Sustained Release of Antimicrobial Peptides from Biodegradable Polymers against Oral Pathogens

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## Table of contents

Abstract ............................................................................................................................................. 3

1. Introduction ................................................................................................................................... 4 – 14
2. Material and methods .................................................................................................................... 15 – 23
3. Results .......................................................................................................................................... 24 – 39
4. Discussion ..................................................................................................................................... 40 – 47
5. Conclusions .................................................................................................................................... 48
6. References ..................................................................................................................................... 49 - 55
Abstract

The development of antibacterial drugs to overcome various pathogenic species, which inhabit the oral cavity, faces several challenges, such as salivary flow and enzymatic activity that restrict dosage retention. Owing to their amphipathic nature, antimicrobial peptides (AMPs) serve as the first line of defense of the innate immune system. The ability to synthesize different types of AMPs enables exploitation of their advantages as alternatives to antibiotics. Sustained release of AMPs incorporated in biodegradable polymers can be advantageous in maintaining high levels of the peptides. In this study, several AMPs mimetics were incorporated in two different biodegradable polymers: poly (lactic acid co castor oil) (PLACO) and ricinoleic acid-based poly (ester-anhydride) (P (SA: RA)) for sustained release. The peptide and polymer formulations were tested for antibacterial activity during one week, by turbidometric measurements of bacterial outgrowth, anti-biofilm activity, biocompatibility by hemolysis and XTT colorimetric assays, mode of action by fluorescence-activated cell sorting (FACS) and release profile by a fluorometric assay. The results show that an antibacterial and anti-biofilm effect, as well as membrane disruption, can be achieved by the use of a formulation of lipopeptide incorporated in biodegradable polymer.
Introduction

Endogenous and exogenous antibacterial agents are required to protect organisms against pathogens. There are many types of antibacterial agents which differ in many aspects. Examples include: synthesis (natural or synthetic), effectiveness (broad spectrum or narrow spectrum) and agent target (membrane structure, membrane synthesis, proteins synthesis, metabolism etc.). Therefore, the ideal agent would be selective to the pathogen, non-toxic to the host, non-allergenic, not interfering to the immune system's activity and not creating pathogen resistance.

The oral environment contains a large variety of microorganisms that create the oral microbiota. It evolves from early oral microbial communities in newborn to adult oral microbiota and include some pathogenic species that are involved in several oral diseases [1] such as dental caries, periodontal and endodontic diseases. Oral pathogens such as: *Streptococcus mutans*, *Actinomyces naeslundii*, *Porphyromonas gingivalis*, *Fusobacterium nucleatum* and *Enterococcus faecalis*, differ from one another but share one thing in common and that is the ability to form biofilm. Dental biofilm is a dynamic, constantly active metabolically structure [2, 3].

*Enterococcus faecalis* is a highly resistant, gram positive facultative anaerobic bacterium, which appears as short chains, diplococcus or as single cells. Within the human microflora, it is situated in the gastro-intestinal tract and therefore is a commensal microorganism. It can cause life threatening infections such as: endocarditis, bacteremia, urinary tract infections and meningitis and it appears especially in hospitals where resistance to antibiotics is developed [4]. The wide range of environments in which *E. faecalis* can grow (survive in different kinds of temperature and pH) endows it with the ability to surmount many obstacles. There are many factors that enable *E. faecalis* to be virulent: They can survive a long period of starvation, inhibit the function of immune cells, form biofilm and carry plasmids which code for proteins
such as cytolysin (a protein that causes lysis of red blood cells) and aggregation substance. Moreover, these plasmids can be shared among different species of *E. faecalis*.

In the oral cavity, *E. faecalis* is found within 4-40% of the root canal primary endodontic infections and much more in persistent peri-radicular lesions. Interestingly, primary infections contain many types of bacteria, while secondary infections following root canal treatment, are created from one or few bacterial species. *E. faecalis* prevalence in failed root canal treatment is nine times higher than in primary infections (24-77%) [5]. Even though *E. faecalis* constitute just a small portion of the dentin tubules flora, it is mainly responsible for these lesions due to its virulence factors. Its size enables *E. faecalis* to colonize within the dentinal tubules and to create biofilm with higher resistance to antibacterial agents. The biofilm mode of growth not only gives bacteria effective protection against the host's defense system but also makes them more resistant to a variety of disinfecting agents used as oral hygiene products or in the treatment of infections [6]. It is important to apply the biofilm concept to endodontic microbiology to understand the pathogenic potential of the root canal microbiota as well as to form the basis for new approaches for disinfection. Successful treatment of these diseases depends on biofilm removal as well as effective killing of biofilm bacteria [6]. Moreover, the serum helps the bacteria to bind to type 1 collagen. Unfortunately, potent antimicrobial root canal agents as calcium hydroxide were found not effective enough against *E. faecalis* [5].

Antimicrobial peptides (AMP's) are conserved evolutionary components of the innate immune system and are ribosomally produced in different kinds of unicellular organisms, plants and highly evolved animal species. AMPs are potent and efficient against a wide range of pathogens due to their structure and charge. AMPs can be suitable candidates as a therapeutic agent and perhaps replace conventional antibiotics. AMPs are one of the key factors that enable humans to stay healthy [7].
In general, antimicrobial peptide’s main activities include an ability to kill microbes without specificity for a specific pathogen. AMPs also activate inflammatory cells and recruit them to enhance the immune response due to their ability to draw these cells to the injured tissue from the blood stream (chemotaxis) [8]. These peptides exhibit broad-spectrum activity against a wide range of microorganisms including Gram-positive and Gram-negative bacteria, protozoa, yeast, fungi and viruses [9].

Hundreds of AMPs were identified in various kinds of organisms including bacteria, insects, plants, animals and humans [9]. In humans, the first synthesis of these peptides (enteric defensins) starts at 13.5-17 weeks after gestation [7], so that the fetus is born with AMPs. The AMPs are secreted from many kinds of cells such as epithelial surfaces, where there is a direct contact with pathogens (oral cavity, gastro-intestinal tract, skin etc.), glandular structures and phagocytic cells like neutrophils [10].

The peptides families differ in their amino acid sequence (8-100) and dimensional structure. Their electric charge is positive due to amino acids such as Arginine, Lysine and Histidine (in acid environment). The peptides contain 50% hydrophobic residues that provide an amphipathic character to the molecule allowing the peptides' function. Furthermore, these peptides are relatively small and have different secondary structures that include: α-helical linear peptides (like LL-37), Cysteine rich AMPs which create disulfide bonds (like defensins), β-sheet peptides, AMPs rich in regular amino acid and AMPs with rare modified amino acid [9].

The functions and mode of actions of AMPs are a direct derivative of their structure and electric charge. Their complex mechanism, is not fully understood. Despite the controversy, there is a consensus that these peptides selectively disrupt the cell membranes due to their amphipathic structural arrangement [9]. In addition, recent studies had shown that they also have the ability to penetrate into the cell, bind to intracellular molecules (like DNA, RNA and different proteins) and by that inhibit cell-wall synthesis, nucleic acid synthesis, protein synthesis or inhibit
enzymatic activity [11, 12]. It is essential to take in consideration that AMPs can display different molecular mechanisms, depending on their concentrations. Based on the classical amphipathic α-helical or β-sheet structures of the AMPs, several hypotheses were suggested that could explain the mechanism of membrane permeation. These models vary from classical “barrel stave” transmembrane pore formation mechanism (characterize peptides that can cross the lipid bilayers regardless of the charge of the phospholipid head-groups) to a very general mechanism of membrane destabilization via “carpet” model that can involve the generation of “toroidal” pores, channel aggregates, or more complex structures, depending on the length and the sequence of the peptide [13]. However, it is known that the first step in the selection of target cells is governed by the electrostatic interactions between the positively charged side chains of the amino acids and the negatively charges components of the microbial cell wall, mainly lipopolysaccharides (LPS or endotoxin) in the outer membrane of Gram-negative bacteria, or lipoteichoic acid (LTA) in Gram-positive bacteria. Furthermore, AMPs perform many activities relating to immunomodulation, including the induction of chemokine and cytokine production, alteration of gene expression in host cells and inhibition of proinflammatory responses of host cells to bacterial components such as lipopolysaccharide (LPS) [14].

Current explanation for the preferential activity of the AMPs against bacteria but not against mammalian cells is based on the difference between the two membranes. Bacterial membranes are organized in such a way that the outermost leaflet of the bilayer, the surface exposed to the outer world, is heavily populated by lipids with negatively charged phospholipid headgroups. In contrast, the outer leaflet of the membranes of plants and animals is composed principally of zwitterionic matter, lipids with no net charge, which should reduce the binding capacity of the cationic AMPs. Most of the lipids with negatively charged headgroups are segregated into the inner leaflet, facing the cytoplasm [15, 16]. So, the AMPs create strong electrostatic and hydrophobic interactions with more affinity to bacterial membranes.
With all that in mind, the advantages of AMPs are the fact that they have a non-specific but selective antimicrobial mechanism of action and because of that, bacteria have difficulty developing resistance against them. Despite that, there are several resistance strategies to avoid AMP's function such as altering net surface charges [17], transporting AMPs into the cytoplasm and degrading them [18] and exporting AMPs by efflux pumps [19]. The promise of antimicrobial peptides lies in their broad efficacy. Indeed, several antimicrobial peptides or synthetic derivatives have reached clinical trials in different phases. However, none have been approved yet by the FDA [20, 21].

The four major groups of human AMPs are cathelicidins (LL-37), defensins, histatins and protegrins. In the oral cavity the expression of these peptides can be constitutive or inducible. LL-37 and α-defensins (types 1, 2, 3 and 4) can be detected in neutrophils that migrate from the junctional epithelium and β-defensins are expressed usually in keratinocytes from the gingival and sulcular epithelium. That kind of dispersion is designated to create synergism to protect the host [20, 22]. In humans, six α-defensins and three β-defensins (and θ-defensins) have been identified. β-defensins are synthesized by epithelial cells. They were initially identified in bovine tracheal epithelia and subsequently in other epithelia and species. The β-defensins are either produced constitutively or induced in inflamed regions. They are synthesized with a signal and pro-peptide and are secreted into biological fluids. All defensins have a compact peptide structure that contains three disulfide bonds. The α- and β-defensins differ in primary sequence and in the placement of the disulfide bonds [23]. β-defensins show both anti-fungal and antibacterial action. Human β defensin 3 (hBD3) (Fig 1) is consistently more active against both bacteria and fungi, with hBD-2 next and hBD-1 last in activity. Defensins act synergistically with other antimicrobial agents such as lysozyme and other antibiotics. This makes them particularly attractive as potential therapeutic agents. In addition to their antimicrobial activity, the defensins have the ability to act as signals that mediate between the innate and the adaptive immune system. They are expressed in the gingiva,
tongue, salivary glands and other oral regions. They are present in oral inflammatory conditions, oral carcinomas and some cell lines derived from oral carcinomas [22].

HBD3 was previously reported as being a highly potent antibacterial AMP against *E. faecalis*. It inhibits *E. faecalis* growth and biofilm formation and it also neutralizes the activity of *E. faecalis* lipoteichoic acid (LTA) that is located within the cell wall of gram-positive bacteria and contact with host cells and initiate an inflammatory response [24, 25, 26, 27].

Another potent antibacterial family is the native lipopeptides which differ from AMPs in that they are non-ribosomal product only in bacteria and fungi during cultivation on various carbon sources. Moreover, native lipopeptides are non-cell-selective and therefore toxic to mammalian cells [28]. Nonetheless, daptomycin (type of lipopeptide), which is active against Gram-positive bacteria, was recently approved by the FDA for the treatment of skin infections caused by *Staphylococcus aureus* [29]. Most native lipopeptides consist of short (six to seven
amino acids) linear or cyclic peptide sequence, with either a net positive or a negative charge, to which a fatty acid moiety is covalently attached to the N-terminus. Compared to AMPs, resistance to lipopeptides is generally rare [30]. Similarly to AMPs, most native lipopeptides act via two major mechanisms: (1) inhibiting the synthesis of cell wall compounds such as (1, 3)-β-D-glucan or chitin, and (2) inducing membrane lysis [31]. Attempts have been made to produce synthetic AMPs. The basic idea of synthetic AMPs is to recruit all the structural advantages of the native AMPs to build improved antibiotic agents, as a result of bacterial resistance which have developed these years [32, 33]. Industrial purposes require that the peptides will be small and with simple structure. Therefore, considerable research has been devoted to optimize peptide length combined with a simple design. Thousands of AMPs and lipopeptides have been previously synthesized (see the UNMC database http://aps.unmc.edu/AP/mail.php ); examples include: ultra-short lipopeptides and an amphipathic α-helical antimicrobial peptide (Amp-1D) (table 1). Amp-1D is 15 amino-acid peptide. It was shown that there is an importance to the diastereomer form of the peptide regarding their structure, function and interaction with model membranes and intact bacteria. Whereas the all L-amino acid peptides were highly hemolytic, had low solubility, lost their activity in serum, and were fully cleaved by trypsin and proteinase K, the diastereomers were non-hemolytic and maintained full activity in serum [34]. The lipopeptides presented are composed of only four amino acids conjugated to aliphatic acids chain (16C). Ultra-short lipopeptides are amphiphilic molecules mimicking detergents, in which one side is hydrophilic (the peptide side) and the other one is hydrophobic (the fatty acid side). Therefore, they can form a hydrophobic core through self-association. However, the tendency to oligomerize is offset by the resulting proximity of the positively charged peptide moiety. For this reason, the hydrophobic moiety needs to be a long fatty acid, usually comprising at least 14 carbons. The finding that a single lysine attached to a palmitic acid (16 carbon atoms) is not active supports the notion that the activity of these lipopeptides is not determined solely by the hydrophobic aliphatic chain, but also requires a specific amino acid sequence. Elongation of the peptide
chain above four amino acids can allow the shortening of the conjugated fatty acid [35]. The ability of the lipopeptides to oligomerize in solution protect them from proteolytic degradation compared with peptides that do not oligomerize. Therefore, the existence of antimicrobial lipopeptides as aggregates may be an advantage in vivo where resistance to proteolytic degradation can influence the half-life of the peptide and its efficacy [31]. It has been shown in previous studies that these ultra-short lipopeptides are potent against a variety of microorganisms. Importantly, despite their short peptidic chain length, their mode of action involves the disturbance of the membrane like native AMPs [28]. Moreover, studies have revealed that fatty acids can compensate for the length of the short peptidic chain. Acylation of synthetic or natural AMPs with fatty acids has been proven to be a useful approach to improve their antimicrobial and antifungal activity. This effect is due to changes in the overall hydrophobicity of these molecules, which affects both their oligomerization, organization in solution and their affinity to membranes. It was found that substituting only one of the four amino acids is sufficient to create molecules with different biological functions. For example, broad spectrum compounds can become selective toward Gram-positive bacteria and fungi only [36]. Another example is the bacterial LPS, which is known for inducing oligomerization of peptide molecules. Because of the large size of the aggregates it is difficult for the peptides to diffuse through the LPS-leaflet into the target cytoplasmic membrane, and therefore they lose activity against Gram-negative bacteria. However, the length of the fatty acid and the amino acid composition of the peptide chain can control aggregate formation and ease their dissociation. Therefore, finding the correct fatty acid and peptide sequence is important for the peptides’ ability to traverse the outer LPS barrier. In previous studies, the in vivo activity of the ultra-short lipopeptides was analyzed in mice models for fungal infection. Moreover, one of the lipopeptides
was more efficient than amphotericin B at nontoxic doses [31].

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino acid sequence</th>
<th>MW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amp -1D</td>
<td>LKLLKKLLKKLLKLL-NH₂</td>
<td>1805.49</td>
</tr>
<tr>
<td>C16-KGGK</td>
<td>CH₃(CH₂)₁₄CO-KGGK- NH₂</td>
<td>626.47</td>
</tr>
<tr>
<td>C16-KKK</td>
<td>CH₃(CH₂)₁₄CO – KKK- NH₂</td>
<td>640</td>
</tr>
<tr>
<td>C16-KAAP</td>
<td>CH₃(CH₂)₁₄CO - KAAP - NH₂</td>
<td>654</td>
</tr>
<tr>
<td>C16-KLLL</td>
<td>CH₃(CH₂)₁₄CO – KLLL - NH₂</td>
<td>738</td>
</tr>
</tbody>
</table>

# The underlined amino acids are D-enantiomers.

In this study hBD-3 and these AMPs mimetics were examined for their antibacterial activity. Herein we focused on developing a slow release therapeutic method. A slow releasing therapeutic can be advantageous in maintaining consistently high peptide levels. This may be especially advantageous in the intra-oral environment and in the intra root canal dentin tubules where there are constantly microorganisms that are threatening the health of teeth and neighboring tissues. Basically, in dentistry there are several challenges in drug delivery such as retention of dosage from dilution of drug concentration by salivary flow and enzymatic action that can cleave certain peptides. Several sustained-release delivery devices already exist in dental practice since the clearance time of most drugs from the oral cavity is rapid due to the salivation and food. Moreover, it is known that the drug must remain in the mouth for a prolonged period since caries are chronic disease based on bacterial infection [37]. Currently, there are slow release devices of fluoride (restorative materials, pellets, bio-adhesive tablets and varnishes) and antibacterial materials like chlorhexidine.
Integration of the structural and functional properties of peptides and proteins with the versatility of synthetic polymers has gained significant interest in material design and application. These polymeric systems have several advantages over conventional methods, such as ease of manufacturing, ease of administration, biodegradability, and the ability to alter release profiles of the incorporated agents [38, 39]. Peptides and proteins have unique structures that convey their ability to function in specific biological activities. Hybrid molecules of peptides conjugated to polymers can be used for various applications with the advantages of being resistant to enzymatic cleavage and less cytotoxic to human cells [40]. Peptide-synthetic polymer conjugates, also referred to as biohybrid medium, consist of biologically relevant peptides and synthetic polymers, aiming to combine the advantages of the two components, namely biological function (biological component) and process-ability (synthetic component). A slow release mechanism can enable high concentration maintenance of therapeutic agents for prolonged periods of time. Biodegradable polymers poly (lactic acid co castor oil) (PLACO) and ricinoleic acid-based poly (ester-anhydride) P (SA-RA) (Fig 2) were synthesized and tested as delivery mediums for this purpose. Fatty acid incorporation in biodegradable polymers provides flexibility, low melting point and hydrophobicity so that the drug is released in a predictable and a controlled manner. The fact that these polymers degrade into natural compounds, turns them environmentally friendly, biocompatible and useful for drug delivery and as implantable devices. For this reason, these polymers have gained tremendous attention in the past two decades for biomedical application. These are attractive candidates materials for short-term medical applications, like sutures, drug delivery devices, orthopedic fixation devices, wound dressings, stents, etc. [41]. For example, P (SA-RA) may be injected via 22-gauge needle and become gel upon contact with aqueous medium, both in vitro and in vivo [42]. Moreover, in previous studies it was shown that P (SA-RA) can be degraded for a few weeks under in vitro physiological conditions while constantly
releasing an incorporated drug for more than 2 weeks. They were also stable to sterilization by γ-irradiation, which is essential within dental practice [43].

A novel formulation was tested for both antibacterial activity and slow release. The AMPs and the lipopeptides were inserted manually into the biodegradable polymers in a certain, constant concentration.
Materials and methods

Test materials

Antimicrobial peptides

Human recombinant β-defensin 3 (hBD3) (GIINTLQKY CRVRGGRCAV LSCLPKEEQIGKCSTRGRKC CRRKK) was obtained from PeproTech (Lot # 0108210, Rocky Hill, NJ, USA).

Five different synthetic AMPs were tested. Amphipathic α-helical antimicrobial peptide (AMP-1D) and four ultra-short lipopeptides: C16-KGGK, C16-KKK, C16-KAAK and C16-KLLK, were synthesized and purified as previously described [28, 34].

Biodegradable polymer synthesis

Poly (lactic acid co castor oil 30:70) (PLACO) and ricinoleic acid-based poly (ester-anhydride) (P(SA-RA)) were synthesized as previously described [40, 42, 43, 44, 45]. In brief, PLACO was synthesized by ring opening polymerization of DL lactide (6g) with a 1% w/w solution of stannous hexanoate as catalyst in castor oil (14g) in a 20 mL ampule. The ampule was heat sealed and kept at 140°C for 48h to form the desired pasty polymer (MW 2300). FTIR and 1H-NMR spectral analysis confirmed the structure and the 3:7 w/w ratio. The poly (ester-anhydride) copolymer of sebacic acid (SA) and ricinoleic acid (RA) at a weight ratio of 3:7 [P(SA-RA) 3:7] was synthesized by transesterification, followed by anhydride melt condensation. In the first step, sebacic acid (SA) is polymerized to PSA with a MW of 20000 or higher using acetic anhydride as activation agent. The formed PSA was reacted with ricinoleic acid (prepared from the hydrolysis of castor oil) at a 3:7 w/w ratio. The formed dimers and trimers of RA-SA or RA-SA-RA were reacted with acetic anhydride to activate the carboxylic acids, followed by polymerization into a polyanhydride at 160°C under a vacuum of 20 mm Hg for 7 hrs. The obtained polymer was pasty at room temperature, with a MW of 13000. Infra-red spectroscopy (FTIR) and nuclear magnetic resonance (1H-NMR) spectral analysis confirmed the structure and the 3:7 w/w ratio.
Formulation of AMP-based biohybrid media

The peptide powder was mixed with a pasty polymer to form a uniform homogeneous paste. A novel formulation of peptide and biodegradable polymer was prepared at a ratio of 100 µg peptide integrated in 100 mg polymer. The two ingredients were mixed manually with a spatula, as previously described [46].

Bacterial strains, cell lines and growth conditions

Preparation of bacterial suspensions

*E. faecalis* (ATCC # v583), was cultured overnight in 5 mL brain-heart infusion (BHI) (Difco, Detroit, MI, USA) broth supplemented with 2 mg/mL vancomycin (Sigma-Aldrich), at 37 °C under aerobic conditions. *S. mutans* (ATCC # 27351) was cultured similarly in BHI broth supplemented with 2.77 µg/mL bacitracin (Sigma-Aldrich) and 5% glucose (Sigma-Aldrich). *A. naeslundii* (ATCC # 17233) was cultured in Wilkins-Chalgren anaerobe broth (Oxoid Ltd., Basingstoke, Hampshire, England) supplemented with 2% sucrose under anaerobic conditions. *P. gingivalis* (ATCC # 33277) and *F. nucleatum* (ATCC # 1594) were cultured in Wilkins-Chalgren broth under anaerobic conditions. The top 4 mL of each bacterial tube were transferred to a fresh test tube and the optical density (OD) was determined according to the specific experiment.

Antibacterial activity

Agar diffusion test (ADT)

The bacteria examined were incorporated into starvation agarose medium which contained: 50 ml phosphate-buffered-saline (PBS) (Sigma-Aldrich), 0.015 g tryptic soy broth (TSB) (Sigma-Aldrich), 0.4 g agarose (0.8%) (Thermo Fisher Scientific) and one drop of tween (Sigma-Aldrich). Then, we created 2 mm diameter wells in the medium to insert the tested materials. Two kinds of plates were prepared: one plate with different concentrations of the
peptide and one plate with different materials to compare the efficacy between them. The plate was incubated for 3 hours to ensure the diffusion of the materials into the agarose before pouring a nutrient-rich overlay gel, which contained: 50 ml PBS, 3 g TSB and 0.5 g agarose (0.5%). The positive control groups were 5mM HCl, chlorhexidine gel 0.2% (Lacer) and calcium hydroxide solution. The negative controls included: PBS and biodegradable polymers without peptide. After 24 hours of incubation the existence or absence of clear zone in every tested group was observed.

Furthermore, we performed a test in which 100 µl of tested bacteria were pored and smeared equally using Drigalski spatula (Sigma-Aldrich) in a blood agar plate. We placed several round papers (4 mm diameter) in the contour of the plate and dripped 5 µl of the tested solution. After 24 hours of incubation the existence or absence of clear zone in every tested group was observed.

**Minimal inhibitory concentration**

The antibacterial activity of the peptides was examined using the microdilution assay [47]. In brief, the bacterial suspension (at OD 0.3) was diluted at a ratio of 1:1000. Aliquots of 150 µl of bacterial suspension were added to 50 µl of peptide dilutions in PBS (Sigma-Aldrich) (in triplicate for each concentration) in a 96-well plate (Nunc 96 microtiter plates, Roskilde, Denmark). The optical density (595 nm) in each well was recorded every 20 min using a microplate reader (VERSAmax tunable microplate reader, molecular devices, Sunnyvale, CA, USA) at 37 °C for 18-24 hrs. The minimal inhibitory concentration (MIC) was determined as the concentration which inhibited visible growth after 18-24 hrs.
Antibacterial activity of sustained release lipopeptides

A total 10 mg of formulation was placed on the side walls of each of 6 wells in a 96 microtiter plate and then 270 µl of medium (BHI supplemented with vancomycin) were added. Every 24 hrs the medium was collected and transferred to a new set of 6 wells in the same 96-well-plate and fresh medium was added to the 6 original wells containing the tested formulation. After one week, a 10 µl volume of bacterial suspension was added to each of the 6 wells and bacterial outgrowth was recorded. The plate was incubated at 37 °C in a VERSAmax microplate reader and turbidity (OD$_{650}$ nm) changes were recorded, every 20 min for 18-24 hrs.

Antibiofilm activity

Antibiofilm activity was tested on *E. faecalis, F. nucleatum* and *S. mutans* biofilms grown for 72 hrs. Biofilm was formed in microtiter plates (24 well plates for the ATP bioluminescence assay and 96 well plates for the crystal violet biomass assay and confocal laser spectroscopy). Saliva was collected from one donor and DL-Dithiolthreitol (DTT) (Thermo Scientific, Abu-Gosh, Israel) was added to 2.5 mM. The suspension was kept at 4 °C for 10 min and then centrifuged for 15 min at 6,500 x g. The supernatant was transferred to a fresh sterile tube and diluted to 25% with sterile double distilled water (DDW). The diluted saliva was disinfected using a 0.2 µm vacuum-driven filter (0.22 µm, 250 µl, Jet biofil, Belgium). Wells in the microtiter plate were coated with clarified saliva by adding the saliva to the wells for 1 hr at 37 °C (150 µl of saliva in the 24 well plate and 50 µl in the 96 well plate). Unbound saliva was removed and the wells were washed gently with PBS. The polymer peptide formulations were placed on the side walls of the wells and 10 µl of bacterial suspension (prepared as described above) were placed in the center of each well not touching the coated sidewall. The saliva coating was used to cover the entire well surface, followed by formulation placement on the sidewall of the wells. The bacterial inoculum was placed in the center of each well, not touching the formulation. After 1 hr incubation at 37 °C BHI broth was added (1 ml in the 24 well plate and
100 µl in the 96 well plate). BHI broth was added every 24 hrs during 72 hrs. After 3 days, the medium was discarded and the wells were washed gently with PBS. Bacterial metabolism in the attached biofilm was assessed using ATP bioluminescence. Biofilm mass was measured using crystal violet as described below.

**ATP bioluminescence**

Bacterial killing was evaluated by measuring intracellular ATP levels, an energy parameter commonly used as an indicator of cell injury and viability [47]. The 72 hr biofilm formed on the bottom of the wells was scraped using a pipette tip and collected into a set of 15 ml tubes. The cells were then centrifuged (6,500 x g, 5 min), resuspended in 1 ml Lysis Buffer (2mM DTT, 2mM trans 1,2 Diaminocyclohexane NNNN Tetraacetic acid, 0.5 mM EDTA, 1% Triton, 25 mM Tris, 25 mM K₂HPO₄, 10% glycerol) and transferred to a 2 ml micro centrifuge tube containing glass beads (Lysing Matrix tubes, 0.1 mm silica spheres; MP Biomedicals, Eschwege, Germany). The cells were disrupted with the aid of a FastPrep cell disrupter (MP Biomedicals, Irvine, CA, USA). The tube was centrifuged for 10 min (4 °C, 13,400 x g). ATP levels were determined using an ATP bioluminescence assay kit (CLS 2, Roch Diagnostics, Mannheim, Germany). In a 96 microtiter plate designed for luminescence assay (Thermo Scientific, NUNC, 96-well optical Btm Plt white, Rochester, NY, USA) a 100 µl volume of the samples was added to 6 wells for each tested group. Then 100 µl luciferase (from the kit) were added to the same wells. The plate was inserted in a GENios reader (TECAN, Salzburg, Austria) and luminescence was measured using the Magelan program (TECAN, V6.6, 2009). ATP calibration was performed using ATP and luciferase from the kit.

**Crystal violet**

The total biofilm yield was assessed using crystal violet staining as follows. Biofilm fixation was performed using 200 µl methanol (MERCK, Darmstadt, Germany) that were added to each well for 20 min. The biofilm was then stained using 200 µl 1% crystal violet (Merck) for 20 min. Then the wells were washed gently 3x with PBS, and 200 µl of 30% acetic acid
(GADOT, Netanya, Israel) were added to the wells. The acetic acid was transferred to wells of a new 96-well microtiter plate that was placed in a microplate reader and absorbance (OD$_{595}$ nm) was measured.

**Confocal microscopy**

Confocal laser scanning microscopy (CLSM) was used to explore the vitality of bacteria in the different depth layers of the biofilm. Bacteria were stained using a live/dead kit (Live/Dead BacLight viability kit, Molecular Probes, OR, USA) as described before [48]. In brief, wells were washed, incubated for 15 min in a solution containing propidium iodide and SYTO 9 and washed again. To read the results directly, the wells were coated with emulsion oil to prevent dehydration. Fluorescence emission was detected using a Zeiss LSM 410 confocal laser scanning microscope (Carl Zeiss Microscopy, Jena, Germany). Red fluorescence was measured at 630 nm and green fluorescence at 520 nm; objective lenses: x60/oil, 1.4 numerical aperture. Horizontal plane (x-y axes) optical sections were made at 700 µm intervals from the surface outwards and images were displayed individually. The biofilm was quantified by measuring the area occupied by the bacteria with the aid of Image Pro 4.5 software (Media Cybernetics, Rockville, MD, USA).

**Biocompatibility**

**Hemolysis of RBC**

The test was performed as described previously [49], in a final volume of 100 µL PBS containing different concentrations of the lipopeptides and 100 µL sheep red blood cells (RBCs) [final concentration 4% (vol/ vol)]. Hemoglobin release was monitored by measuring the absorbance of the supernatant at 540 nm. The controls for 0% hemolysis (blank) consisted of RBCs suspended in PBS, whereas for 100% hemolysis were RBCs suspended in 1% Triton X-100.
**Colorimetric XTT assay**

Cell viability was tested as previously described [50]. In brief, mouse macrophages RAW-246 were cultured overnight in Dulbecco's Minimum Essential Medium (DMEM, Sigma-Aldrich) supplemented with 10% inactivated fetal calf serum (FCS, Biological Industries, Beit-Ha'emek, Israel), 1% L-glutamine (Biological Industries) and 1% streptomycin (Biological Industries), at 37°C in 5% CO₂. Each formulation of biodegradable polymer and lipopeptide within plastic inserts (Rosenshein, Israel) that were previously sandblasted, was added to eight wells of a 96-well microtiter plate. Then 200 µL of cell suspension were added to the wells and after 24 hrs the XTT assay (Biological Industries) was initiated by the addition of 50 µL activated XTT solution to each well. The microtiter plate was incubated for 2-4 hrs and then monitored by measuring the absorbance of the supernatant at 450 nm in a VERSAmax microplate reader.

**Bacterial membrane disruption**

To evaluate the effect of a lipopeptide on the bacterial cell membrane, cytoplasmic membrane depolarization was measured by the DiOC₂(3) assay and fluorescence-activated cell sorting, as described below. The BacLight bacterial membrane potential kit (Molecular Probes, Invitrogen, Eugene, OR, USA) provides a fluorescent membrane potential indicator dye, 3,30-diethyloxacarbocyanine iodide [DiOC₂(3)], along with carbonyl cyanide 3-chlorophenylhydrazone (CCCP). At low concentrations, DiOC₂(3) exhibits green fluorescence in all bacterial cells. As it becomes more concentrated in healthy cells that are maintaining a membrane potential, the dye self-associates and the fluorescence emission shifts to red. CCCP is included in the kit for use as a positive control because it is a proton ionophore and it eliminates the bacterial membrane potential. All bacterial suspension samples (1 mL) were incubated in Eppendorf tubes for 1 hr. Then the samples were filtered through a cell strainer 70 µL (SPL life scientific, Korea). A 10 µL volume of CCCP was added to the control group and 10 µL of DiOC₂(3) were added to each sample. The samples were kept at room temperature for 30 min before analysis by flow cytometry (BD accuri C6 Flow Cytometer, BD
Bioscience, Becton, Dickinson and Company). Whereas the relative red and green fluorescence intensity varies according to cell size and aggregation, the ratio of red to green fluorescence intensity can be used as a size-independent indicator of membrane potential. The data were analyzed with De Novo FCS Express software.

**Sustained release kinetics**

The release profile of the lipopeptide from the biodegradable polymer was tested by the fluorescamine assay due to its high sensibility and specificity for primary amino groups, as described previously [51, 52]. In brief, 2 mL of assay solution containing 1800 µL peptide released from the polymer in boric acid buffer (Sigma-Aldrich) together with 200 µL of fluorescamine reagent were introduced into a 12 X 75 mm glass tube. A 200 µL volume of formulation was added to each assay. Fluorescamine (Aldrich Chemical, Milwaukee, WI) was prepared in acetone (Fisher Scientific) to a final concentration of 0.1 mg/mL in a glass screw cap tube. The assay buffer was vortexed until the solutions were completely mixed. Samples were transferred to a polystyrene cuvette that was previously cleaned with nitric acid followed by several rinses with deionized water. Fluorescence was measured at ambient temperature with a Spex 1680 spectrofluorometer (λ_{ex} = 390, λ_{em} = 460) and a time-based scan mode with 2 sec integration time. The measurements were corrected for lamp intensity fluctuations and for the background fluorescence from a solution containing buffer and fluorescamine solution. The final amount of peptide released from the polymer was calculated according to calibration curves made before the experiment.
Tooth model

Twenty-four uni-canal teeth were sterilized, their clinical crowns were cut off and each tooth was endodontically prepared with Protaper NEXT (Dentsply). Each tooth was infected with *E. faecalis* and incubated for 24 hrs. First group of canals was irrigated with KGGK peptide in MIC concentration, and second group with Dakin’s solution as control. Third group was dressed with KGGK and P (SA: RA) formulation and fourth group was dressed with calcium hydroxide as control. Canals were dried with sterile paper points and 4 µl of fresh medium (BHI) were inserted. The first and the second group were incubated for 1 hr and CFU test was performed for each tooth. After 48 hrs of incubation the dressed canals were treated in the same manner.

Statistical analysis

The data are presented as the mean and standard deviation of a representative experiment performed in triplicate. Multiple comparisons were calculated with Student’s t-test. The level of significance was p < 0.01.
Results

Antibacterial activity

Agar diffusion test (ADT)

Agar diffusion tests were performed in order to examine the AMPs antibacterial effect. Three tests were performed for each AMP. First test compared the antibacterial effect of each AMP and different known antibacterial agents (like CHX). The second test was performed to examine different AMP concentrations and the third test was performed for the same purpose within blood agar plate. After incubation, the existence or absence of clear zone in every tested group was observed and photographed. The results were similar to all AMPs examined in the study. Fig 3 is an example photographed for KLLK AMP. Bacterial growth inhibition was seen for HCl, CHX, calcium hydroxide (positive controls) and P (SA: RA) polymer but not with the different AMP concentrations.

Fig. 3. Agar diffusion test - *E. faecalis* growth in the presence of AMP KLLK. The bacteria examined were incorporated into three different agar plates as described above. Wells were notched from the agar medium to insert the tested materials (A, B). One plate with different materials to compare the efficacy between them (A) and plates with different concentrations of the peptide (B, C). Positive controls included: HCl, CHX and calcium hydroxide. Negative control included PBS. After 24 hours of incubation the existence or absence of clear zone in every tested group was observed.
Minimal inhibitory concentration

The MIC for each of the tested lipopeptides against the tested microorganisms are presented in Table 2. Growth of *E. faecalis* was not inhibited by hBD3 at concentrations of up to 20 µg/ml. Amp-1D did not affect bacterial growth at concentrations of up to 25 µg/ml. C16-KGGK, C16-KKK, C16-KAAK and C16-KLLK completely inhibited bacterial growth at concentrations ranging between 5 and 25 µg/ml. The most potent AMP was the lipopeptide C16-KGGK that caused complete growth inhibition at 5 µg/ml (Fig. 4). Thus, all further formulations regarding *E. faecalis* inhibition, were tested using the C16-KGGK lipopeptide. Surprisingly, at concentrations below 5 µg/ml the growth of *E. faecalis* was not inhibited by C16-KGGK but was rather accelerated.

Table 2. MICs of the AMPs tested [µg/mL]

<table>
<thead>
<tr>
<th>Amino acid sequence</th>
<th><em>E. faecalis</em></th>
<th><em>S. mutans</em></th>
<th><em>F. nucleatum</em></th>
<th><em>P. gingivalis</em></th>
<th><em>A. naeslundii</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>hBD3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GIINTLQKY CRVRGGRCAV</td>
<td>&gt;20</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LSCLPKEEQI GKCSRGRKC</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CRRKK</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Amp-1D</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LKLLLKKLLKKLLL-NH₂</td>
<td>&gt;25</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C16-KGGK</td>
<td>CH₃(CH₂)₁₄CO-KGGK-NH₂</td>
<td>4-5</td>
<td>6-12.5</td>
<td>12.5-25</td>
<td>12.5-25</td>
</tr>
<tr>
<td>C16-KKK</td>
<td>CH₃(CH₂)₁₄CO - KKK-NH₂</td>
<td>6-12.5</td>
<td>12.5-25</td>
<td>4-5</td>
<td>6-12.5</td>
</tr>
<tr>
<td>C16-KAAK</td>
<td>CH₃(CH₂)₁₄CO - KAAK-NH₂</td>
<td>12.5-25</td>
<td>&gt;25</td>
<td>12.5-25</td>
<td>&gt;25</td>
</tr>
<tr>
<td>C16-KLLK</td>
<td>CH₃(CH₂)₁₄CO - KLLK-NH₂</td>
<td>6.25-12.5</td>
<td>6-12.5</td>
<td>12.5-25</td>
<td>6-12.5</td>
</tr>
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<td></td>
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</tbody>
</table>
Fig. 4. *E. faecalis* growth is inhibited by C16-KGGK but not by hBD3. Growth of *E. faecalis* was measured in the presence of increasing concentrations of hBD3 (A) or of the lipopeptide KGGK (B). Percent growth inhibition was calculated compared with that of untreated bacteria during the logarithmic phase of the non-treated bacteria. Generation time was calculated from each curve using the section representing the exponential growth phase (C).

### Table 1

<table>
<thead>
<tr>
<th></th>
<th>hBD3</th>
<th></th>
<th>KGGK</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Concentration (µg)</strong></td>
<td><strong>% reduction in growth</strong></td>
<td><strong>Generation time (h/doubling)</strong></td>
<td><strong>Concentration (µg)</strong></td>
<td><strong>% reduction in growth</strong></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>3.025</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>-21.34 ± 0.04</td>
<td>3.504</td>
<td>2</td>
<td>-26.07** ± 0.03</td>
</tr>
<tr>
<td>10</td>
<td>-21.95 ± 0.02</td>
<td>3.059</td>
<td>4</td>
<td>-39.27 ± 0.07</td>
</tr>
<tr>
<td>15</td>
<td>-2.89 ± 0.06</td>
<td>3.152</td>
<td>5</td>
<td>NG*## ± 0.006</td>
</tr>
<tr>
<td>20</td>
<td>-24.82 ± 0.08</td>
<td>3.01</td>
<td>6.25</td>
<td>NG*## ± 0.006</td>
</tr>
</tbody>
</table>

* a calculated at logarithmic phase at each tested condition ** P<0.01 compared with control without AMP, determined using Student’s t-test * No growth
The different bacteria showed varying susceptibility to various lipopeptides. The most potent lipopeptide against *E. faecalis* causing complete growth inhibition was KGGK. The most effective lipopeptides against *S. mutans* were C16-KGGK and C16-KLLK. KKKK exhibited antibacterial activity at low concentrations against *F. nucleatum*, *P. gingivalis* and *A. naeslundii*.

**Sustained release and antibacterial activity**

Release of the C16-KGGK lipopeptide from biodegradable polymers was monitored over one week in two modes. In the first, the antibacterial action of C16-KGGK released into the medium that came in contact with the formulation every 24 hrs was measured (Fig. 5 A, B). In the second, the bacteria were added to the wells with C16-KGGK that was released from the polymer and accumulated for one week (see Fig. 5C). The anti-*E. faecalis* activity of C16-KGGK released from each formulation was reflected by: (I) the final endpoint optical density of the treated bacteria, which was lower than that of the untreated ones; (II) the outgrowth slope (generation time, Fig. 5C), which was more moderate in the treated bacteria.

The anti-*E. faecalis* activity in the medium exposed to the formulation for an entire week (Fig. 5 D, E) generated a longer generation time, especially with P (SA: RA). Bacteria treated with the PLACO formulation exhibited a 27% reduction in growth and those treated with P (SA: RA) a 60% reduction compared with the non-treated bacteria.
Fig. 5. Growth inhibition of *E. faecalis* by KGGK released from P (SA: RA) or from PLACO. The side walls of 6 wells from line A of a 96 microwell plate were coated with the tested formulation (100 μg peptide + 100 mg polymer, ratio 1:1000). Fresh medium was added to the first line of wells and was transferred every 24 hrs to a new line below for a week. Then the bacteria were added to the tested wells and the plate was incubated at 37°C in a VERSAmax microplate reader and OD$_{650}$ in each well was followed automatically for 20 hrs. (A, C) KGGK + PLACO. (B, C) KGGK + P (SA: RA) (D, E) weekly release of both formulations. Percent growth inhibition calculated compared with that of the non-treated bacteria during the logarithmic phase of the non-treated bacteria. Generation time was calculated from each curve using the section representing the exponential growth phase (C, E).
The antibacterial activity was examined for the most potent lipopeptides against *F. nucleatum* and *S. mutans*, as shown in the MIC experiment (Fig. 6). Lower final optical densities and milder growth curve slopes for all the bacteria treated with the formulated peptides were recorded. The most significant antibacterial effect was observed between 24-48 hrs, except for the KKK and P(SA-RA) formulation, where it was evident between 0-24 hrs.

**Fig 6. Growth inhibition of bacteria by lipopeptides released from P (SA: RA) or from PLACO.** The tested formulation 100 μg peptide + 100 mg polymer, ratio 1:1000 was evaluated for its antibacterial effect every 24 hrs during 1 week. (A, B) C16-KGGK formulations against *F. nucleatum* and (C, D) KLKK formulations against *S. mutans*.

**Anti-biofilm effect**

**Crystal violet dye**

Crystal violet was used to stain and measure biofilm mass so that inhibition of biofilm formation in the presence of C16-KGGK formulated with P(SA-RA) (Fig. 7A) or PLACO (Fig 7B) or in the presence of the soluble tested AMPs (Fig. 7C) could be determined. A significant anti-biofilm effect was obtained with C16-KGGK using both formulations but not with the soluble...
C16-KGGK (Fig. 7 A, B). The vehicle formulation itself does not possess anti-biofilm activity. From the other peptides tested in suspension, only C16-KKK showed anti-biofilm activity (Fig. 7C).

**Fig. 7. Effect of the antimicrobial peptides on the development of *E. faecalis* biofilms.** *E. faecalis* biofilms were grown in 96 microtiter plate wells for 72 hrs in the presence of KGGK formulated with P (SA: RA) (A), or formulated with PLACO (B) or with soluble peptides (C). Ef represents the non-treated bacteria, KGGK + Ef - bacteria treated only with peptide, formulation + Ef - bacteria treated with polymer and peptide and Ef + polymer - bacteria treated only with polymer control. The biofilm was stained with 1% crystal violet measured at OD 595 nm. The optical density of the polymers alone without the bacteria was subtracted from the results of the biofilm that came in contact with the formulation and the polymer.
ATP bioluminescence assay

The level of ATP indicates the active metabolism of a cell. ATP levels in *E. faecalis* biofilms treated with soluble C16-KGGK were decreased compared with the untreated control (*E. faecalis* alone) (Fig. 8). P (SA: RA) formulation (without C16-KGGK) reduced bacterial viability. As opposed to P (SA: RA), PLACO had the reverse effect and the luminescence values were much higher than that of the positive control. These findings led to the question whether the luminescence values are derived from bacterial number, the metabolic status or both. In addition, a similar experiment was performed in which the biofilm was first grown for 48 hrs and then the tested materials were added to verify if C16-KGGK can affect an already constructed biofilm. The results were similar to those above (where the materials were added immediately after inoculating the bacteria). This may indicate that the formulation and the peptide (each) have an anti-metabolic effect even after the biofilm is formed.

![Graph showing ATP bioluminescence assay results](image.png)

*Fig. 8. Effect of KGGK incorporated in biodegradable polymer on ATP in *E. faecalis* biofilm.* Biofilm was exposed to the formulation for 72 hrs and ATP was measured as described in Materials and Methods. *Ef* - represents the untreated bacteria as control, KGGK - bacteria treated only with peptide; formulation - bacteria treated with sustained release peptide; polymer - bacteria treated only with polymer as control.

*P<0.01 compared to control without AMP, determined using Student’s t-test*
Bacterial vitality

To test the vitality of the bacteria within the biofilm by a different, independent method, live/dead staining followed by confocal microscopic analysis was performed. The differences between the four tested groups are clearly evident for both P (SA: RA) and PLACO incorporated C16-KGGK (Fig. 9). Soluble C16-KGGK induced death in the biofilm bacteria (Fig. 9 A-B, E. faecalis + KGGK). However, C16-KGGK in both formulations was more effective than the soluble peptide alone. The P (SA: RA) polymer had a strong inhibitory activity against biofilm formation as seen by the reduction in bacterial load.
The anti-biofilm effect against uni-strain biofilms is shown in Fig 10. P (SA: RA) was effective against all the uni-strain biofilms. Formulations containing both the lipopeptides and the biodegradable polymers exhibited a higher antibacterial effect than the non-formulated lipopeptides and CHX.

**Fig. 9. Live/dead assay.** *E. faecalis* came in contact with the examined materials for 72 hrs to form biofilm. The medium was discarded and the wells were washed gently with PBS. The live bacteria were stained with green dye, the dead bacteria were stained with a red dye. A 5 ml volume of each dye from the dead/live dying kit was added to 450 µl PBS using an Eppendorf and 30 µl of the solution were added in each well. Images were taken using an Olympus confocal microscope (A, B). The black column represents the dead bacteria, the white column represents the live bacteria. The biofilm was quantified by measuring the area occupied by the bacteria with the aid of Image Pro 4.5 software (Media Cybernetics) (C, D).

**Fig 10. Biofilm growth inhibition by lipopeptide incorporated in biodegradable polymers.** The antibiofilm effect was evaluated with the use of a dead/live dying kit against a 72 hrs formed biofilm. The live bacteria were stained with a green dye, the dead bacteria were stained with a red dye. Results are shown for *E. faecalis, F. nucleatum* and *S. mutans*. The control group included 0.025% CHX.
Biocompatibility

Hemolysis of RBC

The results of the hemolysis assay are presented in Fig 11. Lipopeptides C16-KGGK, C16-KKK and C16-KLLK caused high-level hemolysis at the higher concentrations and low-level hemolysis occurred at lower concentrations.

Fig 11. Lipopeptide hemolysis assay. All four lipopeptides: C16-KGGK, C16-KKK, C16-KAAK and C16-KLLK were tested for hemolysis in sheep RBC, at concentrations of 5, 10, 20, 50 and 100 µg/ml. Insignificant hemolysis was detected at the MICs. The control group was considered complete hemolysis.

* P<0.01 compared with control without lipopeptide, determined using Student’s t-test
Colorimetric XTT assay

PLACO and P(SA-RA) were analyzed with the XTT. The viability of the RAW cells decreased significantly following P (SA-RA) exposure, whereas the PLACO polymer did not affect cell viability compared with that of the control (Fig 1A). Applications of all four formulations (peptides with PLACO) to the cells resulted in high percentages of cells survival with a minimal decrease in viability vs that of the control. The C16-KKK, C16-KGGK and C16-KAAK formulations led to lower cell survival than the formulation containing C16-KLLK (Fig 1B).

![Graph A](image1)

![Graph B](image2)

* P<0.01 compared with control without lipopeptide, determined using Student’s t-test

**Fig 12. Formulation biocompatibility by the XTT assay.** Mice macrophages RAW-246 were cultivated in wells of a 96 well microtiter plate. Each polymer was tested. PLACO exhibited the highest cell survivability (A). Each formulation containing lipopeptide and PLACO polymer was tested. All the formulations showed high cell survivability. C16-KLLK exhibited the highest cell survival vs the C16-KKK, C16-KGGK and C16-KAAK formulations (B).
Bacterial membrane disruption

Compared with the untreated bacteria, contact with C16-KGGK, increased bacterial staining with DiOC2 seen as a shift to the left (red emission) in the flow cytometry presented in Figure 13A and indicating membrane disruption. The ratio between the red and green emission was calculated for each test group (Fig 13B). The treated bacteria presented lower ratios vs the control, indicating that the bacterial membrane was permeated. CCCP, which was designated to disrupt the cell membrane, did not show depolarization activity against the tested bacteria, *E. faecalis*. 
Fig 13. The bacterial membrane is disrupted by lipopeptide after a 1 hr exposure. A representative experiment showing lipopeptide C16-KGGK and its interaction with the *E. faecalis* bacterial membrane. Fluorescence-activated cell sorting was used to measure cytoplasmic membrane depolarization and to determine membrane disruption. At high cytoplasmic concentrations, the DiOC₂(3) self-associates and the green fluorescence emission (FL1-A axis) shifts to red (FL3-A axis). The bacteria were stained with DiOC₂(3), exhibiting green fluorescence (FL1-A) with a shift to red emission shift as the dye molecules self-associate at the higher cytosolic concentrations caused by the larger membrane potential (FL3-A). Left shift of the bacteria exposed to KGGK is shown in the dot plot (A). The red/green ratio is lower for the bacteria exposed to C16-KGGK, indicating that the bacterial membrane was disrupted and thus revealing the lipopeptide antibacterial mechanism (B).

* P<0.01 compared with control without lipopeptide, determined using Student ’s t-test
Sustained release kinetics

The release profile of C16-KGGK lipopeptide from P (SA: RA) and PLACO polymers during one week is shown in Fig 14. The sustained release of C16-KGGK incorporated in PLACO peaked after about 24 hrs, whereas C16-KGGK incorporated in P (SA: RA) peaked after 72 hrs.

Fig 14. Lipopeptide release profile from biodegradable polymer. A representative release profile of C16-KGGK lipopeptide from P (SA: RA) and PLACO polymers evaluated at 1, 8, 24, 48, 72, 96 and 168 hrs. C16-KGGK release from PLACO peaked after about 24 hrs, whereas the release from P (SA: RA) peaked after 72 hrs. The amount of accumulated peptide released from the polymer was calculated according to calibration curves made before the experiment.
Tooth model

Antibacterial activity of KGGK and KGGK formulation with P (SA: RA) was tested using a root canal treatment model. The materials tested were placed in endodontically treated canals by two traditional clinical ways: irrigation and capping. Live bacterial counts (CFU/ml) were performed for each tooth and compared with non-treated tooth. KGGK irrigation did not present an antimicrobial effect, whereas the formulation showed bacterial growth inhibition (Fig. 15).

Fig 15. Antibacterial effect within endodontically treated tooth model. CFU assay was performed on 24 uni-canal teeth. 20 teeth were infected with *E. faecalis* and then treated with different materials: calcium hydroxide, KGGK + P (SA: RA) formulation as capping materials, Dakin’s solution and KGGK as irrigation materials. The third dilution is presented in this test.
**Discussion**

Antimicrobial peptides are one of nature's solution against bacterial invasion [7]. Their nonspecific mode of action, which is based on physical membrane disruption, is effective against various bacteria and is less likely to induce bacterial resistance than antibiotics. Recently, synthetic AMPs mimicking these strategic antibacterial agents have been gaining interest. To exploit the advantages of AMPs, improved mimetic AMPs were synthesized [32, 33]. Industrial considerations require that the peptides be small and of simple structure. Therefore, considerable research has been devoted to optimize peptide length combined with a simple design, such as: ultra-short lipopeptides. The ultra-short lipopeptides described here, C16-KGGK, C16-KKK, C16-KAAK and C16-KLKL, composed of only four amino acids conjugated to an aliphatic acid chain (16C, palmitate), were synthesized and tested. Studies have revealed that fatty acids are able to compensate for the length of a short peptide chain. Acylation of synthetic or natural AMPs with fatty acids has proved to be a useful approach for improving their antimicrobial and antifungal activity. This effect is due to changes in the overall hydrophobicity of these molecules, which affects their oligomerization, organization in solution and affinity for membranes [36].

Combining sustained release and an antimicrobial compound holds many advantages and has proved itself in the past. In this study a potent antimicrobial agent was identified against *E. faecalis* and other oral pathogens, and then incorporated in two candidate biodegradable polymers. First, agar diffusion tests were performed to evaluate the antibacterial effect of each AMP (Fig 3). No bacterial inhibition was observed, suggesting the AMPs have no efficacy. We assumed that the AMPs positive charge did not allow their diffusion to the medium. Thus, we turned to other assays to examine the AMPs effectiveness. Then, the MIC for each tested lipopeptide against the bacterial pathogens was determined (Table 2). Each bacterium was sensitive to a different lipopeptide. Certain lipopeptides, such as C16-KGGK, were more efficient against the Gram-positive cocci, whereas other lipopeptides, such as C16-KKK, were more potent against the Gram-negative bacteria. This phenomenon might be explained by
differences in membrane structure. It is possible that certain amino acids have a great affinity for specific components, like the lipopolysaccharide (LPS) in the bacterial membrane of Gram-negative bacteria or lipoteichoic acid in Gram-positive bacteria [14].

The most efficient of the six investigated antimicrobial peptides specifically against *E. faecalis* was the lipopeptide C16-KGGK. We focused on *E. faecalis* as an example of a pathogen that causes severe nosocomial infections and as an example of a strongly forming biofilm bacterium. *E. faecalis* can grow and survive in a wide range of environments (wide range of temperatures and pH) enabling it to surmount many obstacles [4]. Interestingly, root canal treated teeth are about nine times more likely to harbor *E. faecalis* than are primary infections. *E. faecalis* has been found in root canal-treated teeth in 30% to 90% of the cases. This frustrating rate of post treatment disease is mainly attributed to the limitations of the present technology that offers no tool to combat intra-canal infection following the cleaning and shaping stage of the endodontic treatment [5].

The tested antibacterial peptides were first assayed in suspension against planktonic *E. faecalis* (Fig 4). Although hBD3 was previously reported as being a highly potent antibacterial AMP against *E. faecalis* [24, 25, 26, 27], in the present study it showed an antibacterial effect against *E. faecalis* only when used at high concentrations. This may be due to the differences in *E. faecalis* strains and the hBD3 chemical synthesis. As hBD3 is a costly peptide, high concentrations are predestined to be irrelevant as a conventional therapeutic agent and thus were not tested further. As mentioned previously, screening of the AMPs' MICs demonstrated that the C16-KGGK lipopeptide was the most potent against *E. faecalis* and it was further investigated and formulated into biodegradable polymers. Interestingly, in some experiments at low concentrations bacterial growth was not inhibited but rather accelerated. As this phenomenon may compromise the antimicrobial effect, further investigation of the peptides' mode of release is required. The exact mechanism of this opposite outcome is unknown, but the main assumption is that somehow the bacteria overcome lower concentrations of the lipopeptide and show accelerated growth compared with the untreated bacteria. This
phenomenon needs to be considered when dealing with the amount of peptides that are released from the polymer. The new biohybrid medium incorporating C16-KGGK results in an anti- \textit{E. faecalis} effect when tested against planktonic bacteria (Fig 5). Indeed calculation of bacterial number (using a calibration curve) revealed that the final bacterial load was lower by one order of magnitude in the treated wells. Additionally, the slope of the curve representing the bacterial growth rate (generation time) was more moderate in the treated bacteria, showing that the peptide is released into the medium. The generation time of the bacteria treated with each of the formulations and especially with P (SA: RA) was longer compared with that of the non-treated bacteria.

Next, we focused our evaluation on the most potent lipopeptides against the other oral pathogens. The new biohybrid formulation, polymer incorporating lipopeptide, resulted in an antibacterial effect when tested against bacteria in suspension (Fig 6). This was reflected by both the lower final optical density of the treated bacteria (total growth mass) and the growth rate, measured as the slope compared with that of the untreated bacteria. The most significant antibacterial effect was evident in all experiments after 24-48 hrs of release. It can be assumed that the greatest amount of lipopeptide is released in this time window.

The oral bacterial species tested in this study grow naturally in biofilms within the oral cavity, especially \textit{E. faecalis} within the root canal. Moreover, \textit{E. faecalis} is known to form biofilms that greatly increase its resistance to phagocytosis, antibodies and antimicrobials [5]. Therefore, in the second part of the study the anti-biofilm effect was tested. Three approaches were used to test the activity of the soluble AMPs and the new controlled release formulations against \textit{E. faecalis} biofilm. In the first, crystal violet was used to stain and measure biofilm mass (Fig 7). In the second, an ATP bioluminescence assay was performed and used as a viability indicator (Fig 8). In the third, the vitality of bacteria grown in a biofilm was tested using a dead/live stain (Fig 9). All three experiments revealed inhibition of biofilm formation when \textit{E. faecalis} was exposed to the novel formulation. The three parameters examined were the amount of biofilm, its metabolic state and bacterial viability. Interestingly, the formulations were effective against
a biofilm in the process of formation (developing biofilm) and against an established biofilm (mature biofilm). This is an important finding considering the fact that mature biofilm is much harder to treat. Moreover, we specifically tested the formulations’ potency against ATCC v583 strain due to its high known resistance to several antibiotics (among them vancomycin), compared to other strains such as ATCC 29212 [53]. It can be suggested that a formulation that was shown to be active against ATCC v583 is likely to be potent against other *E. faecalis* strains.

To determine the antibiofilm effect of other formulations against other oral pathogens, a live/dead staining assay was performed on the selected bacteria with the most effective lipopeptides found against them (Fig 10). In all experiments, the biohybrid formulation inhibited biofilm formation, whereas there was no significant effect on the bacteria exposed to the base lipopeptides, indicating that the biofilm prevails over the lipopeptide itself. Consequently, it is likely that the formulation allows continuous sustained release of the antibacterial agent and an anti-biofilm effect. Moreover, it was found that formulations containing P (SA: RA) polymer have an added value, since it results in an antibacterial effect by itself.

Although the tooth model assay was difficult to standardize, it can be clearly seen that the formulation had similar antibacterial effect to that of the calcium hydroxide, which is a known clinically capping material in dentistry and has anti-infective activity (Fig 15). However, the formulation antibacterial activity did not reach the activity of the Dakin’s solution, which is also a known clinically irrigation material in endodontics.

The next step was to examine the biocompatibility of the tested lipopeptides as an essential stage of their characterization as therapeutic agents. Two assays were performed. The first (the hemolysis test) tested the lipopeptides and polymers by themselves (Fig 11), and the second (the colorimetric XTT assay) tested the polymers and the formulation containing PLACO and the lipopeptides (Fig 12). Both experiments showed that within the effective concentrations, the lipopeptides alone and the formulations were biocompatible and safe.
These results coincide with the results of other, previously performed, hemolysis tests mentioned in the literature [28]. However, in the colorimetric XTT assay P (SA: RA) alone was not found biocompatible and in the hemolysis assay, PLACO showed a high-level of hemolysis. Because PLACO renders the liquid medium cloudy, it can be assumed that it interfered with the absorbance measurements and distorted the experimental results. In this study, we used a monocyte cell line to test the cytotoxicity of the tested compounds. In the biocompatibility literature there are many lines used for cytotoxic tests, including monocytes, epithelial cells and fibroblasts [54]. Monocytes and macrophages are known to play a critical role in the biological response to materials [55], and consequently in the chronic inflammatory response. In comparison with peripheral blood monocytes, the cell lines are more suitable for cytotoxic screening due to their stability and less variation in their response [56]. Nonetheless, before clinical application further in vitro and in vivo tests are necessary to ensure the safety of their use.

A recent study showed that lipopeptides like C16-KGG, tend to aggregate in solution due to their hydrophobic residues, and alter the intrinsic order of the lipid bilayer upon binding. The cationic lysines make contact with the anionic head-groups of the phosphatidylglycerol lipids, suggesting a model for binding and insertion [57]. In previous studies, scanning electron microscopy revealed bacterial membrane permeation by lipopeptides [28]. In the present investigation, to assess the lipopeptide mode of action and better understand its mechanism, a DiOC$_2$ (3) assay was performed (Fig 13). It is known that AMPs operate through membrane disruption, as found here for the tested lipopeptides. Indeed, membrane permeation was detected in this experiment. CCCP did not show depolarization activity against this specific E. faecalis. Previous studies have discussed this issue and it was found that CCCP does not inhibit the efflux pumps of the bacteria, thereby contributing to their resistance [58]. However, more extensive tests should be performed to understand the specific mode of action and the liaison between such lipopeptides and the bacterial membrane.
The delivery of peptides and proteins by polymeric carriers for extended periods of time has been a challenge because of their instability. Although there are more than 200 peptides and proteins in clinical use and clinical development, there are only a few long-acting drug delivery systems. Luteinizing hormone-releasing hormone (LHRH) and somatostatin delivery systems based on poly (lactic acid), which deliver these agents for months after a single injection, are still the main delivery formulations, developed three decades ago. The challenges of peptide delivery have been reviewed extensively [59, 60]. These novel formulations have broad applications, from cancer immunotherapy to dentistry. There is a wide range of carriers, including lipids, liposomes, nanoparticles and micelles. In the oral cavity, modification of peptides by hydrophobic fatty acid residues or amphiphilic block copolymers has been acknowledged as a useful strategy for protein delivery. In the field of dentistry, polymeric particles and micelles are applicable for binding minerals to the tooth surface, delivering AMPs over a prolonged period of time and thus inhibiting the growth of oral pathogen biofilm in the presence of the saliva pellicle layer [61]. The first polymer for the delivery here was synthesized by ring opening polymerization of DL-lactide onto castor oil that served as co-catalyst for alcohol groups. The second polymer was synthesized by insertion polymerization process that guaranteed alternating ester-anhydride polymer structure. These two polymers are pasty and the incorporation of vulnerable peptides is by gentle mixing without any solvent, heat or sheer stress. Consequently, the activity of these peptidic antimicrobial agents was not affected when incorporated into the delivery system.

As antibacterial agents need to overcome salivary flow and enzymatic cleavage to be sufficiently potent, a sustained release therapeutic can be advantageous in maintaining consistently high peptide levels for local treatment [38, 39]. This must be especially efficient in the intra-oral environment and in the intra root canal dentin tubules where microorganisms such as *E. faecalis* are always present and threaten the integrity of teeth and neighboring tissues. Several sustained release delivery devices are already used in dental practice, as the clearance time of most drugs from the oral cavity is rapid and most oral diseases are of a
chronic nature [37]. This prompted us to examine four ultra-short lipopeptides, which were incorporated in two selected biodegradable polymers, for sustained release. Peptides and proteins have unique structures that convey their ability to participate in specific biological activities. The fact that these polymers are degraded into natural compounds, renders them environmentally friendly, biocompatible, useful for drug delivery and suitable as implantable devices. The polymer candidates which contain fatty acids have several advantages over other biodegradable polymers such as: flexibility, low melting point, improved handling and provide better degradation and release profiles [41]. As previously reported, biodegradable polyanhydrides and polyesters are useful materials for controlled drug delivery. They have a hydrophobic backbone with hydrolytically labile anhydride and/or ester that may be hydrolyzed to dicarboxylic acids and hydroxy acid monomers when placed in an aqueous medium. Fatty acids are suitable candidates for the preparation of biodegradable polymers, as they are natural body components and hydrophobic, and thus may retain an encapsulated drug for longer time periods when used as drug carriers [40]. Moreover, it was shown that these polymers are biocompatible [45]. Two different polymers were tested as delivery media and led to different results in their activity and mode of action. In the sustained release experiments, PLACO showed similar bacterial kinetic growth curves whereas P (SA: RA) did not, indicating that the two have separate modes of release. Furthermore, in the ATP bioluminescence assay, PLACO presented higher levels of luminescence and accordingly higher levels of ATP, suggesting that this polymer elevates the metabolic state of the biofilm, compared with P (SA: RA) which had the opposite effect. In the live/dead assay, the main difference between the two polymers is that in P (SA: RA) a larger amount of dead bacteria appeared, reinforcing our previous findings that P (SA: RA) itself may be an antibacterial agent. Thus, P (SA: RA) is apparently a more suitable delivery medium for this purpose. However, the XTT assay showed that P (SA: RA) decreased the cell viability, suggesting that perhaps this polymer is not suitable due to lack in biocompatibility.
The two biodegradable polymers exhibited different releasing profiles in the fluorescamine assay (Fig 14). Interestingly, in this experiment in the early hours of release, the lipopeptide levels were low. In previous results, accelerated bacterial growth was found at AMP concentrations lower than the MIC. This results can be explained by the release profile of the lipopeptide from the biodegradable polymer and by the possibility that in the early hours of lipopeptide release from the polymer, the bacteria can overcome the antibacterial effect if it does not reach the appropriate MIC. Both studies serve to underline the importance of lipopeptide release kinetics. The fluorescamine assay showed the release profile of C16-KGGK from P(SA-RA) and PLACO. After 48 hrs 65% of the lipopeptide was released from PLACO and 15% was released from P(SA-RA). However, these results are not in agreement with the results of the antibacterial activity found in the controlled-release lipopeptides assay in which the most significant antibacterial effect was seen after 24-48 hrs release. This experiment should be performed with other lipopeptides for a better understanding. Although it appears that after one week most of the lipopeptide is released from the polymer; further experiments should be performed for longer periods.
Conclusions

Synthetic AMPs were shown to have an effective antimicrobial activity against *E. faecalis*. A peptide that allows selective killing of *E. faecalis* would be a good candidate for endodontic treatment. Moreover, this lipopeptide when formulated in a biohybrid polymer medium has an increased antibiofilm effect. Thus, the novel effective formulation presented here can be advantageous in root canal treatment for the prevention of endodontic failure due to *E. faecalis*.

Four essential parameters for biohybrid formulations were introduced here: antibacterial activity, biocompatibility, mode of action and release profile. Based on the presented results, it seems reasonable to assume that the biohybrid formulation containing lipopeptides and biodegradable polymer may be a potential candidate for antimicrobial use in the oral cavity. The advantages of these formulations stem for their reinforced antibacterial activity, ease of manufacture and their ability to cope with a challenging oral environment. Nonetheless, as *in vitro* studies have strict limitations, clinical assumptions should be made with maximum precaution.

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