity of two recognized antifungal peptides, KP and L18R, against planktonic *E. faecalis* cells. Moreover, both peptides proved to reduce the biomass of *E. faecalis* biofilm onto polystyrene plates, while a qualitative biofilm inhibition was demonstrated on hydroxyapatite (HA) disks by confocal laser scanning microscopy (CLSM) [29]. KP is a decapeptide derived from the sequence of the variable region of a single-chain recombinant anti-idiotypic antibody that represents the functional internal image of a wide-spectrum yeast killer toxin [30]. KP showed a remarkable activity against taxonomically unrelated pathogens, including protozoa, fungi, bacteria and viruses [31]. L18R was synthesized on the basis of the sequence of immunoglobulin gene J (locus heavy, IGHJ2). L18R proved to display a strong fungicidal activity in vitro and to be therapeutic against *Candida albicans* experimental infection in *Galleria mellonella* [32].

The aim of this study was to better characterize the effect of KP and L18R on *E. faecalis* early stage biofilm on polystyrene plates and mature biofilm on HA disks. Their efficacy on mature biofilm was compared with $Ca(OH)_2$, a commonly used endodontic dressing. Both peptides proved to be effective against *E. faecalis* biofilm, while lacking cytotoxic activity against in vitro cultured mammalian cells. Based on the obtained results, L18R is envisaged as a promising candidate for the development of new strategies for endodontic infection control.

2. Results

2.1. Cytotoxic effect of the investigated peptides

KP and L18R were tested for their cytotoxic activity against eukaryotic cells by the MTT assay. At all the tested concentrations (up to 400 μg/mL), mean absorbance values were generally higher than the ones of control cells in the absence of peptides, although no statistically significant difference was observed. In Table 1, results of the cytotoxicity assay are expressed as % cell viability (control in the absence of peptides, 100% viability).

Table 1. In vitro cytotoxic activity of the investigated peptides against L929 cells.

Concentration (µg/ml)	Cell viability (%) after treatment with KP	Cell viability (%) after treatment with L18R
400	104 ± 17	127 ± 24
200	121 ± 15	99 ± 20
100	145 ± 34	102 ± 0
50	120 ± 39	120 ± 16

2.2. *Activity of KP and L18R against E. faecalis biofilm onto polystyrene plates*

The capability of KP and L18R to interfere with *E. faecalis* biofilm formation onto polystyrene plates was investigated by Alamar Blue assay. Both peptides proved to reduce biofilm metabolic activity in a dose-related manner (Figure 1). The half maximal effective concentration (EC_{50}) value for KP were 123.3 µg/ml (confidence interval 79.41-191.6), corresponding to 1.235×10^{-4} M (confidence interval 0.795-1.920). L18R proved to be more active, showing an EC_{50} value of 32.77 µg/ml (confidence interval 27.72-38.73), corresponding to 1.585×10^{-5} M (confidence interval 1.340-1.873).

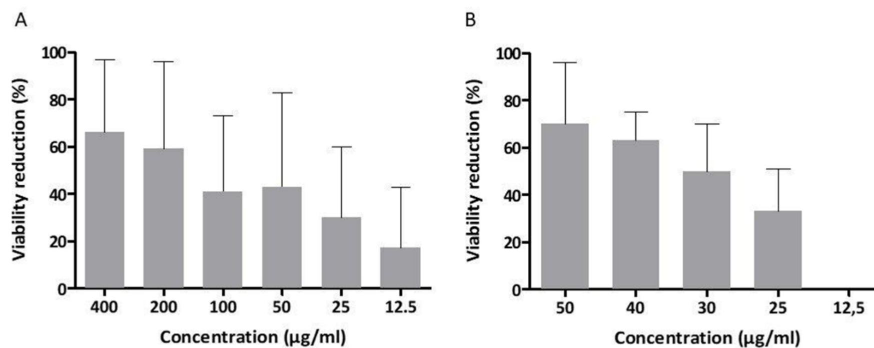


Figure 1. Effect of the investigated peptides against *E. faecalis* ATCC 29212 biofilm. Biofilm metabolic activity was determined by Alamar Blue assay after treatment with different concentrations of (**A**) KP; (**B**) L18R. Results are reported as percent reduction of biofilm viability as compared to untreated samples. Data are presented as mean ± SD of at least 3 independent experiments.

2.3. *Activity of KP and L18R against E. faecalis biofilm on hydroxyapatite disks*

The activity of KP and L18R against 48 h-old *E. faecalis* biofilm grown HA disks was assessed by CLSM and scanning electron microscopy (SEM) and compared with samples treated with a saturated $Ca(OH)_2$ endodontic dressing solution. CLSM images (Figure 2, A-C) and 3D reconstruction (Figure 2, D) of untreated *E. faecalis* biofilms on HA disks showed mainly viable cells organized in a homogeneous and robust biofilm layer. As compared with the untreated controls, biofilm grown on HA disks and exposed to $Ca(OH)_2$ saturated solution (Figure 2, E-G), 100 µg/mL KP (Figure 2, I-K) and 50 µg/mL L18R (Figure 2, M-O) showed a consistent number of dead cells, along with less adhering bacteria for samples treated with $Ca(OH)_2$ and L18R, as evidenced by 3D reconstruction images (Figure 2, panels H, L, and P for $Ca(OH)_2$, KP, and L18R, respectively). The reduction of biofilm thickness was particularly evident after treatment with L18R.

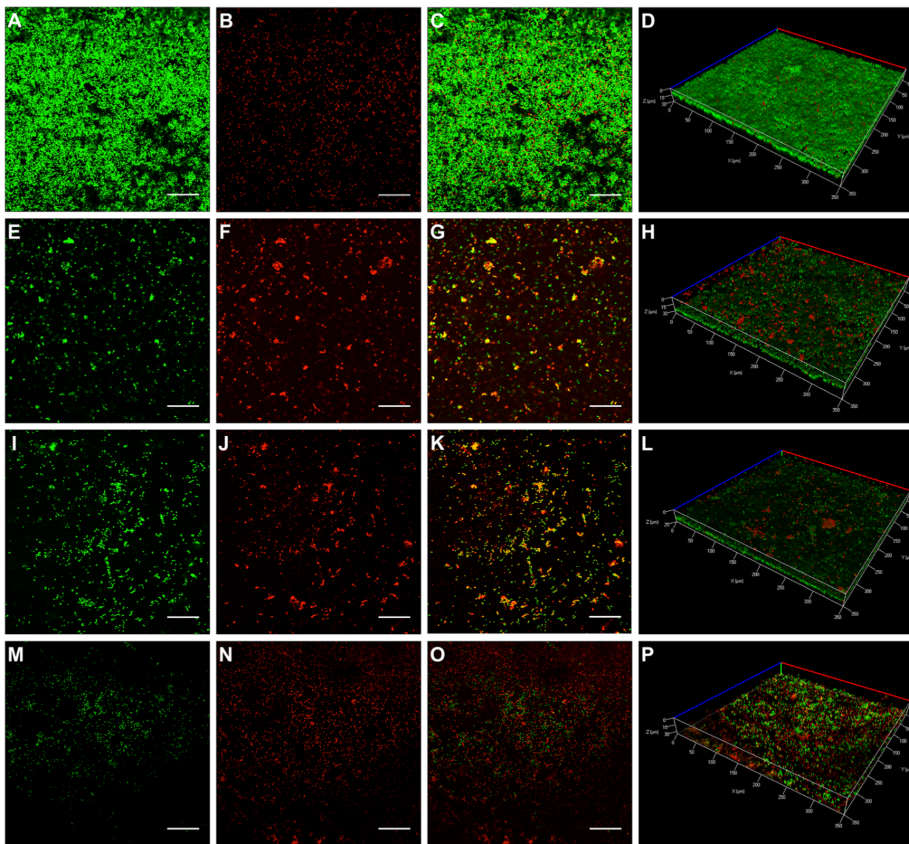


Figure 2. Representative CLSM images of *E. faecalis* biofilms on HA disks. Images of 48 h-old *E. faecalis* ATCC 29212 biofilm were acquired by CLSM after exposure to H_2O (control, **A-C**), $Ca(OH)_2$ saturated solution (**E-G**), KP (100 μg/mL, **I-K**) and L18R (50 μg/mL, **M-O**) for 24 h. In each line is shown the same field (single focal plane). From the left to the right: SYTO-9 (green, viable cells), propidium iodide (red, dead cells), merged image of viable and dead cells. 3D reconstruction of the selected fields (full thickness, merged images) are shown in panels **D** (control), **H** (exposed to $Ca(OH)_2$ saturated solution), **L** (exposed to KP), and **P** (exposed to L18R). Notably, in L18R-treated sample the 3D reconstruction shows a reduced thickness of the biofilm in comparison to untreated control. Bar = 50 μm.

A quantitative analysis of the fluorescence intensities (FI), performed on 3D CLSM reconstruction of four random fields from each disk, revealed a detaching effect of $Ca(OH)_2$ and L18R treatments. In fact, the sum of total FI (viable and dead cells) was clearly reduced from 42.93 ± 4.95 for controls, to 24.18 ± 10.70 and 10.87 ± 4.33 for $Ca(OH)_2$ and L18R, respectively. A different effect was observed after KP treatment, since total FI resulted 76.88 ± 12.

The proportion of dead bacteria in treated biofilm is shown in Figure 3. A significant increase of dead cells followed treatment with L18R and $Ca(OH)_2$. Notably, in biofilm treated with L18R the percentage of dead cells was significantly higher than that obtained after treatment with the conventional endodontic dressing. KP treatment caused an increase, although not significant, in dead cells.

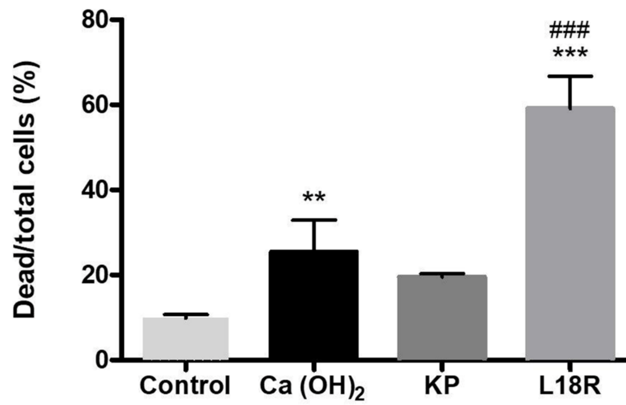
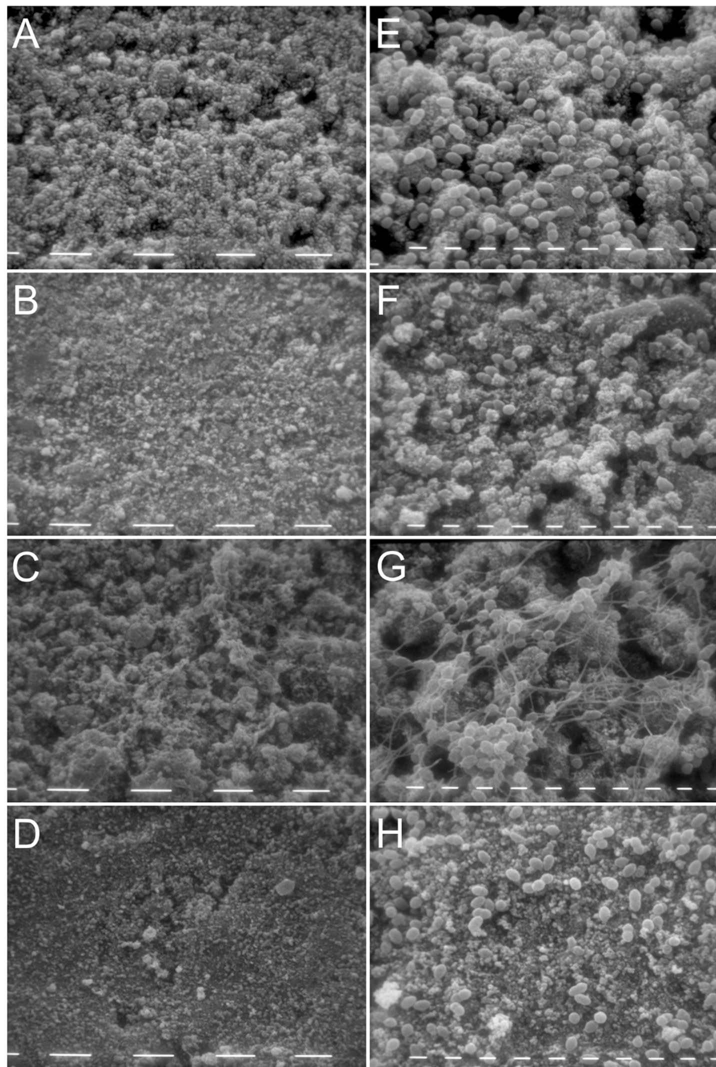


Figure 3. Dead cells (%) after treatment of *E. faecalis* biofilm pre-grown on HA disks. Values were obtained by 3D CLSM analysis of 48 h-old *E. faecalis* ATCC 29212 biofilms after exposure to H_2O (control), $Ca(OH)_2$ saturated solution, KP (100 µg/mL) and L18R (50 µg/mL) for 24 h. Red and green fluorescence intensity measure was carried out with Imaris 7.2 on four random fields of each disk. The results are expressed as means ± SD (**, $p < 0.01$ vs control; ***, $p < 0.001$ vs control; ###, $p < 0.001$ vs $Ca(OH)_2$).

These results were confirmed by SEM images, which showed a lower number of cells in biofilm grown on HA disks treated with $Ca(OH)_2$ and L18R as compared to untreated control sample (Figure 4). L18R treatment caused the highest detaching. In KP-treated samples, a network of fibril-like structures on adhering cells was observed.



162

163 **Figure 4.** Representative scanning electron microscopy (SEM) images of *E. faecalis* biofilms on HA
164 disks. Images of 48 h-old *E. faecalis* ATCC 29212 biofilm were acquired by SEM after exposure to
165 H_2O (control; **A** and **E**), $Ca(OH)_2$ saturated solution (**B** and **F**), KP (100 µg/mL; **C** and **G**) and L18R
166 (50 µg/mL; D and H) for 24 h. Magnification 1250 × (**A**-**D**, bar = 10 µm) and 5000× (**E**-**H**, bar = 1
167 µm).

168 **3. Discussion**

169 In the root canal environment, the ability of *E. faecalis* to resist harsh environmental
170 conditions and form biofilms on the inner surface of the tooth canals makes its elimina-
171 tion extremely difficult [21]. These features can explain the high prevalence of this bacte-
172 rium in secondary and persistent endodontic infections [17].

173 In a previous published preliminary study, in order to look for new alternative and
174 nontoxic antibacterial substances to improve root canal disinfection, the effects of the
175 synthetic peptides KP and L18R against planktonic cells and *E. faecalis* biofilm onto pol-
176 ystyrene plates in early stage development have been assessed [29]. The results demon-
177 strated a good antibacterial activity of both peptides against *E. faecalis* planktonic cells at
178 micromolar concentrations. L18R proved to be the most effective with an EC_{50} value of
179 3.624 × 10^{-7} M, while KP EC_{50} resulted to be 4.520 × 10^{-6} M. KP and L18R were also shown
180 to interfere with *E. faecalis* biofilm formation by reducing biofilm mass. As for the activi-
181 ty against planktonic bacteria, L18R resulted more effective than KP in biofilm inhibi-
182 tion. On the basis of these promising results, new investigations were done to better
183 characterize the effect of both peptides on *E. faecalis* biofilms.

184 In the present work, KP and L18R activity against *E. faecalis* onto polystyrene plates
185 was confirmed by the assessment of their ability to reduce biofilm metabolic activity.
186 Notably, the comparison of the results obtained in this assay with those of the previous-
187 ly described experiment that evaluated the reduction of biofilm mass [29] showed a sim-
188 ilar behaviour for both peptides. In fact, taking into consideration, as an example, the
189 concentration of 50 µg/ml, KP caused a reduction of biofilm mass of approximately 35%
190 and of biofilm viability of approximately 43%. Similarly, L18R, at the same concentra-
191 tion, caused a reduction of biofilm mass and viability of approximately 73% and 70%,
192 respectively.

193 KP and L18R were effective against *E. faecalis* biofilm at concentrations higher than
194 against planktonic cells, as commonly found with conventional antibiotics. This behav-
195 ior suggests a mechanism of action, during the early phases of *E. faecalis* biofilm for-
196 mation, not distinct from the killing activity against planktonic bacterial cells. On the
197 contrary, some peptides, like the human cathelicidin LL-37, are able to inhibit and dis-
198 perse preformed bacterial biofilms at concentrations lower than or equal to concentra-
199 tions effective against free-floating cells, implying an action on biofilm-specific targets
200 rather than ubiquitous microbial structures [33].

201 From previous studies on yeasts, it has been hypothesized that the first step of KP
202 killing activity is an interaction with cell-wall glucan-like structures [31]. For L18R, a di-
203 rect penetration via an energy-independent pathway involving stable or transient desta-
204 bilisation and peptide folding on the lipid portion of membrane was shown [32], indi-
205 cating that the activity of this peptide may involve different mechanisms of action. Pos-
206 sible multi-modal mechanisms of action would render the peptides potentially advan-
207 tageous in targeting different biofilm sub-populations. The mechanism of action of the
208 investigated peptides against bacterial cells is not known, and further studies will be
209 necessary to elucidate it.

210 Notably, both peptides proved to be nontoxic to murine fibroblasts at active con-
211 centrations, in agreement with previous studies that demonstrated the lack of detectable
212 toxicity in vitro to other cell lines, erythrocytes, and peripheral blood mononuclear cells
213 [31,32,34]. As for other AMPs, the selective antimicrobial action of KP and L18R may be
214 explained by the cationic nature of these molecules, that promotes the interaction with
215 the negatively charged membranes of bacteria and not with the zwitterionic membranes
216 of mammalian cells [35]. Conversely, for other endodontic dressing of clinical use, such
217 as $Ca(OH)_2$ and cresol, a certain degree of toxicity against host cells was demonstrated
218 [36,37].

219 In the preliminary study, the antibacterial effects of KP and L18R were tested in a
220 model of endodontic infection consisting in pre-formed *E. faecalis* biofilm grown on den-
221 tin-mimicking hydroxyapatite disks, in comparison with a saturated $Ca(OH)_2$ endodon-
222 tic dressing solution. This model, that represents a simplification of the clinical reality of
223 the infected root canal, was used for a qualitative CLSM analysis [29].

224 In the present study, the same proved in vitro model, was subjected to a quantita-
225 tive CLSM and qualitative SEM analysis which showed deep alterations in the architec-
226 ture and spatial distribution of the treated biofilms, with the highest detaching effect

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induced by L18R. In KP treated samples, SEM images revealed a network of fibril-like structures on adhering cells, confirming the self-assembling properties previously shown by the peptide when challenged against yeast cells [31,38]. L18R treatment did not involve the formation of fibril-like structures on biofilm, accordingly to previous observations on *C. albicans* cells [32]. It has been previously described that KP molecules easily dimerize in solution, due to the formation of disulfide bridges, and, with time, KP dimers self-assemble giving rise to fibril-like aggregates that can be visualized by transmission electron microscopy. Moreover, KP aggregates are readily formed in the presence of soluble 1,3-β-glucans and after incubation with *C. albicans* cells exposing 1,3-β-glucans on their surface [38]. Notably, this peculiar property of KP, able to confer protection against proteases, has been associated to KP therapeutic activity in vivo against experimental fungal infections. Further studies are needed to establish which surface components on *E. faecalis* cells are able to induce KP assembly in a fibril-like network. The peculiar aggregation of KP in the presence of the targeted microorganisms could explain the data obtained by the quantitative analysis of FI in 3D reconstructions of CLSM images, i.e. the sum of total FI (viable and dead cells) which resulted higher for KP-treated sample (76.88 ± 12) in comparison to control (42.93 ± 4.95). It is conceivable that KP fibril-like network may hinder the detachment of bacterial cells (viable and dead) during biofilm washing procedures. Likewise, the above mentioned difference observed for KP activity against *E. faecalis* biofilm onto polystyrene plates between the reduction of biofilm mass (35%) and of biofilm viability (43%), although not very high, could have the same explanation.

The determination of the viability profile of *E. faecalis* cells allowed to detect a significantly higher ratio of dead to total cells in L18R-treated samples compared to control and to samples treated with $Ca(OH)_2$ (Figure 3). These findings suggest the potential of L18R for *E. faecalis* biofilm treatment, showing its possible benefit over the established inter-appointment medicament $Ca(OH)_2$, and confirm the already reported partial resistance to the commercial endodontic dressing [39,40].

Further aspects, such as the spectrum of anti-biofilm activity of L18R, its activity against multispecies consortia and the synergistic interaction with other peptides, which may represent a greater challenge towards biofilm eradication, should be investigated. Nonetheless, the obtained results indicate L18R as a promising candidate for further development as an anti-biofilm agent to be used, alone or in combination with classic endodontic dressings, as an innovative intracanal medicament to reduce endodontic failures.

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4. Materials and Methods

4.1. Peptides and bacterial strain

KP (AKVTMTCSAS, molecular mass 998.17) was synthesized in its active dimeric form by NeoMPS (PolyPeptide Group, Strasbourg, France), while L18R (LLVLRSLGPWHPGHCLLR, molecular mass 2068.1) was synthesized at the CRIBI Biotechnology Center (University of Padua, Italy), as previously described [32,34]. Peptides were solubilized in DMSO (starting solution, 20 mg/mL) and diluted prior to use. In all experiments, controls (without peptides) contained DMSO at the proper concentration.

The reference *E. faecalis* ATCC 29212 strain was maintained in Brain Heart Infusion Agar (BHA; Sigma-Aldrich, St. Louis, USA) plates. Subcultures were made two times a week.

4.2. Peptide cytotoxicity assay

Cytotoxicity of the peptides against L929 murine fibroblasts was assessed by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Cells cultured in Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich, St. Louis, USA) with 10% fetal bovine serum, 100 U/ml of penicillin, and 100 μg/mL of streptomycin,

were seeded in a 96-well microplate (100 μL/well, 4 × 10^5 cells/mL) and incubated for 24 h at 37 °C in 5% CO_2 atmosphere. Cells were then treated for 24 h with the peptides at 50, 100, 200 and 400 μg/mL in DMEM with 2% FBS. Cells in medium without peptides were used as control. The medium was discarded and 100 μL of MTT at the concentration of 0.5 mg/ml in serum-free DMEM were added in each well. After 4 h of incubation at 37 °C, 100 μL of the proper reagent (10% Triton-X 100 in acidic isopropanol 0.1N HCl) were added to solubilize formazan crystals formed following the reduction of MTT by viable cells and absorbance was measured at 540 nm. Each assay was run in triplicate. Results, from two independent experiments, were expressed as percentage of viable cells in comparison to control.

4.3. Treatment of E. faecalis biofilm formed on polystyrene surfaces

The effect of KP and L18R was investigated on early stages of biofilm developed on polystyrene plates, as previously described [29]. Briefly, *E. faecalis* cells (7.5 × 10^6 cells/mL, 200 μL/well) were incubated for 90 min at 37 °C, then planktonic bacteria were removed and adherent cells were exposed to serial concentrations of peptides for 5 h at 37 °C. Cells incubated in water served as control. After washing, the plates were further incubated at 37 °C for 48 h, then evaluation of biofilm metabolic activity was performed by Alamar Blue (CellTiter-Blue, Invitrogen, Carlsbad, USA) assay. The plates were washed with PBS before addition of 200 μl/well of the cell viability reagent. After 1 h incubation at 37 °C the fluorescence was measured using a microplate reader (EnSpire® Multimode Plate Reader, PerkinElmer, Waltham, USA), setting excitation at 570 nm and emission at 585 nm. Each assay was run in triplicate. Four independent experiments were performed. Results were expressed as percentage of biofilm viability reduction with reference to untreated control (100% viability). EC_{50} values were calculated using Graph Pad Prism 4.01 software.

4.4. Treatment of E. faecalis biofilm formed on hydroxyapatite disks

In order to evaluate the activity of KP and L18R on mature biofilms, an in vitro model of root canal infection on hydroxyapatite (HA) disks was employed, as previously described [29]. Sterilized HA disks were placed in wells of flat-bottom 24-well plates and inoculated with 500 μL of a 7.5 × 10^6 cells/ml bacterial suspension prepared as previously described. After 48 h of incubation at 37 °C, the medium was gently washed off and the disks were treated with 500 μL/well of a saturated solution of $Ca(OH)_2$ endodontic dressing, KP (100 μg/mL), L18R (50 μg/mL), or sterile water (control) for 24 h at 37 °C. The effect of the treatments on the biofilm preformed on HA was assessed by confocal laser scanning and scanning electron microscopy. Two independent experiments were performed.

4.4.1. Confocal laser scanning microscopy

For each treatment, half of the disks was examined by CLSM to determine the biofilm architecture and the viability of bacteria. After washing with PBS, bacteria on HA disks were stained using 500 μL of a live/dead kit (LIVE/DEAD FilmTracer™ Biofilm Viability Kit, Invitrogen, Paisley, UK) solution, containing two component dyes (0.3% SYTO-9, 0.3% propidium iodide), according to the manufacturer's instructions. After 20 min, the disks were washed again and fluorescence emission was detected using a LSM 510 Meta scan head integrated with the Axiovert 200 M inverted microscope (Carl Zeiss, Jena, Germany). The excitation/emission wavelengths were 480/500 nm for the SYTO-9 live cell stain and 490/635 nm for the propidium iodide dead cell stain. The samples were observed using a 40 × NA1.3 oil immersion lens and four random fields were scanned in each sample. A stack of 80–100 slices in 0.5 μm step sizes was captured along the Z-axis from the top to the bottom of the biofilm. CLSM images were acquired and three-dimensional (3D) reconstructions were produced using the microscope manufacturer's software (Axiovision module inside 4D release 4.5, Carl Zeiss, Jena, Germany).

The ratio of red fluorescence intensity (FI) to green-and-red FI, calculated with the Imaris 9.5.0 software (Bitplane AG, Zurich, Switzerland), indicated the proportion of dead cells for treatment groups.

4.4.2. Scanning electron microscopy

The remaining half of HA disks was processed for SEM. Briefly, the disks were washed with PBS and dried at room temperature for 15 minutes. The samples were then fixed with a solution of glutaraldehyde 2.5 % in 0.1 M sodium cacodylate for 1 h at room temperature, dehydrated in graded series of ethanol (25%, 50%, 75%, 90%, 100 %; 30 minutes between each passage), immersed in absolute acetone and subjected to critical point drying. The disks were mounted on aluminium stubs, and covered with a 60 nm gold film using a metal sputtering device. The samples were observed using a Philips 501 microscope equipped with a Nikon Coolpix digital camera for acquisition of the images.

4.5. Statistical analysis

Statistical analysis was performed using Prism 4.01 (Graph Pad software, San Diego, USA). ANOVA test followed by Tukey post-hoc was used for multiple comparisons. Values of $p < 0.05$ were considered significant.

Author Contributions: Conceptualization, Giovanni Mergoni and Laura Giovati; Formal analysis, Giovanni Mergoni and Laura Giovati; Investigation, Giovanni Mergoni, Pio Bertani, Tecla Ciociola and Laura Giovati; Methodology, Giovanni Mergoni and Laura Giovati; Project administration, Maddalena Manfredi and Stefania Conti; Resources, Giovanni Mergoni and Laura Giovati; Supervision, Maddalena Manfredi; Validation, Giovanni Mergoni, Maddalena Manfredi and Laura Giovati; Visualization, Giovanni Mergoni and Laura Giovati; Writing – original draft, Giovanni Mergoni and Laura Giovati; Writing – review & editing, Stefania Conti. All authors have read and agreed to the published version of the manuscript.

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

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