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# **ANTIMICROBIAL AND ANTI-INFLAMMATORY ACTIVITY OF BRANCHED PEPTIDES AS PROMISING ANTIBIOTICS FOR TREATING DRUG-RESISTANT BACTERIA**

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# INDEX

**ABSTRACT** **PAG. 1**

---

**INTRODUCTION** **PAG. 2**

---

1. ANTIMICROBIAL RESISTANCE
  - 1.2 TYPES OF AMR
  - 1.3 SOURCE OF AMR
    - 1.3.1 CONSUMPTION OF ANTIBIOTICS IN HUMANS
    - 1.3.2 FOOD-PRODUCING ANIMALS AND AGRICULTURE
    - 1.3.3 ENVIRONMENT
  - 1.4 MANAGEMENT OF AMR
2. ORAL MICROBIOTA
  - 2.1 ORAL BIOFILM
  - 2.2 PERIODONTAL DISEASE
  - 2.3 ENDODONTIC INFECTIONS
  - 2.4 ASSOCIATION OF ORAL INFECTIONS WITH CARDIOVASCULAR DISEASES
3. ANTIMICROBIAL PEPTIDES
  - 3.1 MECHANISM OF ACTION OF AMPs
  - 3.2 IMMUNOMODULATORY ACTIVITY OF AMPs
  - 3.3 ADVANTAGES OF AMPs
  - 3.5 CHALLENGES TO THE PEPTIDE AS THERAPEUTIC USE
4. M33L
  - 4.1 IN VITRO ANTIMICROBIAL ACTIVITY OF M33L AND M33D
  - 4.2 ANTIBIOFILM ACTIVITY OF M33L AND M33D
  - 4.3 IN VIVO ACTIVITY OF M33L AND M33D
  - 4.4 PEPTIDES DERIVED FROM NATURAL HOST DEFENSE PEPTIDES Dermaseptin, Mastoparan, Cathelicidin, Anoplin

**OBJECTIVES** **PAG. 25**

---

**MATERIALS AND METHODS** **PAG. 26**

---

- 1.1 PEPTIDE SYNTHESIS
- 1.2 PEPTIDE STRUCTURE PREDICTION
- 1.3 HUMAN SERUM PROTEASE STABILITY
- 1.4 BACTERIAL STRAINS
- 1.5 MIMIMUM INHIBITORY CONCENTRATIONS
- 1.6 ANTIBIOFILM ACTIVITY
- 1.7 BACTERIAL MEMBRANE INTERACTION
- 1.8 LPS-PEPTIDE BINDING ASSAY
- 1.9 DENTIN SLICE MODEL
- 1.10 CELL CULTURES
- 1.11 MACROPHAGES STIMULATION
- 1.12 HUMAN CARDIAC FIBROBLASTS MODULATION
- 1.13 SCANNING ELECTRON MICROSCOPY
- 1.14 TRANSMISSION ELECTRON MICROSCOPY
- 1.15 SKIN INFECTION MODEL
- 1.16 EUKARYOTIC CELLS VIABILITY ASSAY
- 1.17 HEMOLYTIC ACTIVITY
- 1.18 STATISTICAL ANALYSIS

## **RESULTS**

**PAG. 35**

- 
- 1 EXPERIMENTS WITH M33D AND M33i/l
    - 1.1 PEPTIDE STRUCTURE PREDICTION
    - 1.2 HUMAN SERUM PROTEASE STABILITY
    - 1.3 MIMIMUM INHIBITORY CONCENTRATIONS
    - 1.4 ANTIBIOFILM ACTIVITY
    - 1.5 BACTERIAL MEMBRANE INTERACTION
    - 1.6 LPS-PEPTIDE BINDING ASSAY
    - 1.7 DENTIN SLICE MODEL
    - 1.8 MACROPHAGES STIMULATION AND HUMAN CARDIAC FIBROBLASTS MODULATION
    - 1.9 EUKARYOTIC CELLS VIABILITY ASSAY AND HEMOLYTIC ACTIVITY
  - 2 EXPERIMENTS WITH BAMPs
    - 2.1 PEPTIDE STRUCTURE PREDICTION

2.2 HUMAN SERUM PROTEASE STABILITY	
2.3 MIMIMUM INHIBITORY CONCENTRATIONS	
2.4 ANTIBIOFILM ACTIVITY	
2.5 BACTERIAL MEMBRANE INTERACTION	
2.6 LPS-PEPTIDE BINDING ASSAY	
2.7 SCANNING ELECTRON MICROSCOPY AND TRANSMISSION ELECTRON MICROSCOPY	
2.8 EUKARYOTIC CELLS VIABILITY ASSAY AND HEMOLYTIC ACTIVITY	
2.9 SKIN INFECTION MODEL	

<b>DISCUSSION</b>	<b>PAG. 49</b>
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<b>REFERENCES</b>	<b>PAG. 53</b>
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# ABSTRACT

Antimicrobial resistance poses a severe and escalating global health challenge, reducing the effectiveness of conventional antibiotics and complicating the treatment of infectious diseases. Antimicrobial peptides (AMPs) offer promising alternatives due to their activity against multidrug-resistant pathogens and unique mechanisms of action.

In this context, oral infections such as periodontal and endodontic diseases cause chronic inflammatory conditions linked to a dysbiosis of the oral microbiome and are associated with systemic inflammatory diseases, such as cardiovascular disorders. Conventional antibiotics are often ineffective due to the presence of antibiotic-resistant species within oral biofilms, such as *Enterococcus faecalis* in persistent endodontic infections. Moreover, the localized nature of these infections necessitates alternative treatments to minimize antibiotic use and reduce resistance development.

The tetra-branched AMPs M33-D and its analogue M33i/l were studied for their potential in treating endodontic infections. M33-D and M33i/l demonstrated strong antibacterial activity against a wide range of oral pathogens, including antibiotic-resistant strains, with MICs of 0.3–1.5  $\mu$ M. They rapidly permeabilized bacterial membranes, disrupted biofilms, and reduced *E. faecalis* colonies in dentin slices—a model for root canal irrigation—by up to 20-fold.

Additionally, both peptides exhibited immunomodulatory effects, neutralizing bacterial lipopolysaccharide (LPS) and reducing the release of pro-inflammatory cytokines (IL-1 $\beta$ , TNF- $\alpha$ , IL-6) in LPS-stimulated murine macrophages and human cardiac fibroblasts. This dual activity suggests their potential to prevent systemic complications, such as atherogenesis. Furthermore, both peptides showed low toxicity to eukaryotic cells and minimal haemolytic activity, making them suitable candidates for intracanal irrigation.

Beyond M33-D and M33i/l, a novel library of antimicrobial peptides was explored, providing insights into their antimicrobial efficacy, mechanisms of action, and therapeutic potential. Notably, the peptide BAMP2 showed potential for treating local infections in a mouse skin infection model.

These findings underscore the potential of AMPs as innovative alternatives to traditional antibiotics for localized infections and as a strategy to combat antimicrobial resistance.

# INTRODUCTION

## 1.1 ANTIMICROBIAL RESISTANCE

Antimicrobial resistance (AMR) is the ability of microorganisms, such as bacteria, viruses, fungi, and parasites, to survive and proliferate despite being exposed to antimicrobial agents (e.g., antibiotics, antivirals, antifungals, or antiparasitics) that would normally inhibit or kill them. AMR arises due to selective pressure from the overuse or misuse of antimicrobial agents in both human and veterinary medicine, as well as in agriculture. Under such pressure, susceptible bacteria are killed or inhibited, while resistant strains survive and multiply, eventually becoming dominant in the microbial population.<sup>1-3</sup> In recent years, AMR has become a major global public health threat, leading to increased morbidity, mortality, and healthcare costs, while also straining economic resources. Currently, AMR is responsible for nearly 700,000 deaths worldwide each year, a figure which is estimated to increase to up to 10 million deaths annually by 2050.<sup>4,5</sup>

## 1.2 TYPES OF AMR

Resistance can be categorized into two main types: intrinsic and acquired. Intrinsic resistance refers to the natural ability of certain bacteria to resist specific antibiotics. This ability is a permanent characteristic of the bacterial species and results from structural or functional features.

6

The most common bacterial mechanisms involved in intrinsic resistance include:

- Reduced permeability of the outer membrane, which limits antibiotic access to intracellular targets, as commonly observed in Gram-negative bacteria, where the outer membrane acts as a barrier to many drugs.<sup>6,7</sup>
- Efflux pumps, which actively expel antibiotics from the bacterial cell before they can reach their target, thus limiting the intracellular concentration of the drug.<sup>6,7</sup>
- Absence of the antibiotic targets, meaning some bacteria naturally lack the molecular structures, such as specific enzymes or proteins, that antibiotics typically bind to.<sup>6</sup>

Many bacterial species exhibit antibiotic resistance also through the production of specific enzymes. For instance, *Klebsiella pneumoniae* is intrinsically resistant to ampicillin due to the synthesis of beta-lactamases, enzymes that hydrolyze beta-lactam antibiotics, rendering them ineffective.<sup>8</sup> Similarly, *Enterococcus* species produce altered penicillin-binding proteins (PBPs), which reduce the binding affinity of beta-lactams.<sup>9</sup> Lastly, *Pseudomonas aeruginosa* exhibit resistance to beta-lactams via its periplasmic  $\beta$ -lactamases and reduced membrane permeability, thus limiting antibiotic entry.<sup>10</sup>

On the other hand, acquired resistance occurs when previously susceptible bacteria develop resistance through genetic changes.<sup>6</sup> One of the key processes driving AMR is genetic mutations, which can occur naturally through vertical gene transfer, where resistant traits are passed from parent to daughter cells during replication. These spontaneous mutations can alter antibiotic targets or metabolic pathways, reducing drug efficacy.<sup>6,7</sup>

However, horizontal gene transfer (HGT) plays a more crucial role in spreading resistance across bacterial populations. Through mechanisms such as transformation, transduction, and conjugation, bacteria can acquire resistance genes from unrelated species or strains.<sup>2</sup>

Transformation is an active mechanism by which free DNA present in the environment, typically derived from dead bacteria, is taken up into the cytoplasm and incorporated into bacterial chromosome.<sup>2,11,12</sup> That's the case of recombination of foreign DNA from *Streptococcus mitis* to *Streptococcus pneumoniae*, conferring penicillin resistance via the formation of mosaic genes.<sup>12</sup>

Transduction is a gene transfer mechanism mediated by bacteriophages. During their replication cycle, bacteriophages can mistakenly incorporate fragments of the host bacterium's DNA into their viral particles. When the phage infects a new bacterial cell, it transfers this bacterial DNA, potentially introducing genes that confer antibiotic resistance.<sup>2,12</sup> For instance, the *mecA* gene, responsible for methicillin resistance, can be transferred via transduction to methicillin-susceptible *Staphylococcus aureus*, leading to the development of methicillin-resistant *S. aureus* (MRSA).<sup>2</sup>

Finally, conjugation is a transient fusion between two bacteria where the transfer of genetic material, such as plasmids and transposons, including the genes that encode for antibiotic resistance, is transferred from donor to recipient bacteria via a conjugation bridge.<sup>2,6,11</sup> This allows for the rapid dissemination of resistance factors, such as plasmids carrying multiple antibiotic resistance genes, leading to the emergence of multidrug-resistant pathogens.<sup>5</sup> For example, MRSA has been identified to acquire vancomycin-resistant genes via conjugation from vancomycin-resistant enterococci (VRE) during co-infection.<sup>2</sup>

Acquired resistance is particularly concerning because it can spread rapidly within bacterial populations, especially in environments where antibiotics are frequently used, leading to the emergence of multidrug-resistant pathogens, thus amplifying the challenge of controlling AMR.<sup>2,5</sup>

### 1.3 SOURCE OF AMR

Antibiotic resistance is a natural phenomenon in which bacteria evolve to withstand the effects of antibacterial compounds. This process has been accelerated by the extensive and often inappropriate

use of antibiotics in humans and animals, creating significant evolutionary pressure and facilitating the emergence of resistant bacteria.<sup>2,13</sup>

In agriculture, especially in livestock and aquaculture, antimicrobials are often administered at subtherapeutic levels, which greatly contributes to the rise of resistance. These resistant bacteria can spread to humans through the food chain, direct contact with animals, or environmental contamination. The situation is made worse by the large, interconnected human population and the frequent misuse of antimicrobials in healthcare settings.<sup>4,5</sup>

Identifying and regulating the importance of environmental factors in the genesis and progression of AMR is crucial to effectively combat AMR globally.<sup>5</sup>

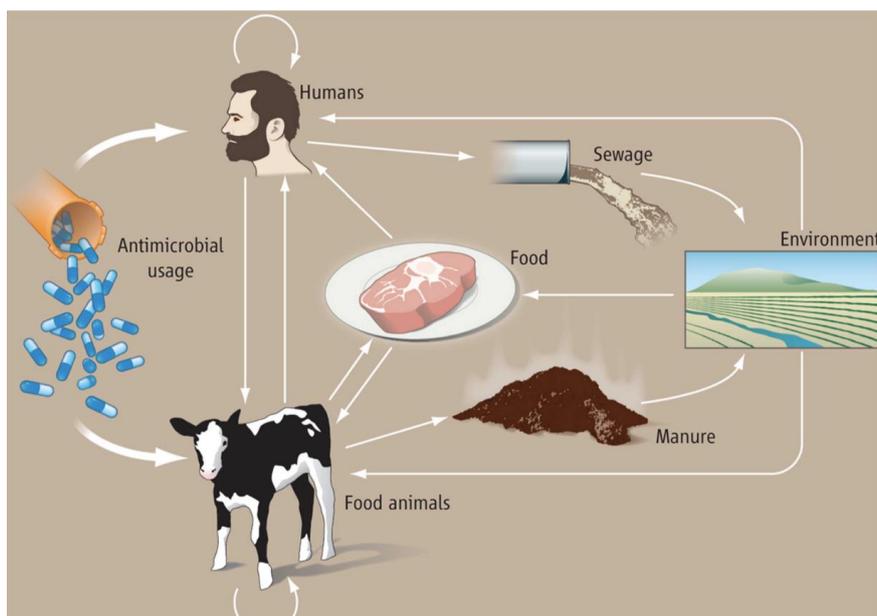


Fig.1 Routes of transmission of AMR between farm animals, the environment, and humans.<sup>14</sup>

### 1.3.1 CONSUMPTION OF ANTIBIOTICS IN HUMANS

Epidemiological studies have shown a causal relationship between the overuse of antibiotics and the development of microbial resistance. Despite repeated warnings from health organizations, the misuse and overuse of antibiotics persist at alarming levels globally.<sup>13</sup> For instance, antibiotics are often prescribed for viral infections like fever and flu, even though they are only effective against bacterial infections. This unnecessary exposure contributes to the development of resistance.<sup>2</sup>

It is estimated that nearly 50% of antibiotic prescriptions are inappropriate. In many countries outside Europe and North America, non-prescription antibiotic use remains common. Additionally, 20–30% of European patients receive antibiotics during hospitalization, with hospital wastewaters becoming hotspots for horizontal gene transfer, which facilitates the spread of antibiotic resistance and virulence factors among various bacterial species.<sup>15</sup>

### 1.3.2 FOOD-PRODUCING ANIMALS AND AGRICULTURE

The use of antibiotics in livestock farming has seen a significant rise in many developing countries, driven by the growing demand for animal proteins. Originally intended to prevent and treat infections in animals, antibiotics are now widely used randomly for various purposes, including treating animal diseases, preparation of animal feed for growth promotion, as well as for disease prevention. This practice is more prevalent in developing countries to gain more income from the food animal farms and due to lack of regulatory government policies, which has been a major contributor to human AMR.<sup>13,15</sup>

The extensive use of antibiotics in farming results in elevated levels of residues in waste, as a significant portion—between 30% and 90%—of the antibiotics given to animals is expelled through manure and urine, subsequently contaminating the environment. This contamination, along with the presence of antibiotic residues in animal-derived products, such as muscle, liver, fat, milk, and eggs, contributes to the spread of AMR. In fact, humans consume antibiotics through food, raising the chances that resistant genes may be passed from animals.<sup>2,15</sup>

Among bacterial species that have been detected in manure, compost, soil and water samples there are *Salmonella* serovars and *Campylobacter* spp; *Escherichia coli*, enterococci and *Staphylococcus* spp., some of which can cause human and animal diseases.<sup>12,15</sup>

Agriculture's role in the rise of AMR is also concerning. The use of metals, such as copper applied as a bactericide or fungicide, as well as the natural presence of metals in certain regions, can promote resistance. Even nitrogen fertilizers commonly used in farming can alter soil microbiomes, affecting the abundance of antibiotic resistance genes.<sup>12</sup> Additionally, agricultural runoff introduces antibiotic residues into water bodies and soil, further spreading antimicrobial resistance genes among environmental bacteria.<sup>4</sup>

### 1.3.4 ENVIRONMENT

AMR is increasingly recognized as an environmental concern, with natural ecosystems such as soil, water, and air serving as reservoirs for resistant bacteria, that can persist, multiply, and transfer resistance traits. This process is worsened by human activities that introduce antibiotics and resistant bacteria into the environment through sources like hospital waste, animal farming, and agricultural runoff.

Even though some antibiotics degrade quickly, their continuous release leads to the accumulation of residues that persist in ecosystems, creating selective pressure. Contaminated environments also facilitate the transfer of resistance genes between animals, humans, and microbial populations. For

example, antibiotic residues found in soil and water can promote the development of resistance in environmental bacteria, which may then pass these traits to human pathogens through direct contact or through food and water supplies. This interaction between environmental and clinical AMR presents significant challenges for public health, as it speeds up the global spread of resistance. <sup>4,15</sup>

#### 1.4 MANAGEMENT OF AMR

To address the growing issue of AMR, national action plans have been implemented based on a 'One Health' approach, which recognizes the link between human, animal, and environmental health and introduces intervention strategies to contain the spread of AMR. <sup>14,16</sup>

A crucial step is the responsible use of antibiotics in both healthcare and agriculture. They should be prescribed only for bacterial infections and never for viral illnesses. In agriculture, their use must be restricted to treating infections, avoiding their use for growth promotion or disease prevention in healthy animals.

Another essential strategy is enhancing infection prevention. By improving hygiene practices, sanitation, and vaccination programs in both human healthcare and animal farming, the need for antibiotics can be reduced by stopping infections before they spread.

Additionally, raising awareness among the public, healthcare providers, and farmers about the risks of AMR is crucial. Public awareness campaigns can inform people about the importance of completing prescribed antibiotic courses and the risks associated with misuse. Healthcare professionals and veterinarians should receive training in antimicrobial stewardship to ensure they prescribe antibiotics wisely. <sup>14,16,17</sup>

Finally, efforts are being made globally to improve the effectiveness of current antibiotics and to find new treatments.

One promising method involves altering the chemical structure of antibiotics to enhance their binding capabilities and increase their ability to penetrate cell membranes. For instance, this approach has notably improved the efficacy of vancomycin against Van-A resistant *Enterococcus*.

Another successful strategy is combination therapy, where antibiotics are chosen based on the susceptibility patterns of microbes to create a synergistic effect, thereby restoring bacterial sensitivity and enhancing treatment results.

In addition, researchers are increasingly looking into biopharmaceuticals as alternative options in the battle against AMR. Among the most promising advancements are antimicrobial peptides, which can disrupt bacterial membranes and potentially lead to their complete destruction. Peptides like polymyxin B, polymyxin E, and gramicidin S have shown strong clinical effectiveness, particularly

against Gram-negative bacteria, and they have a low likelihood of developing resistance, making them valuable candidates in the search for new antibacterial agents.<sup>2</sup>

In conclusion, the One Health approach promotes collaboration among human health, veterinary medicine, and environmental sectors, as antibiotic residues in water and soil from agriculture can contribute to resistance. By tackling AMR through coordinated efforts across these fields, we can slow the rise of resistant bacteria and maintain the effectiveness of antibiotics for future generations.

14,16,17

## 2. ORAL MICROBIOTA

The oral microbiome is a complex and diverse community of microorganisms that plays a vital role in maintaining human health. It includes more than 700 different bacterial species, along with viruses and fungi, all living in various ecological niches within the mouth, such as the teeth, tongue, cheeks, and gingival crevices.<sup>18</sup>

Each part of the oral cavity offers a unique microenvironment, resulting in specialized microbial communities that interact with one another and with host tissues. These interactions are crucial for maintaining oral homeostasis, as the microbiome contributes to nutrient processing, immune modulation, and pathogen inhibition. For example, the oral microbiota assists in digestion by breaking down complex molecules, which helps with nutrient absorption.<sup>19</sup>

Furthermore, commensal bacteria serve as a defense against pathogens by occupying spaces that could otherwise be colonized by harmful microorganisms. However, when the balance of this microbiome is disrupted—due to factors like poor oral hygiene, diet, smoking, or antibiotic use—a dysbiotic state can occur, leading to diseases such as dental caries, periodontitis, and even systemic conditions like cardiovascular disease and diabetes.<sup>19,20</sup>

The process of acquiring and establishing the oral microbiota begins at birth, influenced by genetics, the environment, and early exposure to microbial communities. This initial setup plays a crucial role in determining an individual's oral health throughout their life. Therefore, understanding the composition, function, and dynamics of the oral microbiome is essential for developing effective strategies for preventing and treating both oral and systemic diseases.<sup>20</sup>

## 2.1 ORAL BIOFILM

A biofilm is a complex community of microorganisms, such as bacteria, fungi, and viruses, that attaches to surfaces and is surrounded by a self-produced matrix of extracellular polymeric substances (EPS). This matrix consists of polysaccharides, proteins, lipids, and extracellular DNA, all of which create a protective and stable environment for the microbes.<sup>21,22</sup>

The formation of a biofilm occurs in several interconnected stages:

- **Initial Attachment:** Free-floating (planktonic) bacteria first adhere to a surface. This initial attachment can be either temporary or permanent, facilitated by bacterial structures such as pili and flagella.
- **Microcolony Formation:** After attachment, the bacteria start to multiply, forming microcolonies. During this stage, they reinforce their attachment through cell-to-cell interactions and the secretion of EPS.
- **Maturation:** The microcolonies continue to grow and develop into a more structured biofilm. The production of EPS increases significantly, creating a protective matrix that surrounds the bacterial cells and facilitates nutrient exchange and waste removal within the biofilm.
- **Dispersion:** In the final stage, some bacterial cells detach from the biofilm and revert to a planktonic state, enabling them to colonize new surfaces and initiate the biofilm formation process once more.<sup>22,23</sup>

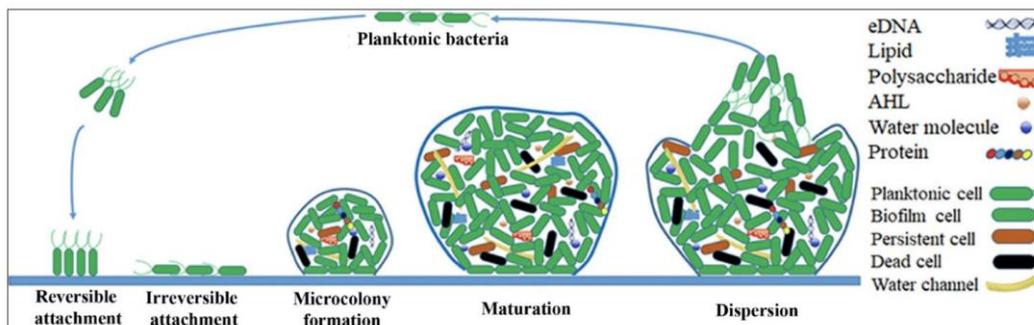


Fig.2 Steps of biofilm formation.<sup>22</sup>

Many bacteria can switch between a free-floating planktonic state and a biofilm state. In their planktonic form, bacteria usually show relatively high rates of growth. However, the biofilm state is considered to be the more natural and prevalent form. Several factors explain why bacteria prefer the biofilm state.<sup>24</sup>

Primarily, biofilms serve as protective barriers, safeguarding bacteria from environmental stressors like extreme temperatures, pH changes, and exposure to harmful substances. This matrix-enclosed structure also inhibits the effectiveness of antibiotics and disinfectants, increasing bacterial resistance

and complicating treatment of infections. Within a biofilm, bacteria can enter a slower metabolic state, which further enhances their resilience to antimicrobial agents.

Moreover, biofilms allow bacteria to capture nutrients from their surroundings, improving nutrient availability, particularly in nutrient-deficient environments.

The close proximity of cells within the biofilm encourages horizontal gene transfer, enabling bacteria to share genetic material, including genes that confer antibiotic resistance, thus supporting adaptation and evolution. Biofilms also support quorum sensing—a communication system that synchronizes gene expression and collective behaviors, allowing bacteria to respond as a community to changes in their environment.

All together, these factors make biofilms a beneficial survival mechanism, enabling bacteria to endure and flourish in a variety of challenging environments.<sup>24</sup>

Regarding the oral cavity, biofilms develop on various surfaces, including teeth, gums, and tongue. A well-known example is dental plaque, a multi-species biofilm that forms on tooth surfaces. Dental plaque begins with the initial colonization of early bacteria, such as *Streptococcus* species, which create an environment that supports the growth of other bacteria.<sup>25</sup>

This biofilm is made up of a variety of microorganisms, including species of *Streptococcus*, *Actinomyces*, *Fusobacterium*, and *Porphyromonas*. When in a state of homeostasis, the microbial community in oral biofilm sustains a balanced ecosystem that promotes oral health and prevents harmful species from taking over.

However, if homeostasis is disrupted, the balance can shift, allowing opportunistic pathogens to thrive. This change can result in oral health issues like dental caries and periodontal disease. For instance, during dysbiosis, there is an increase in acid-producing bacteria, such as *Streptococcus mutans*, which lowers the pH and leads to the demineralization of tooth enamel, resulting in caries.

Conversely, the overgrowth of anaerobic bacteria like *Porphyromonas gingivalis* during dysbiosis is linked to inflammation and periodontal disease. Thus, it is essential to maintain homeostasis in the oral biofilm for good oral health, while dysbiosis poses a risk for the progression of disease.<sup>25–28</sup>

In summary, while biofilms are a natural component of the oral ecosystem, they can pose risks to human health if they disrupt the balance of the microbiome or become pathogenic. Due to their dense, protected structure, biofilms are highly resistant to immune responses and antimicrobial treatments. When oral biofilms grow unchecked, they can result in dental caries, gingivitis, and periodontal disease. Additionally, pathogenic bacteria from these biofilms can enter the bloodstream, potentially leading to serious systemic health issues, including cardiovascular disease and diabetes.<sup>18</sup>

## 2.2 PERIODONTAL DISEASE

Periodontitis is a chronic inflammatory condition that affects the structures supporting the teeth, mainly the gums, periodontal ligament, and alveolar bone. It begins with the accumulation of dental plaque on the teeth and below the gumline. If not cleaned away, plaque can harden into calculus (tartar), which further irritates the gums. This accumulation triggers an immune response that, in vulnerable individuals, results in persistent inflammation of the gums and surrounding tissues.

The primary cause of periodontal damage is the inflammatory and immune responses from the host to the buildup of microbial plaque and its enzymes, including lipases, proteases, and nucleases. Additionally, individual factors play a significant role, such as genetic susceptibility, lifestyle choices, such as a diet lacking in vitamin C, D, and other essential micronutrients, as well as tobacco use, and various systemic diseases, including osteoporosis, atherosclerosis, and diabetes, which can worsen the onset and progression of periodontal disease.

The progression of periodontitis is characterized by symptoms such as gum redness, swelling, and bleeding, along with the development of periodontal pockets around the teeth, where harmful bacteria like *Porphyromonas gingivalis* and *Tannerella forsythia* prosper. These bacteria disturb the balance of the oral microbiota, resulting in dysbiosis and intensifying the inflammatory response.

The progression of periodontitis is effectively illustrated in Figure 3, which shows the transition from a healthy periodontal state to a diseased condition. It highlights how the loss of homeostasis leads to the initial acute inflammation mediated by leukocyte infiltration. It also emphasizes the delicate balance between pro-resolution mediators, which reduce immune cell recruitment, and the consequences of dysbiosis and persistent pro-inflammatory states, ultimately contributing to chronic pathology.<sup>29</sup>

If not treated, periodontitis can lead to the gradual destruction of the periodontal ligament and alveolar bone, causing tooth mobility and ultimately tooth loss.

In addition to its effects on oral health, periodontitis has been associated with broader health problems, as the bacteria and inflammatory substances involved in the disease can enter the bloodstream, potentially leading to cardiovascular diseases, respiratory infections, and complications in conditions such as diabetes and rheumatoid arthritis.

This systemic effect highlights that periodontitis is not just a dental issue but also a crucial factor in overall health. Therefore, effective prevention and treatment methods, including proper oral hygiene, regular dental visits, and, when necessary, periodontal therapy, are vital for maintaining oral health and minimizing related systemic health risks.<sup>25,26,30</sup>

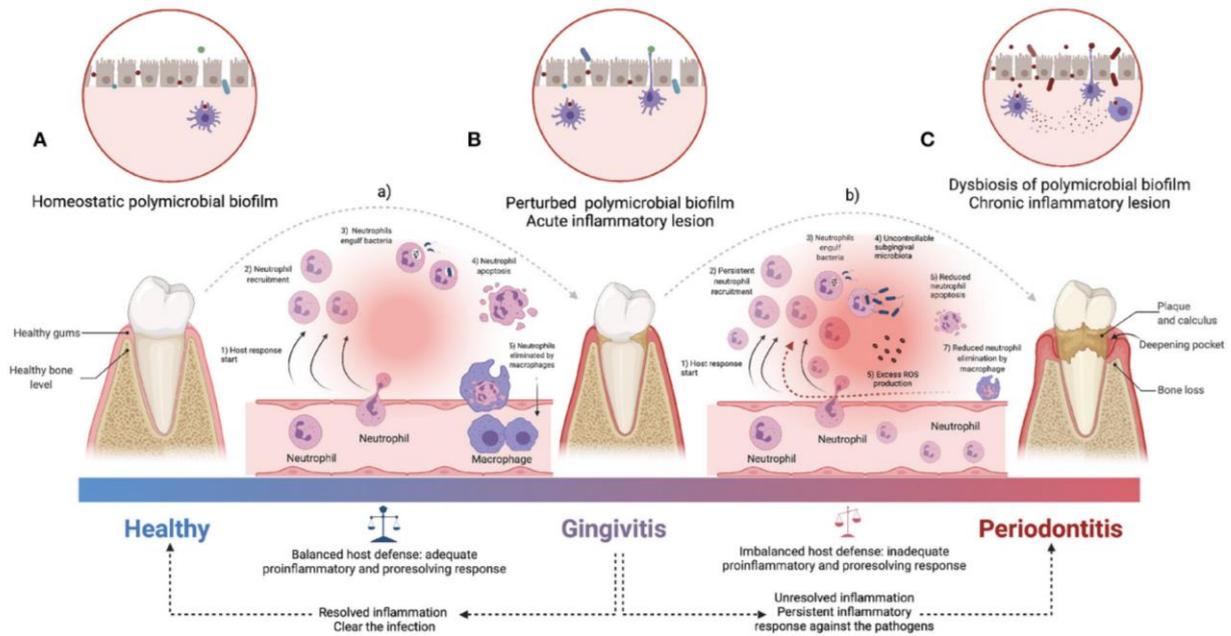


Fig.3 Pathogenesis of periodontitis.<sup>29</sup>

## 2.3 ENDODONTIC INFECTIONS

Endodontic infection refers to a microbial invasion of the dental pulp and surrounding periapical tissues, resulting in inflammation and potential damage to these structures.<sup>31</sup> The dental pulp is the innermost soft tissue of the tooth, consisting of nerves, blood vessels, and connective tissue that provide nourishment and sensory function. It is surrounded by dentin, a mineralized tissue that supports the tooth structure and transmits sensations to the pulp. Enamel encases the dentin in the crown of the tooth, acting as a hard, protective outer layer.<sup>32</sup>

The etiology of endodontic infections involves microbial invasion, typically occurring when the dental pulp, which is usually a sterile tissue, becomes exposed to bacteria. This colonization typically arises through dental caries, trauma that exposes the pulp, or cracks in the tooth that enable bacteria to penetrate the dentin and pulp. Factors such as untreated pulp necrosis and inadequate endodontic treatment can also contribute to the infection process. Once bacteria penetrate the pulp, it can lead to inflammation and subsequent infection, compromising the health of the surrounding periapical tissues.<sup>31,33,34</sup>

If left untreated, this can progress to periapical lesions, which are inflammatory responses at the root apex, potentially compromising the bone structure surrounding the tooth.

Endodontic infections can be categorized in primary endodontic infections, which happen when bacteria invade the pulp and subsequently spread to the surrounding necrotic tissues, and persistent

endodontic infections, that arise from microorganisms that resist cleaning methods employed during treatment and can thrive in the confined environment of treated root canals.<sup>35</sup>

In the initial stages of a primary endodontic infection, microorganisms penetrate the pulp due to dental caries, trauma, or cracks, establishing a microbial community. This phase is characterized by the presence of facultative anaerobes, such as *Streptococcus* and *Lactobacillus*, which can survive in both oxygen-rich and oxygen-poor environments and are commonly linked to dental caries.

As the infection progresses, the pulp chamber becomes increasingly anaerobic due to tissue necrosis and bacterial oxygen consumption. This environment favors the growth of strict anaerobes, such as *Porphyromonas*, *Prevotella*, and *Fusobacterium*. These bacteria are known for their virulence factors, which contribute to inflammation and tissue damage, resulting in more severe symptoms.

The diversity of microbes also increases, resulting in an intricate biofilm structure within the root canal. This biofilm is made up of various bacterial species that are embedded in a self-produced matrix, which shields them from antimicrobial agents and the immune response of the host. As the infection advances, certain bacteria can produce enzymes that degrade host tissues, further promoting necrosis and inflammation.

Current treatments for endodontic infections mainly focus on root canal therapy, which is designed to clean, disinfect, and shape the root canal system before sealing it to prevent any bacterial reinfection. This process includes the mechanical removal of necrotic tissue and bacteria, followed by the use of chemical irrigants to enhance disinfection. Sodium hypochlorite is the most commonly used irrigant in endodontics, valued for its strong antimicrobial and tissue-dissolving properties. Other disinfectants, such as chlorhexidine and EDTA, may also be used to improve the treatment's effectiveness. The aim is to reduce the microbial load and create a favorable environment for healing, ultimately preserving the tooth and preventing recurrence of infection.<sup>35-37</sup>

However, despite these measures, completely eliminating bacteria is often not possible.

Endodontic therapy can fail due to microbiological, anatomical, procedural, and host-related factors. A primary reason is the persistent intracanal infection, often caused by bacteria such as *Enterococcus faecalis*, which can survive treatment due to their resistance to disinfection and ability to endure extreme conditions, including nutrient scarcity. These bacteria are especially proficient at forming biofilms, which protect them from antimicrobial agents, making complete eradication challenging.

Additionally, the complex anatomy of root canals, including lateral canals and dentinal tubules, can prevent adequate cleaning and disinfection, leaving residual bacteria that contribute to reinfection. Secondary infections can also arise if bacteria are introduced into the root canal system after treatment, often due to poor sealing or contamination during the procedure.

Furthermore, chemical irrigants like sodium hypochlorite can be limited in their effectiveness by factors like canal anatomy and biofilm resistance.

Finally, the patient's immune response and overall health status, including conditions like diabetes or immunosuppression, can influence the healing process and increase the risk of treatment failure. Procedural errors, such as inadequate shaping, cleaning, or filling, may also create voids or improperly treated areas where bacteria can survive and cause recurrent infections. Altogether, these factors make endodontic therapy success dependent on precise technique, effective disinfection, and favorable host conditions.<sup>33,34,36,38</sup>

Furthermore, the systemic implications of unresolved endodontic infections, linking them to broader health concerns.<sup>39</sup> Therefore, even though current therapies are largely effective, the presence of resistant bacteria, biofilms, and complex root canal anatomy contributes to incomplete disinfection and treatment failures, highlighting the need for improvements in endodontic care.

#### 2.4 ASSOCIATION OF ORAL INFECTIONS WITH CARDIOVASCULAR DISEASE

Maintaining a balanced oral microbiome is essential not only for oral health but also for overall systemic well-being. Oral diseases, such as periodontitis and endodontic infections, have been increasingly associated with systemic inflammation, as they can contribute to the onset or progression of conditions like cardiovascular disease (CVD), diabetes, and autoimmune disorders.<sup>29,40,41</sup> Inflammation in the oral cavity begins when bacterial infections activate an immune response. The gingival epithelium releases chemical mediators, such as interleukins, PGE<sub>2</sub>, and TNF- $\alpha$ , which recruit phagocytic cells like neutrophils and macrophages to the infection site. These immune cells have surface receptors, such as Toll-like receptors (TLRs), that allow them to identify bacterial molecules, while the complement system increases the susceptibility of pathogens to phagocytosis. Once in the area, immune cells release pro-inflammatory cytokines, including IL-1 $\beta$ , IL-6, and TNF- $\alpha$ , to further amplify the inflammatory response.<sup>41</sup> This initial immune response aims to eliminate microbes and clear necrotic tissue without damaging surrounding areas. However, persistent bacterial growth or an altered immune response can shift inflammation from an acute to a chronic state, recruiting more immune cells, such as T-cells and monocytes, and producing additional mediators. This prolonged inflammation results in formation of periapical lesions, alveolar bone resorption by osteoclasts, degradation of periodontal ligament fibers by matrix metalloproteinases (MMPs), which contribute to the progressive destruction of periodontal tissue.<sup>29,39,41</sup>

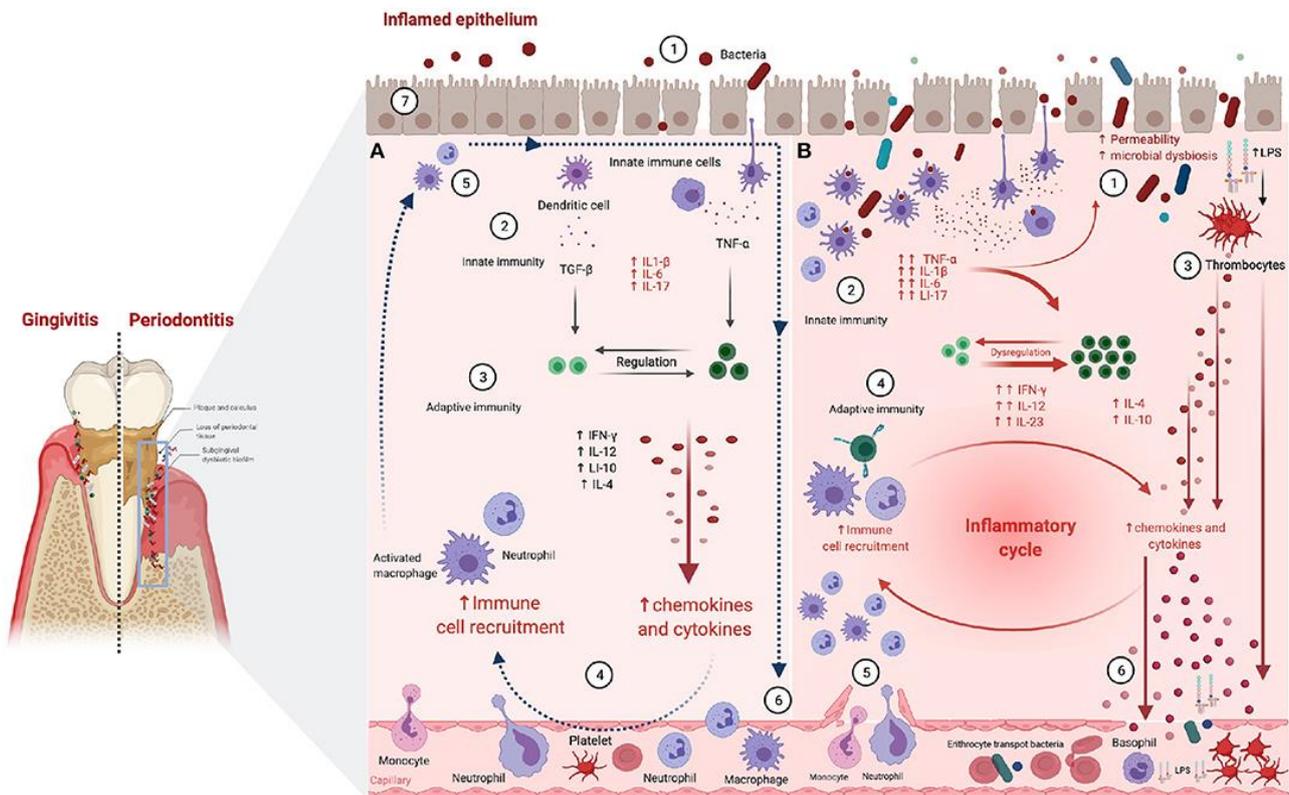


Fig. 4 Periodontitis-associated immune and inflammatory processes. (A) Resolved inflammation and infection clearance. (B) Unresolved periodontal inflammation and persistence of infection.<sup>29</sup>

Cytokines released from an oral infection site, along with bacteria or their toxic byproducts, including lipopolysaccharides (LPS), can enter the bloodstream through ulcerated or damaged periodontal tissues. Once in circulation, these inflammatory molecules and pathogens can potentially cause or worsen damage in distant organs, such as the cardiovascular system. In particular, the presence of pro-inflammatory cytokines can contribute to atherosclerosis, a condition characterized by the buildup of plaque in the arterial walls. For instance, IL-1 $\beta$  and TNF- $\alpha$  increase the expression of vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), promoting leukocyte adhesion to the endothelial surface. This leads to the recruitment of monocytes to the vessel wall, where they differentiate into macrophages and take up oxidized low-density lipoprotein (LDL) particles, transforming into foam cells, which are a hallmark of atherosclerotic plaque formation. This process may lead to arterial blockages, thereby increasing the risk of cardiovascular events, such as heart attack and stroke.<sup>40-42</sup>

Additionally, cytokines like TNF- $\alpha$  and IL-6 promote oxidative stress in the vascular wall by inducing the production of reactive oxygen species, further damaging endothelial cells and contributing to vascular permeability. This oxidative stress, along with the inflammatory effects of IL-1 $\beta$  and IL-6, contributes to the destabilization of atherosclerotic plaques. MMPs, enzymes activated by inflammatory signals, degrade extracellular matrix components within the plaque, weakening its fibrous cap and increasing the likelihood of rupture. Plaque rupture is a critical event that can result

in thrombosis, potentially leading to life-threatening conditions like myocardial infarction.<sup>43</sup> Moreover, studies have found oral bacteria like *Porphyromonas gingivalis* within atherosclerotic plaques, suggesting that oral pathogens can directly contribute to the development of these plaques.<sup>40,41</sup> IL-6 also contributes to systemic inflammation by stimulating the liver to produce acute-phase reactants, such as C-reactive protein (CRP), which is a biomarker associated with a higher risk of adverse cardiovascular events, like myocardial infarction.<sup>41</sup> Overall, the connection between oral infections and CVD underscores the importance of maintaining oral health, as it may have protective effects against cardiovascular and other systemic diseases.

### 3. ANTIMICROBIAL PEPTIDES

The rapid increase of drug-resistant infections has presented a serious challenge to antimicrobial therapies.<sup>44</sup> In the quest of new antibiotics, antimicrobial peptides (AMPs) have recently raised great interest.<sup>45</sup>

Also called host defense peptides (HDPs), AMPs represent an ancient host defense mechanism against infections in all living organisms, including viruses, bacteria, fungi, plants and animals<sup>46,47</sup> and are essential components of the innate immune system of multicellular organisms, including humans.<sup>48,49</sup>

AMPs are oligopeptides of 5 to 100 amino acids with a positive net charge, mainly due to lysine and arginine residues, and a significant proportion, typically 50%, of hydrophobic residues.<sup>49,50</sup> These features confer an amphipathic conformation, with both hydrophobic and hydrophilic domains, which enables the initial electrostatic interaction with the negatively charged microbial membrane.<sup>44,46,51,52</sup> The conformational flexibility of AMPs is largely attributed to their amphipathic nature. The secondary structure of AMPs, such as  $\alpha$ -helices,  $\beta$ -sheets, or a mixture of both, is formed upon interaction with the targeted microbial membrane.<sup>46</sup> At this point, AMPs penetrate the outer membranes and interact with the negatively charged phospholipids of the inner membranes through electrostatic attraction. This interaction leads to the formation of pores or temporary openings in the cell membranes, which ultimately disrupts membrane integrity, causing the contents of the bacteria to leak out, resulting in microbial lysis and cell death.<sup>51</sup>

### 3.1 MECHANISM OF ACTION OF AMPs

Most vertebrate and invertebrate AMPs display a direct and rapid antimicrobial activity by disrupting the physical integrity of the microbial membrane without specifically interacting with receptors.<sup>53,54</sup>

The structural characteristics and the physiochemical properties of AMPs are essential for their activity, as they selectively attack bacterial membranes without harming eukaryotic cells.<sup>46</sup> This property is attributed to the fact that bacterial membranes display lipids with negatively charged phospholipids such as phosphatidylglycerol and cardiolipin on their surface, while the membranes of eukaryotic cells are composed of zwitterionic phospholipids such as phosphatidylethanolamine, phosphatidylcholine, and sphingomyelin, providing a neutral net charge.<sup>51,55-57</sup>

Moreover, the teichoic acid and LPS in gram-positive cell wall and gram-negative outer membrane, respectively, create an extra electronegativity to the bacterial surface,<sup>58</sup> allowing the initial electrostatic attraction with cationic AMPs.<sup>49,59,60</sup>

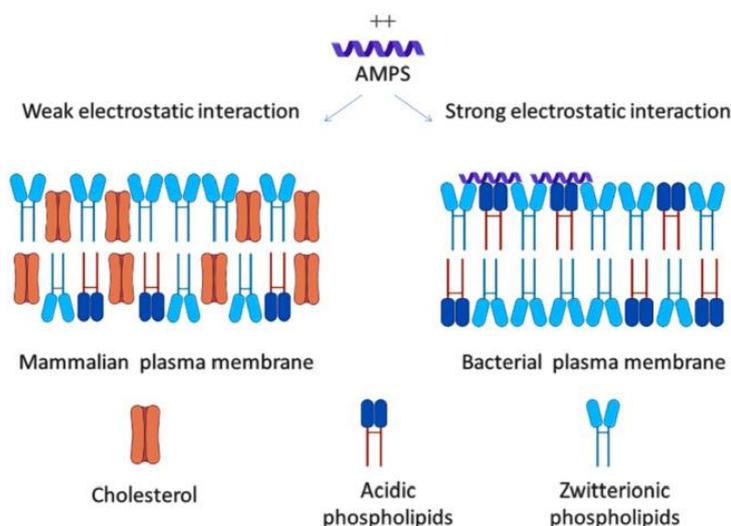


Fig.5 Selectivity of antimicrobial peptides.<sup>61</sup>

Furthermore, an increase in the hydrophobicity of the amino acid sequence of AMPs correlates with its low selectivity and toxicity toward mammalian cells.

After the initial electrostatic interactions, the AMPs accumulate at the surface and self-assemble on the bacterial membrane after reaching a certain concentration.<sup>62</sup> At this stage various models have been proposed to describe the action of AMPs.<sup>60</sup>

In the barrel stave model peptides insert perpendicularly in the lipid bilayer and interact laterally, resulting in the formation of a pore that behaves like a protein ion channel.<sup>60,63,64</sup> In this pore, the peptides are aligned with the hydrophobic side facing the lipid core of the membrane and the hydrophilic regions facing the interior region of the pore.<sup>56</sup> Peptide amphipathic structure ( $\alpha$  and/or  $\beta$

sheet) is essential in this pore formation mechanism as the hydrophobic regions interact with the membrane lipids and hydrophilic residues form the lumen of the channels.<sup>60</sup>

In the toroidal pore mechanism peptides insert perpendicularly in the bilayer and interact only with the head groups of the lipids,<sup>62</sup> affecting the local curvature of the membrane and resulting in the formation of high curvature peptide-lipid toroids.<sup>64</sup>

Finally, in the carpet model the peptides align and accumulate parallel to the surface of the membrane until they reach a critical concentration, leading to changes in the membrane fluidity and removal of small segments of the bilayer through micellization.<sup>60,62,65</sup>

It was originally thought that membrane targeting was the only mechanism of action<sup>60</sup>, but it is now well established that several AMPs can directly translocate across the membrane and accumulate intracellularly, where they target a variety of essential cellular processes such as nucleic acid synthesis, protein synthesis, enzymatic activity, and cell wall synthesis.<sup>54,66</sup>

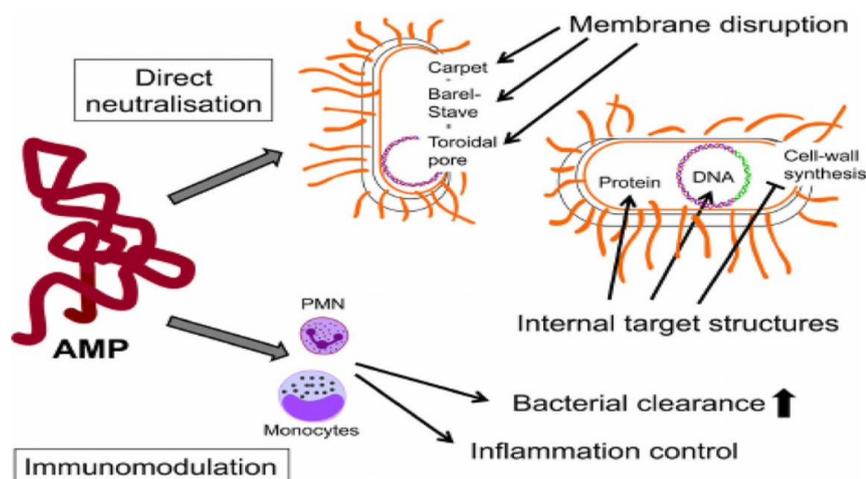


Fig.6 Mechanism of action of AMPs.<sup>67</sup>

### 3.2 IMMUNOMODULATORY ACTIVITY OF AMPs

AMPs play a fundamental immunomodulatory role in both innate and adaptive immunity, bridging the two systems to protect against infections.

In innate immunity, AMPs are stored in granules of immune cells such as neutrophils, macrophages, and epithelial cells. For instance, neutrophils contain defensins that are released to act against pathogens by disrupting cell membranes.<sup>48</sup>

In addition to their antimicrobial properties, AMPs also function as chemoattractants, guiding immune cells to infection sites, where they help coordinate a rapid immune response. For example, cathelicidins attract macrophages to areas of bacterial invasion, where they enhance phagocytosis and promote inflammatory signaling to contain the infection. This dual function not only directly targets

pathogens but also enhances the immune system's efficiency by quickly mobilizing immune cells to act.<sup>68</sup>

AMPs exhibit anti-inflammatory properties by inhibiting the attachment of lipopolysaccharides (LPS) to their receptors, particularly Toll-like receptor 4 (TLR4). This inhibition occurs through strong electrostatic interactions between the positively charged groups of AMPs and the negatively charged components of LPS, leading to a reduction in the release of inflammatory mediators.<sup>69</sup>

They help regulate the release of cytokines, balancing pro-inflammatory (like IL-6 and TNF- $\alpha$ ) and anti-inflammatory responses depending on the context of the infection. This regulation controls how intense and how long the inflammation lasts, which is crucial for preventing tissue damage.<sup>70</sup>

In adaptive immunity, AMPs play a role in recruiting antigen-presenting cells (APCs), such as dendritic cells, to infection sites. This recruitment facilitates the presentation of antigens to T-cells, starting the adaptive response.<sup>71</sup> Additionally, AMPs boost T-cell activity and promote the proliferation of B-cells, which is essential for producing antibodies that neutralize pathogens and establish long-term immunity. This immunomodulatory function underscores their potential in treating infections and various immune-related disorders.<sup>70-72</sup>

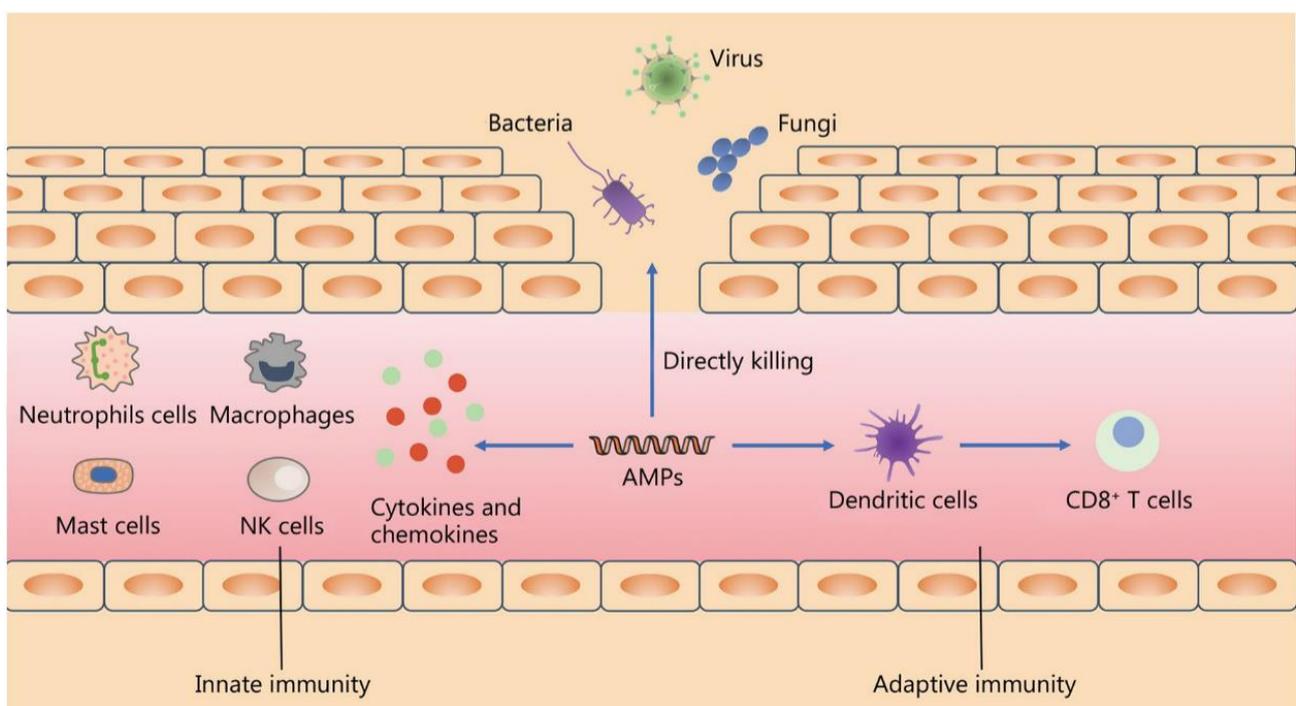


Fig.7 Immunomodulatory activity of AMPs.<sup>71</sup>

### 3.3 ADVANTAGES OF AMPS

AMPs offer several advantages compared to traditional antibiotics, making them a promising candidate to overcome the issue of multidrug resistance.<sup>73</sup> One key benefit of AMPs is their ability to interact with multiple targets on bacterial membranes and within the cell, disrupting vital functions and ultimately leading to cell death. This multi-target mechanism reduces the chances of resistance development.<sup>73</sup> In fact, while conventional antibiotics typically focus on specific pathways, AMPs can simultaneously disrupt cellular membranes and inhibit multiple essential processes. This characteristic allows them to be effective against a broader range of pathogens, including both Gram-positive and Gram-negative bacteria, as well as multidrug-resistant strains.<sup>51,74</sup> Additionally, AMPs demonstrate synergistic effects when combined with other antimicrobial agents, enhancing their effectiveness at non-toxic concentrations.<sup>73,75,76</sup> Another advantage is their low cytotoxicity towards eukaryotic cells, making them safer for therapeutic applications in humans. Beyond their direct antimicrobial properties, AMPs are multifunctional agents with various therapeutic roles, including anti-inflammatory, immunomodulatory, endotoxin-neutralizing activities, and cytotoxic effects on cancer cells, which enhance their potential for pharmacological use. Currently, more than 60 peptides have received approval from the US Food and Drug Administration (FDA), and over 150 peptides are undergoing clinical trials.<sup>73,77,78</sup> Overall, the unique properties of AMPs highlight their potential as a promising alternative to traditional antibiotics in managing infections, particularly in an era marked by increasing antibiotic resistance.<sup>79</sup>

### 3.5 CHALLENGES TO THE PEPTIDE AS THERAPEUTIC USE

Although AMPs offer therapeutic advantages over current antibiotics, they exhibit several challenges that limit their use in clinical settings. A significant issue is their short half-life *in vivo*, as AMPs are prone to rapid degradation by proteolytic enzymes from both the host and bacteria, resulting in quick clearance and a reduced therapeutic window.<sup>49,51,80</sup> This often necessitates frequent dosing to maintain effective levels, which can be both impractical and expensive.

Another concern is toxicity, as AMPs can damage mammalian cells with long-term use, especially at elevated concentrations, leading to cytotoxic effects.<sup>51</sup> For instance, Indolicidin, a 13-residue cationic peptide rich in tryptophan, displays broad-spectrum antimicrobial activity but also has hemolytic properties, limiting its clinical potential.<sup>51</sup> This unintended harm to host tissues restricts the safe therapeutic dosage and presents a considerable obstacle to their clinical application. For these reasons, the majority of AMPs in clinical trials are limited to topical applications.<sup>49,60</sup>

Moreover, AMPs often present poor absorption and low bioavailability when given systemically or orally. Their hydrophilic and cationic characteristics inhibit their ability to penetrate biological barriers, such as the gastrointestinal tract or epithelial tissues, thereby diminishing their effectiveness *in vivo*.<sup>51,80</sup> As a consequence, in the past, pharmaceutical companies have shown little interest in developing peptide-based therapeutics due to their extremely poor ADME (absorption, distribution, metabolism, and elimination) characteristics.<sup>80</sup>

To address these challenges, various strategies have been implemented, including chemical modifications, encapsulation, and innovative delivery systems. For instance, techniques like lipidation, cyclization, and the use of non-natural amino acids can enhance the stability of peptides and make them less vulnerable to proteolytic enzymes.<sup>60</sup> Nonetheless, these modifications may sometimes compromise antimicrobial activity or introduce new issues, such as immunogenicity or changes in pharmacokinetics.<sup>51</sup> Therefore, while AMPs show significant promise, additional research is necessary to effectively navigate these limitations and create molecules that are both potent and safe for clinical applications.

#### 4. M33L

M33 is a synthetic antimicrobial peptide selected from a peptide library tested against *Escherichia coli* to identify sequences with strong antibacterial properties. This library was developed using phage display technology, which helps identify peptides capable of binding to specific bacterial targets.<sup>81,82</sup> The M33 sequence (KKIRVRLSA) is amphipathic and cationic, which is typical for AMPs, but did not show any sequence homology with known AMPs of natural or non-natural origin.<sup>83</sup>

After the initial selection process, the linear sequence of the M33 peptide was optimized and then constructed into a tetrabranching (dendrimeric) structure. This modification was made to improve its stability, enhance its antibacterial effectiveness, and increase its ability to neutralize lipopolysaccharides (LPS), a component of Gram-negative bacterial membranes that can lead to severe immune responses like septic shock.

This branched structure also reduces susceptibility to proteolytic degradation, allowing it to remain active for a longer time in biological environments. Furthermore, the tetrameric design boosts its binding affinity, as each branch can engage with bacterial targets at the same time, resulting in a more powerful antimicrobial effect. Research has also indicated that M33 is both non-hemolytic and non-cytotoxic, meaning it does not damage red blood cells or healthy mammalian cells, which is crucial for therapeutic applications.<sup>82,84,85</sup>

Additionally, M33 has demonstrated effectiveness in neutralizing LPS, suggesting its potential role in treating severe bacterial infections.<sup>82,84,85</sup>

These characteristics make M33 a promising candidate for clinical use, providing direct bactericidal activity while minimizing potential side effects related to cytotoxicity.<sup>82,84,85</sup>

This combination of effectiveness, stability, and safety highlights its therapeutic potential.

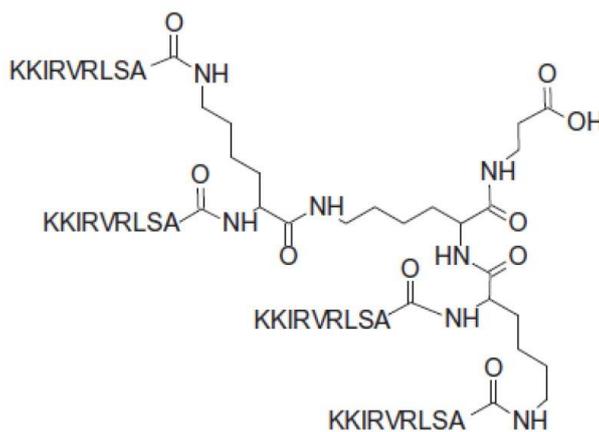


Fig.8 Tetra-branched structure of the antimicrobial peptide M33L<sup>86</sup>

#### 4.1 IN VITRO ANTIMICROBIAL ACTIVITY OF M33L AND M33D

In vitro studies have shown that M33L exhibits strong antimicrobial activity, particularly against Gram-negative bacteria, including *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and various *Enterobacteriaceae*, with MIC values in the micromolar range. Notably, M33L is also effective against multidrug-resistant strains, including those with extended-spectrum  $\beta$ -lactamases and carbapenemases, such as *P. aeruginosa* strains isolated from cystic fibrosis patients.<sup>85</sup>

Additionally, M33L has demonstrated strong antimicrobial activity against clinical isolates of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, two pathogens commonly associated with hospital-acquired infections and known for their antibiotic resistance. Notably, M33L showed comparable or superior efficacy to traditional antibiotics like colistin and tobramycin against these resistant strains.<sup>87-89</sup>

Building on the success of M33L, researchers developed an isomeric form, M33D, by incorporating D-amino acids. While M33L primarily targets Gram-negative bacteria, M33D extends its activity to Gram-positive pathogens, including *Staphylococcus aureus* and *Staphylococcus epidermidis*, with MIC values 4 to 16 times lower than M33L, even against methicillin-resistant and vancomycin-intermediate strains, and also with clinically relevant species like *S. aureus*, *Enterococcus faecalis*, and *Bacillus subtilis*.

As previously observed with M33-L, M33-D exhibits antimicrobial activity against antibiotic-susceptible reference bacterial strains and multidrug-resistant (MDR) strains of clinical origin, showing effectiveness against Gram-negative pathogens like *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*.

Moreover, the incorporation of D-amino acids enhances M33D's stability against proteolytic degradation.

Additionally, M33D exhibits enhanced binding affinities to lipopolysaccharide (LPS) in Gram-negative bacteria and lipoteichoic acid (LTA) in Gram-positive bacteria, which allows it to neutralize bacterial toxins more effectively than M33L.

Collectively, these characteristics make M33D a promising option for treating infections caused by multidrug-resistant pathogens.<sup>83,90</sup>

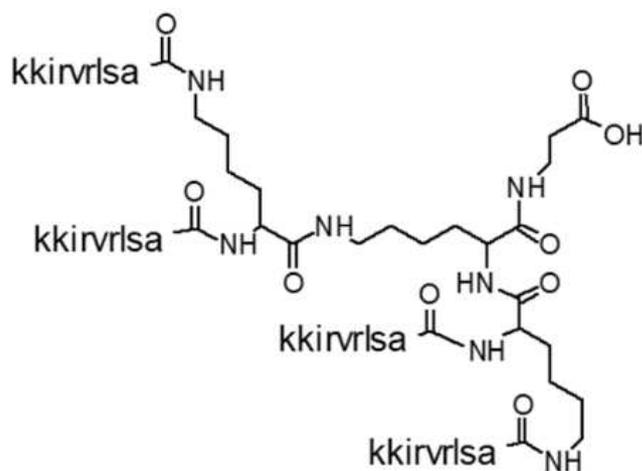


Fig.9 Tetra-branched structure of the antimicrobial peptide M33L<sup>91</sup>

#### 4.2 ANTIBIOFILM ACTIVITY OF M33L AND M33D

The rising need for antimicrobials that can eliminate microbial biofilms prompted the assessment of M33L and M33D against biofilms created by *E. coli*, *P. aeruginosa*, and *S. aureus*. Both peptides exhibited similar effectiveness against Gram-negative bacteria. However, M33D demonstrated greater anti-biofilm activity against the Gram-positive *S. aureus* when compared to M33L. These results underscore the potential of M33D as a more effective choice for targeting biofilms associated with this strain.<sup>83</sup>

#### 4.3 IN VIVO ACTIVITY OF M33L AND M33D

In vivo studies have demonstrated the therapeutic potential of M33L and M33D. The efficacy and toxicity of the novel antibacterial peptide M33L were evaluated in three *Pseudomonas aeruginosa* infection models: sepsis, lung infection, and skin infection. In the sepsis model, mice were treated with M33L (5 mg/Kg) 24 and 72 hours post-infection. M33L showed remarkable results, with a survival rate of 60% in treated mice, compared to those left untreated. In the lung infection model, M33L demonstrated strong antimicrobial activity, successfully reducing bacterial load in the lungs, with 40% of survival compared to untreated mice. Similarly, in the skin infection model, M33-based lotion (10 mg/ml) was spread on the infection site every day, thus leading to a significant reduction in bacterial levels on the skin.<sup>89</sup>

Compared to colistin, which is often used for treating resistant *P. aeruginosa* infections, M33L showed similar or even better effectiveness, particularly at higher doses, while also having significantly lower acute toxicity in mice. Initial assessments indicated that M33L was well tolerated at doses up to 20 mg/kg, with no signs of toxicity. In contrast, colistin showed severe toxicity at just 10 mg/kg, leading to 50% mortality right after administration and 70% within 24 hours. At even higher doses, colistin resulted in 100% mortality, highlighting M33L's promise as a safer treatment option for serious bacterial infections.<sup>89</sup>

Regarding M33D, an in vivo infection model with methicillin-resistant *S. aureus* (MRSA) strain USA 300, the peptide demonstrated strong antimicrobial activity. Mice infected with a lethal dose of bacteria and treated with 5 mg/kg of M33D showed 100% survival after 4 days, while a dose of 2.5 mg/kg resulted in 90% survival. Additionally, in acute toxicity testing, mice treated with single doses of 20 or 25 mg/kg of SET-M33D showed no signs of toxicity over 4 days, highlighting SET-M33D's potent efficacy and safety profile.<sup>90</sup>

#### 4.4 PEPTIDES DERIVED FROM NATURAL HOST DEFENSE PEPTIDES Dermaseptin, Mastoparan, Cathelicidin, Anoplin

Antimicrobial peptides (AMPs) are naturally occurring molecules that can be isolated from various organisms and exhibit potent antibacterial properties. Through chemical modifications, these peptides can be optimized to enhance their activity against multiple bacterial strains, including antibiotic-resistant pathogens, or to reduce their toxicity, making them promising candidates for therapeutic applications.

Dermaseptins are a group of AMPs that were first identified in the skin secretions of Hyloid frogs. They have an amphiphilic structure, characterized by a high content of alanine and lysine residues,

which allows them to effectively disrupt microbial membranes. Known for their broad-spectrum activity, dermaseptins can target both Gram-negative and Gram-positive bacteria, as well as fungi and protozoa. Their strong antimicrobial properties, especially against drug-resistant pathogens, underscore their potential as promising candidates for new therapeutic developments.<sup>92</sup>

Anoplin, which is isolated from the venom of the wasp *Anoplius samariensis*, is a short, amphipathic  $\alpha$ -helical peptide with a straightforward sequence that is rich in lysine and alanine. Despite its simple structure, it shows effectiveness against both Gram-negative and Gram-positive bacteria. Its low toxicity to mammalian cells, along with the possibility of structural modifications like lipidation, enhances its therapeutic potential by improving stability and effectiveness.<sup>93,94</sup>

Mastoparan, obtained from the venom of the wasp *Vespula lewisii*, is another amphipathic  $\alpha$ -helical peptide known for its strong bactericidal activity. It disrupts microbial membranes, leading to cell death, and also triggers mast cell degranulation, which results in the release of histamine and other inflammatory mediators. Although its hemolytic toxicity poses challenges for direct application, researchers are working on modifying mastoparan to decrease its toxicity while maintaining its antimicrobial and immunomodulatory effects.<sup>95</sup>

Cathelicidins are AMPs that play a crucial role in the innate immune systems of various vertebrates, including mammals, birds, fish, and amphibians. They are produced as inactive precursors and become active through cleavage, forming either  $\alpha$ -helical or  $\beta$ -sheet structures. Found in immune cells such as leukocytes or in epithelial tissues, cathelicidins demonstrate broad-spectrum antimicrobial activity against bacteria, viruses, and fungi, while also modulating the immune response. These multifunctional characteristics make them excellent candidates for the development of anti-infective and immunotherapeutic agents.<sup>96</sup>

## OBJECTIVES

Emergence of antibiotic resistance among bacterial pathogens is recognized as a major public health threat, demanding the development of innovative therapies to combat severe infections and safeguard the efficacy of existing drugs. <sup>6,7</sup>

Antimicrobial peptides (AMPs) are emerging as promising alternatives to traditional antibiotics due to their broad-spectrum antibacterial activity, enhanced resistance to proteases, and low cytotoxicity toward eukaryotic cells. Structural modifications, such as branching, further improve their stability against protease degradation and enhance their overall effectiveness, making them a versatile and robust defense mechanism against bacterial pathogens. <sup>44,54,97</sup>

In this thesis, the application of the peptide M33D and its analog M33i/l was evaluated in the context of endodontic and periodontal diseases conditions, often associated with bacterial infections and systemic complications. <sup>91</sup>

Besides, to expand the arsenal against resistant bacteria, a collection of peptides, modeled after natural host defense mechanisms and synthesized in a tetra-branched form, was designed and tested for antibacterial efficacy against a diverse range of bacterial strains.

My research aims to contribute to the development of alternative strategies to address the growing threat of antimicrobial resistance.

## MATERIALS AND METHODS

### 1.1 PEPTIDE SYNTHESIS

The peptides M33D (kkirvrlsa)4K2K $\beta$ A-OH, M33i/1 (kklrvrlsa)4K2K $\beta$ A-OH, BAMP2 (TLLKKVLKAAAK)4K2K, BAMP35 (KLLKRIKKLL)4K2K, BAMP37 (KLLLGKNWKLM)4K2K, BAMP39 (LKKVLKAAAK)4K2K, BAMP45 (INLKKLAKL)4K2K and BAMP49 (GRFKRRFKKL)4K2K were solid-phase synthesized by standard Fmoc chemistry with a Syro multiple-peptide synthesizer (MultiSynTech, Witten, Germany).

Solid-phase synthesis is a common technique for producing peptides. In this method, amino acids are sequentially added to a growing chain that is anchored to a solid surface, such as resin beads.

The resin used was a TentaGel-PHB 4 branch  $\beta$  Ala Wang-type resin (Rapp Polymere, Germany), which carries the branching lysine core in L-form, (Fmoc4-Lys2-Lys- $\beta$ -Ala). The linear homologous (MONO2, MONO35, MONO37, MONO39, MONO45, MONO49 and M33D) were synthesized on TentaGel-PHB Wang-type resin (Rapp Polymere, Germany) following the same procedure.

The N-terminal end of each amino acid is protected by an Fmoc (fluorenylmethyloxycarbonyl) group, which prevents unwanted reactions. To build the peptide, the Fmoc group is removed, exposing the N-terminal so the next amino acid can be added. Amino acids are then sequentially attached one by one, repeating the process of deprotection and coupling until the desired peptide sequence is complete. The side chains of amino acids are protected with appropriate protecting groups.

Once the peptide chain is completely formed, it is deprotected at the side chains and removed from the resin by treatment with trifluoroacetic acid TFA containing triisopropylsilane and water, and precipitated with diethyl ether.

The crude peptide was then purified by reversed-phase chromatography using a Phenomenex Jupiter C18 column (300 Å, 10 mm, 250, 610 mm) as stationary phase and a linear gradient of eluent A (0,1% TFA/water) and of eluent B (acetonitrile) as mobile phase.

Final peptides purity and identity were confirmed by reversed-phase chromatography on a Phenomenex Jupiter C18 analytical column (300 Å, 5 mm), and by mass spectrometry with a Bruker Daltonics Ultraflex MALDI TOF/TOF).

## 1.2 PEPTIDE STRUCTURE PREDICTION

APPTTEST is a novel computational approach that integrates the predictive capabilities of neural networks with structural biology software programs, XPLOR-NIH and CYANA, to predict the tertiary structure of peptides from their primary sequence.

The neural networks are trained using structural data derived from experimentally solved peptide or protein models. These models are sourced from the Protein Data Bank (PDB).

After training, the neural networks can predict structural constraints, which are guidelines that describe how different parts of the peptide are positioned relative to each other.

Neural networks of APPTTEST are constructed using experimental model structures obtained from the Protein Data Bank (PDB) to predict structural constraints, which are used in restrained molecular dynamics simulations to produce a final set of structures (86).

## 1.3 HUMAN SERUM PROTEASE STABILITY

For peptides, a pool of sera from healthy volunteers ( $n = 5$ ) was diluted to 50% with RPMI 1640 medium. Each peptide at a concentration of  $5\mu\text{g/ml}$  was incubated in 50% serum at  $37^\circ\text{C}$  for different time intervals. At the established time, trichloroacetic acid was added at a 7.5% concentration, and the sample was centrifuged at  $13,000\times g$  for 15 min. The resulting supernatant was used for the MALDI-TOF analysis ( $2\mu\text{L}$ ) and for the HPLC ( $250\mu\text{L}$ ) analysis, after being diluted with 1% TFA ( $750\mu\text{L}$ ). Time-zero HPLC and MS-spectroscopy spectra were obtained immediately after mixing the peptide with 50% serum. The presence of the intact peptide was determined in HPLC by spotting and integrating the peptide peak at the correct retention time. The identity of the peptide peak was further also confirmed by MALDI mass spectrometry.

## 1.4 BACTERIAL STRAINS

For M33D and M33i/1, *E. faecalis* ATCC 51299 and *E. coli* TG1 were used as reference strains.

*E. faecalis* post-endodontic treatment (PE) and *E. coli* LC711, featuring resistance to the polypeptide antibiotic colistin, were clinical isolates provided by the Careggi University Hospital and Hospital Le Scotte, respectively.

For BAMP2, BAMP35, BAMP37, BAMP39, BAMP45 and BAMP49, *E. coli* TG1, *P. aeruginosa* ATCC 27853, *K. pneumoniae* ATCC 43816, and *E. faecalis* ATCC 51299 were the reference strains susceptible to all standard antibiotics.

*S. aureus* USA300 was used as a methicillin-resistant reference strain and *E. coli* LC711 was tested as clinical isolate.

*E. coli*-luxCDABE was obtained by transfection of *E. coli* TG1 with the pGEN-luxCDABE plasmid, kindly provided from Harry Mobley (Addgene plasmid # 44918; <http://n2t.net/addgene:44918>; RRID: Addgene\_44918). All bacteria were grown at 35 °C and 90% humidity in their specific medium.

### 1.5 MINIMUM INHIBITORY CONCENTRATIONS (MICs)

MICs of M33D, M33i/l, BAMP2, BAMP35, BAMP37, BAMP39, BAMP45 and BAMP49, were determined in triplicate against reference and clinical strains using a microdilution assay.

A single colony from each bacterial strain was cultured overnight at 37 °C in its appropriate medium. The resulting preculture was then diluted 1:100 in cation-adjusted Mueller Hinton Broth (Sigma-Aldrich) and incubated at 37 °C until reaching an optical density (OD) of 0.15, measured at 600 nm using a densitometer (Densichek, bioMérieux, Marcy-l'Étoile, France).

Microtiter plate wells containing 50 µL of serially diluted peptides were inoculated with an equal volume of 1:100 diluted bacterial suspension, resulting in a final bacterial concentration of  $5 \times 10^4$  CFU per well in a total volume of 100 µL.

The microtiter plate was then incubated at 35 °C for 18–20 h, and MICs were measured either by turbidometry or by visual inspection. Assays were performed in triplicate, and the median MIC values were recorded.

### 1.6 ANTIBIOFILM ACTIVITY

Biofilm inhibition and eradication activities of the peptides were evaluated using two parameters: the biofilm prevention concentration (BPC) and the minimal biofilm inhibition concentration (MBIC).

For the determination of BPC of M33D and M33i/l, an overnight culture of *E. faecalis* PE, *E. faecalis* 51299, *E. coli* TG1, or *E. coli* LC711 was grown to an optical density (OD) of 0.8 in Tryptic Soy Broth (TSB) supplemented with 0.25% glucose. A 100 µL aliquot of this bacterial suspension was added to wells in triplicate in a 96-well U-bottom plate, followed by 100 µL of a twofold serial dilution of M33D or M33i/l. Untreated bacteria served as a positive control. Untreated bacteria were used as positive control. After 24 h of static incubation at 37 °C, wells were washed thrice with PBS and fixed with PFA 4% in PBS (200 µL). Wells were then washed 3 times, and crystal violet (1%) was

added and incubated. Wells were again washed, and the color dissolved with ethanol/acetone (80:20) for OD measurement at 595 nm.

The Minimum Biofilm Inhibitory Concentration (MBIC) assay followed the same protocol as the BPC, except the bacterial suspension (200  $\mu$ L) was added directly to wells and incubated for 24 h at 37 °C to allow biofilm formation prior to peptide treatment. In fact, after incubation, the supernatant was removed, and 200  $\mu$ L of a twofold serial dilution of M33D or M33i/1 in TSB was added to the wells.

For BPC, 100  $\mu$ L of BAMP2, BAMP37, BAMP39, and BAMP49, was added to the wells of a 96-well plate with peg lids in triplicate, using twofold serial dilutions of each peptide. Overnight cultures of *E. coli* TG1 and *K. pneumoniae* 43816 were diluted 1:100 and grown to an OD of 0.8 in 2xTY and LB, respectively. These cultures were further diluted 1:1000, and 100  $\mu$ L was added to each well. Untreated bacteria were used as positive controls. The plate was incubated at 37 °C and 110 rpm for 24 h. After incubation, the pegs were rinsed with sterile PBS, transferred to a fresh 96-well F-bottom plate containing 180  $\mu$ L of bacterial broth, and centrifuged for 20 min at 1400 rpm to remove bacteria from the pegs. The plate was incubated for 4 h at 37 °C, and OD595 was measured.

For MBIC, bacterial suspension was prepared as described above, and 200  $\mu$ L was added to the wells of a 96-well plate with peg lids. After 24 h of incubation at 37 °C and 110 rpm to allow biofilm formation, the pegs were rinsed with sterile PBS and transferred to a fresh 96-well F-bottom plate containing twofold serial dilutions of each peptide in bacterial broth. Plates were incubated for 24 h at 37 °C and 110 rpm, followed by the same rinsing, centrifugation, and OD595 measurement steps as in the BPC protocol.

The BPC is the lowest concentration of peptide that results in 10% lower biofilm formation compared to untreated controls, while MBIC is defined as the lowest peptide concentration that resulted in an OD595 difference of  $\leq 10\%$  compared to the control.

## 1.7 BACTERIAL MEMBRANE INTERACTION

For the evaluation of peptides M33D and M33i/1, *E. faecalis* PE or *E. coli* LC711 cultures were grown overnight at 37 °C, diluted 1:100 in fresh medium, and incubated until reaching an OD of 0.2 (measured with a densitometer). The cultures were centrifuged at 4000 rpm for 10 minutes, and the resulting pellets were resuspended in PBS-glucose to a final concentration of  $1 \times 10^8$  CFU/mL.

Bacterial suspensions were incubated with 5  $\mu$ g/mL propidium iodide (PI) or 5  $\mu$ M SYTOx Green. After gentle mixing, 200  $\mu$ L was added to a black 96-well plate (Optiplate) and preincubated at 37

°C. Fluorescence was measured every minute for 5 minutes until readings stabilized using a plate reader (Victor Nivo, PerkinElmer).

Peptides were added to duplicate wells at 2× and 4× MIC, and fluorescence readings for PI ( $\lambda_{\text{ex}} = 535 \text{ nm}$ ,  $\lambda_{\text{em}} = 617 \text{ nm}$ ) and SYTOx Green ( $\lambda_{\text{ex}} = 504 \text{ nm}$ ,  $\lambda_{\text{em}} = 523 \text{ nm}$ ) were recorded every minute for 100–120 minutes using a plate reader.

The activity of peptides BAMP2, BAMP37, BAMP39, and BAMP49 was evaluated using *E. coli* TG1. Peptides were prepared at final concentrations of 1.5  $\mu\text{M}$  and 3  $\mu\text{M}$ , following the same protocol of M33D and M33i/l.

### 1.8 LPS-PEPTIDE BINDING ASSAY

Peptides M33D, M33i/l, BAMP2, BAMP37, BAMP39, and BAMP49 were diluted in carbonate buffer (pH 9) and used at working concentrations of 10  $\mu\text{g/mL}$ , 5  $\mu\text{g/mL}$  and 1  $\mu\text{g/mL}$  for coating a 96-well ELISA strip plate in which uncoated wells are used as negative controls. The plate was then sealed and incubated overnight at 4° C. After the well contents were aspirated, the plate was washed three times with PBS + 0.05% Tween 20 and then three times with only PBS for removing residues of detergents. Saturation of the plate to block any unspecific site was performed by adding 400  $\mu\text{L/well}$  of 3% milk in PBS and then incubating for 2 h at 37 °C. After washing the plate as previously described, each well is incubated with LPS-Bio (Aurogene srl, Roma, Italy) diluted in PBS-BSA 0,3% at a working concentration of 5 $\mu\text{g/mL}$  or with only PBS-BSA 0,3% as a negative control. The plate was incubated in the dark at 30 °C for 30 min. After washing, 100  $\mu\text{L/well}$  of streptavidin- POD (Sigma-Aldrich, St. Louis, MO, USA) diluted at a ratio of 1:500 in 0.3% PBS-milk was added, followed by incubation in the dark for 30 min at 30 °C. After another washing cycle, 150  $\mu\text{L/well}$  of substrate solution composed of phosphocitrate buffer, TMB, DMSO, glycerol and H<sub>2</sub>O<sub>2</sub>, are added and incubated for 5 minutes. The reaction was stopped with 50  $\mu\text{L/well}$  of HCl 1M and then the plate was read at 450 nm and 650 nm using a microplate spectrophotometer (Multiskan, Thermo Scientific, Waltham, MA, USA).

### 1.9 DENTIN SLICE MODEL

Extracted human molars and premolars were obtained from patients according to a protocol approved by the Clinical Ethics Committee of the Azienda Ospedaliero-Universitaria Senese, COMEC, [N° 7/2021].

Teeth were sliced with a diamond saw at low speed. Slices were then treated with 40% phosphoric acid for 1 min, followed by 5.25% sodium hypochlorite for 10 min in an ultrasound bath.

After autoclaving dentin slices, they were placed in a 24-well plate and challenged with a suspension of *E. faecalis* PE bacteria (OD = 0.8), incubated at 37 °C for 1 h at 110 rpm. The supernatant was removed, and the slices were washed three times with PBS or a solution of the M33D and M33Di/l in PBS at a concentration of 10 µM. Dentin slices were then incubated at 37 °C for 1h in TSB. The supernatant suspension was serially diluted and plated in TSB-agar. CFU were counted after incubation overnight at 35 °C.

### 1.10 CELL CULTURES

For the following experiments, two cell lines were used:

- RAW2647 murine macrophages (ATCC, Rockville, MD, USA) grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with glutamine, fetal bovine serum, penicillin and streptomycin.
- Human cardiac fibroblasts (HCF) (Innoprot P104452) grown in fibroblast basal medium containing fetal bovine serum, fibroblast growth supplement-2, penicillin and streptomycin (Innoprot, Derio, Spain).

In all experiments, cells were grown to 70-80% confluence at 37°C in a 5% CO<sub>2</sub> atmosphere.

### 1.11 MACROPHAGES STIMULATION

RAW264.7 cells (50,000/well) were seeded in a 96-well plate, incubated for 24 hours, and then stimulated with LPS (2 µg/mL) for 18 hours in the presence of M33D or M33i/l peptides (16 µg/mL). Following incubation, supernatants were collected and cytokine levels (IL-1β, TNF-α) were quantified using a Milliplex kit (Merck-Millipore, Molsheim, France) according to the manufacturer's instructions.

### 1.12 HUMAN CARDIAC FIBROBLASTS MODULATION

HCF were plated in a 96-well plate at 500,000 cells/mL and incubated overnight. Cells were treated with LPS (2 µg/mL) alone or in presence of M33D or M33i/l (16 µg/mL) and left overnight at 37 °C. At the same time, a DuoSet ELISA plate (R&D Systems Inc., Minneapolis, MN, USA) was prepared according to the manufacturer's instructions, adding 100 µL/well of the capture antibody. After 24 h,

the DuoSet plate was washed and blocked with 1% BSA in PBS. The HCF supernatant was collected and centrifuged for 5 min at 1200 rpm and added to the DuoSet plate at 100  $\mu$ L/well and incubated for 2h at RT. After washing, the detection antibody was added and left for 2h at RT. Finally, streptavidin-peroxidase was used for detection and the plates were read at 450 nm using a microplate spectrophotometer (Multiskan, Thermo Scientific). The IL-6 concentration was calculated from a standard curve that was linearized by plotting the log of the six concentrations versus the log of the antibody.

### 1.13 SCANNING ELECTRON MICROSCOPY

A single colony of *E. coli* TG1 was grown overnight at 37 °C. The preculture was diluted 1:100 in bacterial broth, grown to OD=1 (Densichek, bioMérieux), then 4 mL of the suspension was centrifuged for 10 min at 4000 rpm. The pellet was resuspended in 2 mL 1 $\times$  PBS containing BAMP2, BAMP37, BAMP39, and BAMP49 (12  $\mu$ M) and incubated for 30 min or 1 h. Untreated bacteria served as controls.

Samples were centrifuged for 5 min at 10000 rpm, and pellets were resuspended in 500  $\mu$ L 1 $\times$  PBS. Aliquots of 20  $\mu$ L were placed on glass coverslips in 24-well plates, fixed with 2.5% glutaraldehyde (2 h, 4 °C), washed with 0.1 M cacodylate buffer (CB), and postfixed with 1% osmium tetroxide (1 h, 4 °C). After dehydration in an ascending alcohol series, tert-butanol was used for freeze-drying. Coverslips were mounted on stubs, coated with 20 nm gold/palladium (60/40) using a Balzers MED010. Sample survey and imaging were performed with an FEI Quanta400 scanning electron microscope operating at an electron acceleration voltage of 20 kV.

### 1.14 TRANSMISSION ELECTRON MICROSCOPY

*E. coli* TG1 culture was grown and treated with BAMP2 as described above for scanning electron microscopy (SEM) sample preparation. Following treatment, each sample was transferred to a 2 mL Eppendorf tube and centrifuged at 10,000 rpm for 5 minutes. The cell pellets were fixed in 2.5% glutaraldehyde in CB for 2h at 4 °C, washed in CB, and postfixed with 1% osmium tetroxide in water for 1h at 4 °C; they were dehydrated in an ascending alcohol series, incubated twice in propylene oxide, infiltrated and embedded in Epon resin (Glycid ether 100, Serva Electrophoresis), and then polymerized in an oven at 60°C for 48h.

Ultrathin sections (65 nm) of the sample were obtained with a Reichert-Jung ultracut E ultramicrotome and collected on 150 mesh copper grids. Section staining was performed with uranyl

acetate and a lead citrate solution. Stained sections were imaged by a transmission electron microscope (FEI Technai G2 SPIRIT, acceleration voltage 100 kV), equipped with a TemCam F216 Tvips CMOS camera.

### 1.15 SKIN INFECTION MODEL

Seven-week-old female BALB/c mice ( $25 \pm 3$  g) were obtained from Charles River Laboratories Italia and acclimatized for four days under conditions compliant with the European Convention for the Protection of Vertebrate Animals and institutional care guidelines.

For infection, *E. coli* (luxCDABE) cultures were grown to  $OD_{600} = 0.8$ , resuspended in PBS, and  $1 \times 10^8$  CFU in 50  $\mu$ L PBS was injected subcutaneously into the shaved dorsum. Mice were divided into two groups: the control group received 50  $\mu$ L sterile saline, while the treatment group received 0.4 mg of BAMP2 (16 mg/kg) in 50  $\mu$ L saline. Treatments were administered subcutaneously at the infection site 2 hours post-infection and repeated every 24 hours for three doses.

Infection progression was monitored via bioluminescence imaging (IVIS Lumina X5, PerkinElmer) under anesthesia. On day four, mice were euthanized with CO<sub>2</sub> following isoflurane anesthesia.

For histological analysis, skin cryosections were fixed in 4% paraformaldehyde (10 min, 2–8 °C), rehydrated in PBS, and treated with 1% horse serum to block non-specific binding. Sections were stained overnight at 2–8 °C with anti-mouse Ly6-G/Ly6-C Alexa Fluor 488 antibody (BioLegend) in 1% bovine serum albumin (BSA). After washing, sections were mounted with ProLong Gold antifade reagent containing DAPI (Molecular Probes) and imaged using a Leica TCS SP5 confocal microscope.

DAPI (364 nm excitation/458 nm emission) and Alexa Fluor 488 (501 nm excitation/523 nm emission) signals were analyzed using ImageJ to quantify total cell counts (DAPI) and monocytes (Ly6-G/Ly6-C), expressed as a ratio of monocytes to total cells.

### 1.16 EUKARYOTIC CELLS VIABILITY ASSAY

RAW264.7 ( $5 \times 10^4$  cells/well) were plated in 96-well plates and incubated for 24 hours at 37 °C with 5% CO<sub>2</sub>. After incubation, the culture medium was aspirated, and 200  $\mu$ L of M33D and M33i/l and BAMP2, BAMP37, BAMP39, and BAMP49, tested at increasing concentrations in fresh medium was added to each well. The plates were further incubated at 37 °C for 4 and 24 hours.

After incubation, cells were fixed with 4% PFA in PBS for 15 minutes at room temperature, stained with 1% crystal violet in water for 30 minutes. Finally, after three washings, the dye was solubilized with ethanol/acetone (80:20). OD at 595 nm was measured using a Bio-Rad microplate reader

(Hercules, CA, USA), and cell viability was determined by comparing treated and untreated controls. Dose-response curves and EC<sub>50</sub> values were calculated using GraphPad Prism 5.03 software.

### 1.17 HEMOLYTIC ACTIVITY

Whole human blood in EDTA was centrifuged at 3500 rpm for 10 min. The pellet of red blood cells was resuspended at a 1:50 ratio in 1× PBS, mixed by inversion, and incubated with serial dilutions of M33D and M33i/l, BAMP2, BAMP35, BAMP37, BAMP39, BAMP45, and BAMP49 from 0,25 to 160 uM for 2h at 37 °C. Data for 100% hemolysis were obtained by adding 0.1% TritonX-100 in water to the cells. The saline solution was used as negative control.

Finally, the plate was centrifuged and the supernatants were transferred to a 96-well F-plate, and absorbance was measured at 405 nm by using a microplate reader. Data for 100% hemolysis was obtained by adding 1% Triton X-100 in PBS to the cells. PBS was used as a negative control. The hemolysis rates of the peptides were calculated with the following equation: Hemolysis (%) = (A peptide – A physiological solution)/(A triton – A physiological solution) 100%, where A is the absorbance.

### 1.18 STATISTICAL ANALYSIS

The data were plotted and analyzed using Excel (Microsoft, Redmond, WA, USA) and GraphPad Prism 5.0 software (GraphPad, San Diego, CA, USA), reported as means ± SD where relevant. Experiments were repeated at least twice.

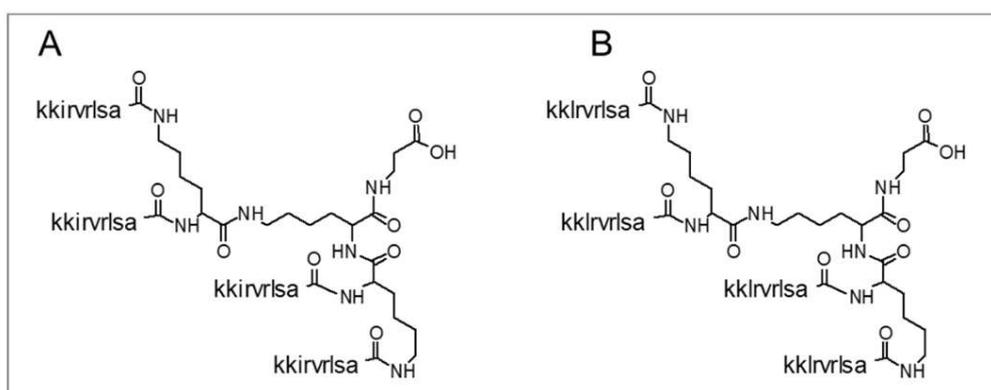
## RESULTS

The thesis work was conducted in two chronological phases: an initial phase focusing on derivatives already available in the laboratory, M33L and M33D, and a subsequent phase involving newly developed derivatives.

### 1. RESULTS FOR M33D and M33i/l

#### 1.1 PEPTIDES STRUCTURE AND STRUCTURE PREDICTION

M33D and M33i/l are synthetic tetra-branched peptides built on a tri-lysine core, which enables the attachment of four identical peptide sequences to the lysine side chains (Figure 10). All amino acids in the sequences are from the D-series, except for the lysine core. M33i/l differs from M33D in the substitution of isoleucine with leucine in its sequences. This substitution was chosen because leucine, having one less stereocenter than isoleucine, reduces the formation of stereochemical byproducts during synthesis with Fmoc-chemistry. Consequently, M33i/l is more cost-effective for industrial production and future development.



*Fig. 10. Structure of the branched antimicrobial peptides, M33D (A) and M33i/l (B)*

The tertiary structure of the linear analogs of M33D and M33i/l was predicted using the computational APPTTEST protocol (Figure 11). Both peptides exhibit a random, non-helical configuration. A top-down view of their structure reveals a distinctly amphipathic nature, with nonpolar aliphatic side chains—such as leucine, isoleucine, valine, and alanine—positioned opposite the polar cationic residues lysine and arginine. This amphipathic arrangement facilitates the peptides' ability to embed into bacterial cell membranes, a critical initial step in disrupting membrane function and ultimately leading to bacterial death.

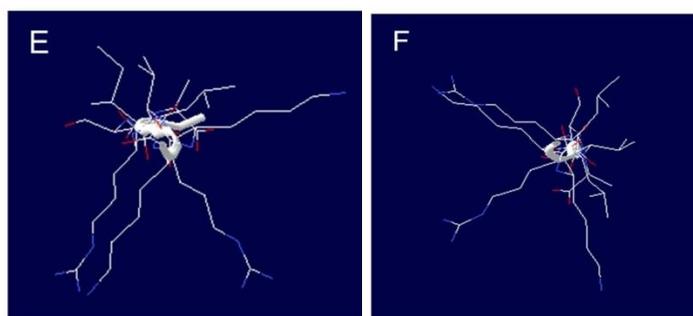


Fig. 11 De novo prediction of peptide tertiary structure by APPTTEST for the linear analogs of M33D (E) and M33i/l (F).

### 1.2 HUMAN SERUM PROTEASES STABILITY

The stability of M33D and M33i/l to proteolytic activity was evaluated by incubating the peptides with human serum for different timepoints. For comparison, the branched isomer synthesized with L-amino acids and its linear L-monomer counterpart (KKIRVRLSA) were included in the same assay. After 72 hours, HPLC analysis showed that 31% of M33D and 25% of M33i/l remained intact. The branched L-isomer (M33L) exhibited a comparable level of stability to the D-isomers, whereas the linear homolog, as expected, was completely degraded within 4 hours due to its unbranched structure and composition of L-amino acids (Figure 12).

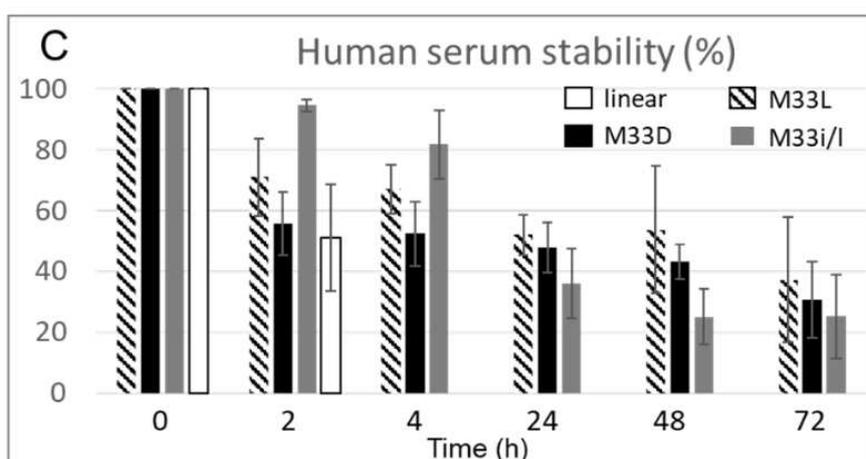


Fig. 12 Stability of M33D and M33i/l to serum proteolytic activity compared to M33D linear homolog, KKIRVRLSA, and to a branched analog in L configuration, M33L.

### 1.3 MIMIMUM INHIBITORY CONCENTRATIONS

MICs of M33D and M33i/l were determined against strains of Gram-negative and Gram-positive bacterial species commonly involved in oral diseases, specifically *Enterococcus faecalis* and *Escherichia coli* (Table 1). For each species, both a reference strain and a clinical isolate were

included in the analysis: *E. faecalis* ATCC 51299 and *E. coli* TG1 as reference strains, and *E. faecalis* PE and *E. coli* LC711 as clinical isolates.

The MIC of both M33D and M33i/l against the reference strain *E. coli* TG1 was 1.5 µM, showing no difference in activity. The clinical isolate *E. coli* LC711, which is resistant to colistin, was also susceptible to M33D with an MIC of 1.5 µM. However, the i/l substitution in M33-D significantly improved activity, lowering the MIC of M33i/l to 0.3 µM.

For *E. faecalis* ATCC 51299, M33D demonstrated a MIC of 3 µM, while M33i/l was more effective, with a reduced value of 1.5 µM. In contrast, both M33D and M33i/l showed the same MIC value of 1.5 µM against the clinical isolate *E. faecalis* PE.

Name	M33D	M33i/l
<i>E. coli</i> TG1	1,5	1,5
<i>E. coli</i> LC711	1,5	0,3
<i>E. faecalis</i> 51299	3	1,5
<i>E. faecalis</i> PE	1,5	1,5

Table 1. MIC values of M33D and M33i/l

#### 1.4 ANTIBIOFILM ACTIVITY

Biofilms significantly contribute to the persistence of bacterial infections, as bacteria within these structures are far more resistant to antibiotics than their free-floating counterparts. Despite their importance, no drugs specifically targeting biofilms are available for clinical use, largely because the molecular mechanisms behind biofilm formation have only recently been better understood.<sup>22,24</sup>

In endodontics, successful root canal treatments aim to eliminate polymicrobial infections and create a seal to prevent reinfection. However, failure to achieve this seal can lead to microbial regrowth and post-treatment complications.<sup>98</sup>

*Enterococcus faecalis* is particularly problematic due to its ability to form resilient biofilms on canal walls, making it highly resistant to antimicrobial therapies.<sup>98</sup>

For this reason, the antibiofilm activity of M33D and M33i/l was evaluated against two bacterial species commonly found in the oral microbiota, *E. faecalis* and *E. coli*. These species, whose biofilms can compromise oral health, were studied using both a reference strain and a clinical isolate as in vitro models.

As shown in Table 2, the biofilm inhibition assay was performed on two *E. faecalis* strains, ATCC 51299 and the PE endodontic isolate, as well as two *E. coli* strains, the TG1 reference strain and the LC711 clinical isolate.

For *E. faecalis*, M33D's BPC matched its MIC for both strains, while M33i/l's BPC was double the MIC for the reference strain and equal to the MIC for the clinical isolate. Both peptides showed an MBIC of 6  $\mu$ M, corresponding to two- and fourfold the MIC for the reference strain and isolate, respectively.

For *E. coli* TG1, the BPC of M33D and M33i/l matched their MIC, while the MBIC was double the MIC. Similarly, against *E. coli* LC711, M33D showed a BPC equal to its MIC and an MBIC twice its MIC. Notably, both peptides exhibited the same BPC (1.5  $\mu$ M) and MBIC (3  $\mu$ M) against LC711, despite M33i/l having a much lower MIC (0.3  $\mu$ M).

Peptide	<i>E. faecalis</i> 51299		<i>E. faecalis</i> PE		<i>E. coli</i> TG1		<i>E. coli</i> LC711	
	M33D	M33i/l	M33D	M33i/l	M33D	M33i/l	M33D	M33i/l
MIC	3	1,5	1,5	1,5	1,5	1,5	1,5	0,3
BPC	3	3	1,5	1,5	1,5	1,5	1,5	1,5
MBIC	6	6	6	6	3	3	3	3

Table 2. M33D and M33i/l biofilm inhibition against *E. faecalis* ATCC 51299, *E. faecalis* PE, *E. coli* TG1, and *E. coli* LC711 with values for MIC, BPC and MBIC.

## 1.5 BACTERIAL MEMBRANE INTERACTION

Cationic AMPs primarily exert their antimicrobial effects by disrupting the permeability and integrity of bacterial membranes. Some AMPs also translocate into the cytoplasm to target intracellular components, making the bacterial membrane a key target for their activity and a critical factor in their effectiveness.<sup>51,53,99</sup>

M33D and M33i/l mechanisms of action of and interactions with bacterial membranes were investigated by monitoring pore formation kinetics using the fluorescent dyes propidium iodide (PI) and SYTOX green in the clinical isolates *E. faecalis* PE and *E. coli* LC711, both oral colonizing species.

These membrane-impermeable dyes bind nucleic acids, producing fluorescence only when the cytoplasmic membrane is critically damaged.

The addition of M33D and M33i/l at 2- and 4-fold the MIC resulted in rapid membrane permeabilization, as evidenced by increased fluorescence within 10–20 minutes, indicating effective pore formation in both bacterial species (Figure 13).

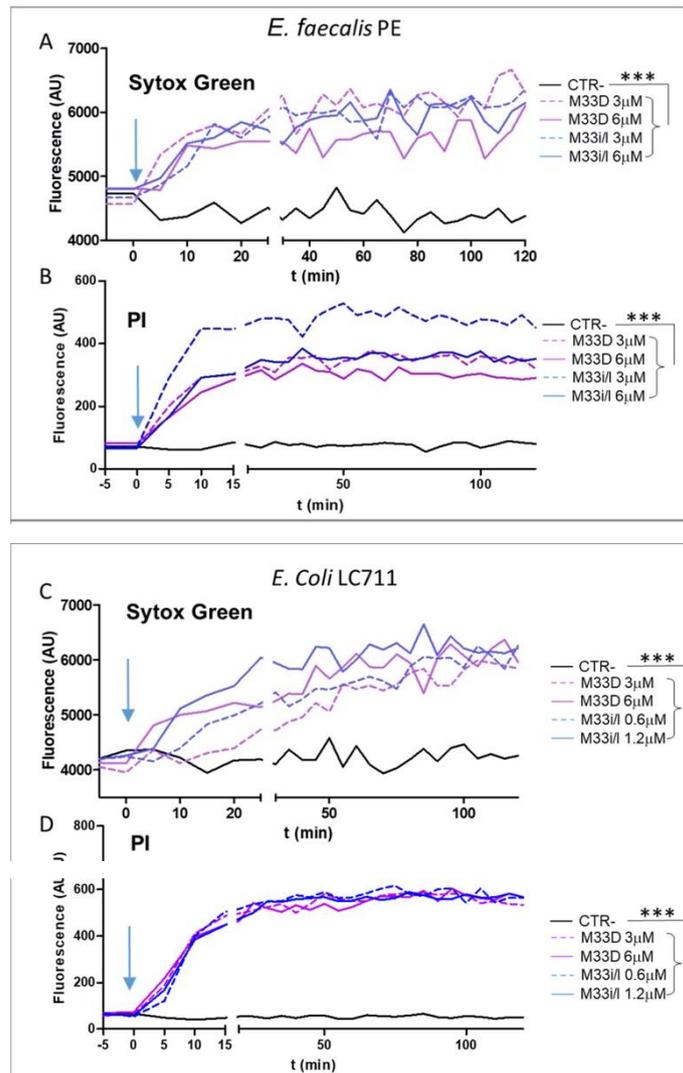


Fig.13 Membrane permeabilization with SYTOx green (A) and PI (B) in *E. faecalis* PE and in *E. coli* LC711 (C, D) after being treated with M33D and M33i/l used at 2- and 4- fold their MIC.

The blue arrow indicates the time when the peptides are added.

\*\*\*  $p < 0.0001$  for both peptides and both concentrations

## 1.6 LPS-PEPTIDE BINDING ASSAY

M33D has demonstrated a potent ability to neutralize lipopolysaccharide (LPS) and lipoteichoic acid (LTA), effectively exerting strong anti-inflammatory effects. By targeting these molecules, M33D significantly reduces the expression of key inflammatory mediators, including cytokines, enzymes, and transcription factors, thereby mitigating the inflammatory response.<sup>90</sup>

For this reason, the neutralization capacity of M33D and M33i/l against LPS, along with their ability to suppress pro-inflammatory cytokine release following LPS stimulation, has been evaluated in RAW264.7 murine macrophages and human cardiac fibroblasts (HCF). Both peptides exhibit a dose-dependent binding to LPS-biotin, as illustrated in Figure 14.

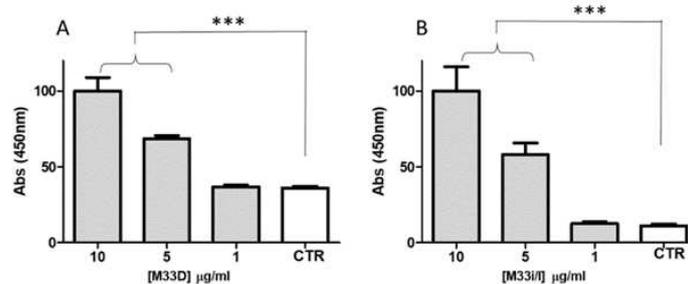


Fig.14 Binding to LPS-bio of M33D (A) and M33i/l (B) ( $n = 6$ , for all groups,  $*** p < 0.0001$ )

### 1.7 DENTIN SLICE MODEL

Root canal therapy, the primary treatment for endodontic infections, involves both mechanical cleaning and chemical disinfection, typically using sodium hypochlorite to eliminate bacteria and seal the canal. However, achieving complete bacterial elimination can often be challenging.<sup>35–37</sup>

Dentin slices were prepared as a model for tooth cavity to assess the potential use of M33-D and M33i/l as antibacterial agents in intracanal irrigation. The goal was to decrease bacterial load and improve restoration results. After sterilizing the slices, they were infected with *E. faecalis* PE and subsequently treated with a 10 µM solution of M33D or M33i/l (Figure 15A). This concentration, significantly above the MIC, was selected because of the localized nature of the irrigation process.

Washing the dentin slices with the two peptides significantly prevented bacterial regrowth (Figure 15B). This led to a statistically meaningful decrease in bacterial load, reducing it to almost one-twentieth of the control level and to a lesser degree compared to the PBS wash.

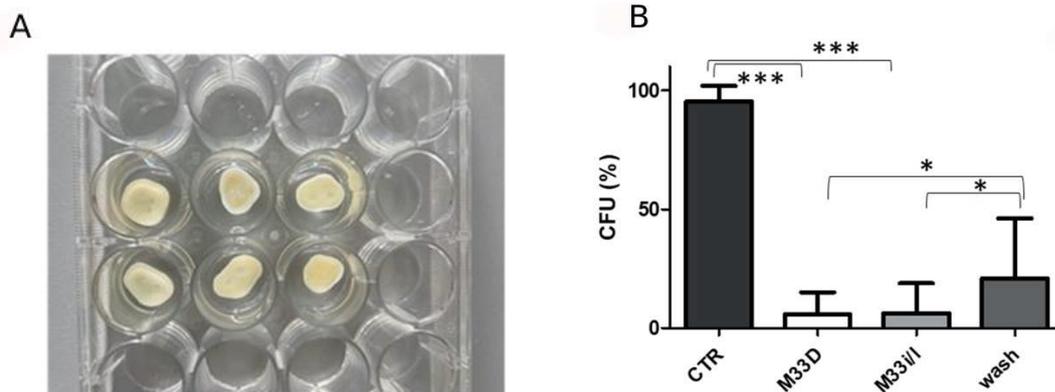


Fig.15. (A) Infected dentin slices of human extracted molar and premolar treated with three washes of M33D or M33i/l (10µM) and then incubated with TSB medium to evaluate bacterial regrowth. (B) Inhibition of bacterial regrowth after washing infected dentin slices with the peptides compared to non-washed controls. Inhibition is expressed in % CFU

$*** p < 0.0001$ ;  $* p < 0.0188$  M33D;  $* p = 0.0423$  M33i/l, CTR  $n = 5$ , M33D and M33i/l  $n = 20$ , washes  $n = 10$

## 1.8 MACROPHAGES STIMULATION AND HUMAN CARDIAC FIBROBLASTS MODULATION.

The anti-inflammatory effects of M33D and M33i/l were assessed through their ability to inhibit pro-inflammatory cytokine release in LPS-stimulated RAW264.7 macrophages and human cardiac fibroblasts (HCF).

Using a bead-based multiplex assay with Luminex technology, LPS stimulation increased IL-1 $\beta$  release in macrophages by 20% compared to untreated cells. M33D and M33i/l reduced this release by 16% and 14%, respectively (Figure 16C).

Similarly, TNF- $\alpha$  release was elevated by 65% following LPS stimulation but was lowered by 13% with M33D and 23% with M33i/l (Figure 16D). In HCF, ELISA assays revealed that M33D and M33i/l reduced IL-6 release triggered by LPS by 50% and 35%, respectively (Figure 16E). These results highlight the peptides' efficacy in modulating inflammatory responses.

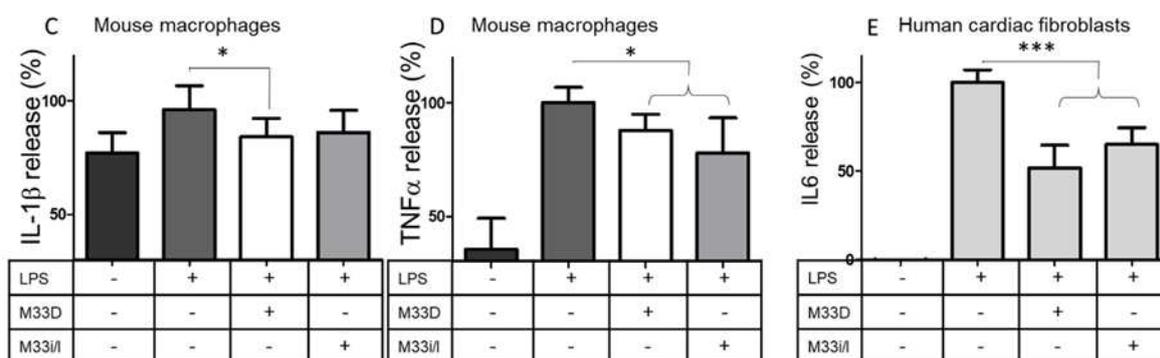


Fig. 16 Inhibition of IL-1 $\beta$  release (C;  $n = 4$  in the first group, 8 in the second, 6 in the third and fourth,  $* p = 0.0348$ ) and TNF- $\alpha$  release (D;  $n = 4$  in the first and second group, 6 in the third and 4 in the fourth,  $* p < 0.05$ ) in LPS-stimulated RAW2647 macrophages and inhibition of IL-6 release (E;  $n = 3$ ,  $*** p < 0.0001$ ) in LPS-stimulated HCF after treatment with M33D and M33i/l.

## 1.9 EUKARYOTIC CELLS VIABILITY ASSAY AND HEMOLYTIC ACTIVITY

The cytotoxicity of M33D and M33i/l was evaluated in RAW264.7 murine macrophages, revealing EC50 values of  $3.0 \times 10^{-5}$  M and  $1.8 \times 10^{-5}$  M, respectively (Fig. 17A). These values are approximately ten times higher than the MIC observed for the tested species. Furthermore, neither peptide exhibited notable haemolytic activity (Figure 17B and C).

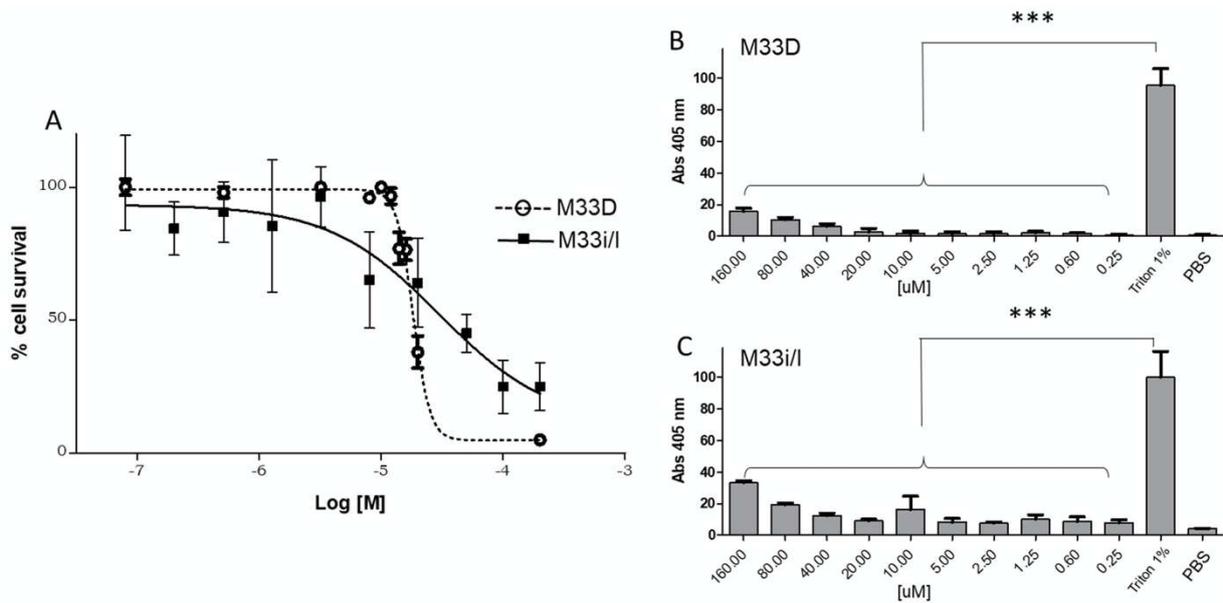


Fig.17. Cytotoxicity of M33D and M33i/l in RAW2647 macrophages (A; cell viability is reported as a percentage of the untreated cells,  $n=3$ . Curves are obtained with GraphPad (nonlinear variable slope)) and haemolytic activity of M33D and M33i/l in red blood cells (B; it is reported as a percentage  $\pm$  SD of 100% obtained with triton 1% in PBS after incubation for 1 h at 37 °C, \*\*\*  $p < 0.0001$  ( $n = 3$ ))

## 2 EXPERIMENTS WITH BAMPs

### 2.1 PEPTIDES STRUCTURE PREDICTION

The APPTTEST protocol was used to analyze peptide sequences, producing 3D models of their tertiary structures in water. BAMP2 and BAMP35 formed amphipathic  $\alpha$ -helices involving most residues (Figure 18A, B), while BAMP39 exhibited an  $\alpha$ -helix only in its central residues (Figure 18C). BAMP49 was fully arranged in an amphipathic  $\alpha$ -helix (Figure 18D), whereas BAMP37 and BAMP45 showed a random linear arrangement.

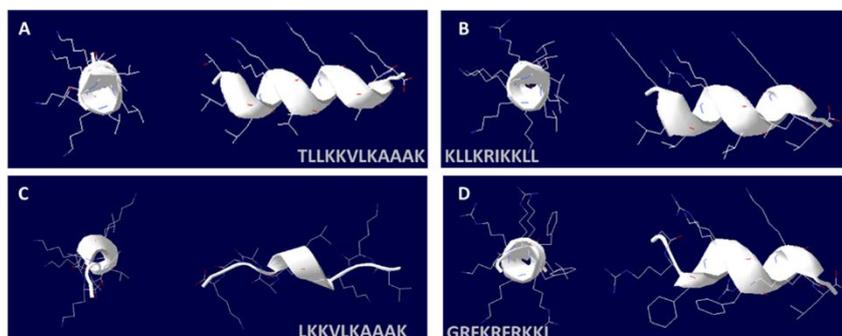


Figure 18 (A–D) Secondary structure calculated for BAMP2, BAMP35, BAMP39, and BAMP49 in a linear form

## 2.2 HUMAN SERUM PROTEASES STABILITY

Analysis using HPLC and MS revealed degradation in all peptides. However, most branched peptides, except BAMP49, remained detectable and intact after 24 hours, while all monomeric analogues were fully degraded within this timeframe. Notably, MONO37 and MONO39 were already hydrolyzed after just 4 hours.

In summary, Branched peptides showed greater stability compared to their monomeric analogues, which degraded rapidly.

## 2.3 MINIMUM INHIBITORY CONCENTRATIONS

The peptides demonstrated greater activity against Gram-negative species. For *E. coli* TG1, MIC values ranged from 1,5 to 6  $\mu$ M. Similarly, MICs against the clinical isolate *E. coli* LC711 ranged from 3 to 6  $\mu$ M, except for BAMP37 and BAMP49, which exhibited lower activity. All peptides were effective against the *P. aeruginosa* reference strain, with MICs between 3 and 6  $\mu$ M. Against *K. pneumoniae* (reference strain), MICs ranged from 6 to 25  $\mu$ M.

Notably, BAMP2, BAMP35, BAMP39, and BAMP45 were active against colistin-resistant *E. coli* LC711. This is significant, as colistin resistance due to acquired mechanisms is increasingly reported in clinical settings.

However, activity against Gram-positive strains, including *E. faecalis* and *S. aureus*, was generally low, with the exception of BAMP35, which showed strong activity (MIC of 3  $\mu$ M) against *S. aureus* USA300 (Table 3).

Name	BAMP2	BAMP35	BAMP37	BAMP39	BAMP45	BAMP49
<i>E.coli</i> TG1	1,5	6	3	1,5	0,7	6
<i>E.coli</i> LC711	3	6	>25	6	6	>25
<i>P.aeruginosa</i> ATCC 27853	6	6	3	6	3	6
<i>K.pneumoniae</i> 43816	6	6	25	12.5	12	25
<i>S.aureus</i> USA 300	>25	3	>25	>25	>25	>25
<i>E.faecalis</i> 51299	25	25	>25	>25	>25	>25

Table 3. Minimum Inhibitory Concentrations of BAMPs

## 2.4 ANTIBIOFILM ACTIVITY

In *E. coli* TG1, BAMP2 and BAMP37 exhibited BPC equal to their MICs, with BAMP2 effectively removing preformed biofilms at twice the MIC (Table 4). BAMP37 had an MBIC eight times its MIC, while BAMP39 and BAMP49 showed BPCs four times their MICs but weaker MBICs (50  $\mu$ M). In contrast, *K. pneumoniae* 43816 biofilms were more resistant to all BAMPs (Table 4).

	<i>E.coli</i> TG1				<i>K.pneumoniae</i> 43816			
	BAMP2	BAMP37	BAMP39	BAMP49	BAMP2	BAMP37	BAMP39	BAMP49
MIC	1,5	3	1,5	6	6	25	12,5	>25
BPC	1,5	3	6	25	50	50	>50	50
MBIC	3	25	50	50	50	50	50	50

Table 4. Antibiofilm activity of BAMPs

## 2.5 BACTERIAL MEMBRANE INTERACTION

In the case of BAMP2, BAMP37, BAMP39 and BAMP49, the membrane damaging effect of these peptides against *E. coli* TG1. The peptides increased membrane permeability within 10 minutes of incubation at twice the MIC, showing clear dose-dependent effects. At higher concentrations, fluorescence quickly plateaued after 10 minutes, while at the MIC, this effect stabilized after 40–50 minutes (Figure 19).

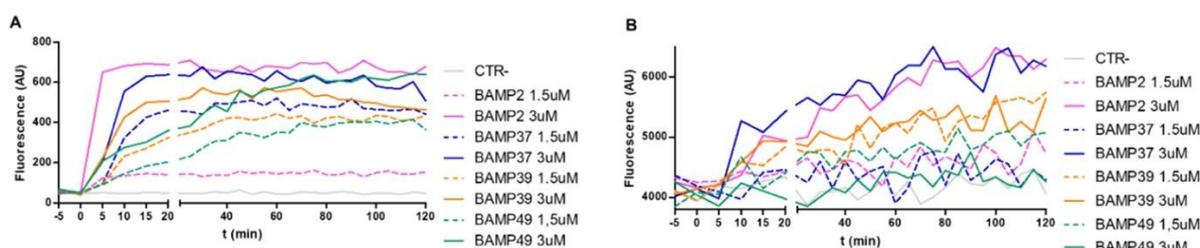


Fig. 19 Cytoplasmic membrane permeability of *E. coli* on treatment with the BAMPs at the MIC and twice the MIC. Fluorescence due to binding of PI (A) and Sytox green (B) fluorescent probes with DNA was measured at 535–617 and 480–523 nm excitation and emission wavelengths, respectively, with a plate reader. The data is expressed as means ( $\pm$ SD) of three independent repeats in triplicate.

## 2.6 LPS-PEPTIDE BINDING ASSAY

BAMPs, like many natural AMPs, are rich in cationic amino acids, giving them a positive charge that facilitates their attraction to negatively charged bacterial surface components like LPS and LTA. This electrostatic interaction contributes to AMPs' bacterial selectivity over eukaryotic cells. Binding to

LPS is particularly important for reducing inflammation, as AMPs can neutralize LPS and prevent its toxic effects, especially during sepsis.

All peptides demonstrated dose-dependent binding to LPS, as shown in Figure 20. This was confirmed using an ELISA assay with LPS-biotin and streptavidin-peroxidase (POD) on peptide-coated wells.

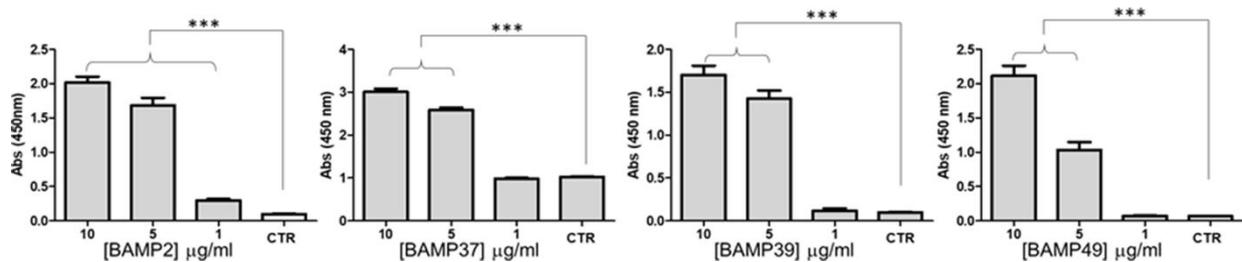


Fig. 20 Binding of BAMPs to LPS was tested by ELISA with LPS-biotin and streptavidin-POD. \*\*\* $p < 0.0001$ ,  $n = 12$ .

## 2.7 SCANNING ELECTRON MICROSCOPY AND TRANSMISSION ELECTRON MICROSCOPY

Scanning electron microscopy (SEM) was used to observe the effects of BAMP2, BAMP37, BAMP39 and BAMP49 on *E. coli* membranes. After 30 and 60 minutes of incubation at twice the MIC, the bacteria showed membrane damage, surface wrinkling, cell enlargement, and loss of smoothness compared to untreated controls (Figure 4C). While the effects were similar at both time points, they occurred more frequently after 60 minutes.

Transmission electron microscopy (TEM) of *E. coli* treated with BAMP2 for 60 minutes revealed damage to the outer membrane, including loss of its double-layer integrity and blister formation (Figure 4D).

The mechanism of action of BAMPs involves three key steps: (1) strong binding to LPS through multivalent interactions, (2) insertion into the membrane via their hydrophobic face due to their amphipathic structure, and (3) disruption of membrane integrity, ultimately leading to cell death (Figure 4E).

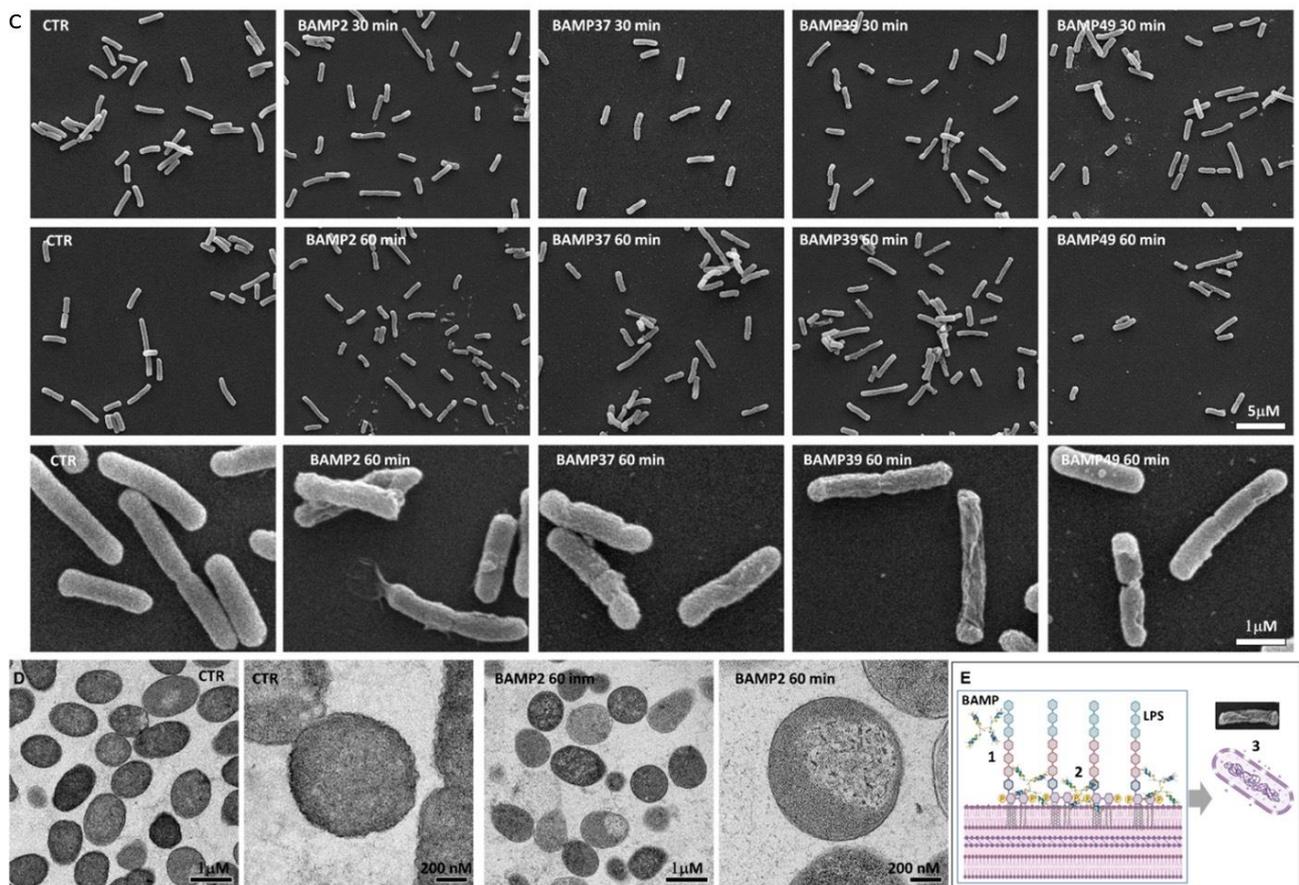


Fig.21 (C) SEM images of *E. coli* treated with the peptides for 30 and 60 min and (D) TEM images of *E. coli* treated with the peptide BAMP2, where the loss of outer membrane double layer and blisters are visible. Model of the BAMPs 3-step mechanism of action: 1- electrostatic interaction of positive charges on the peptide and LPS, 2-embedding into the membrane, thanks to amphipathic helix structure, and 3-loss of membrane functionality

2.8 EUKARYOTIC CELLS VIABILITY ASSAY AND HEMOLYTIC ACTIVITY  
 BAMP2 caused hemolysis at 5  $\mu\text{M}$ , while its shorter analogue, BAMP39, showed no hemolytic activity even at concentrations ten times its MIC. BAMP35 was more toxic, causing hemolysis at just 1.25  $\mu\text{M}$ . BAMP45 and BAMP49 triggered hemolysis at around 15  $\mu\text{M}$ , whereas the mastoparan derivative BAMP37 showed no hemolytic activity (Figure 23A-F).

The toxicity of these peptides was further tested on RAW264.7 murine macrophages as a eukaryotic model. Cell viability was assessed using a colorimetric assay after 4 and 24 hours of incubation at various peptide concentrations (Figure 23G, H). Although the rate of cell death was similar at both time points, 100% cell killing was only observed after 24 hours. Among the peptides, BAMP39 was the least toxic, requiring 200  $\mu\text{M}$  to achieve complete cell death, with an LC50 of  $5.455 \times 10^{-5}$  M. In comparison, BAMP2, BAMP37, and BAMP49 were more toxic, with LC50 values of  $4.405 \times 10^{-7}$ ,

$1.000 \times 10^{-5}$ , and  $1.258 \times 10^{-5}$  M, respectively, and induced complete cell death at concentrations ten times lower than BAMP39.

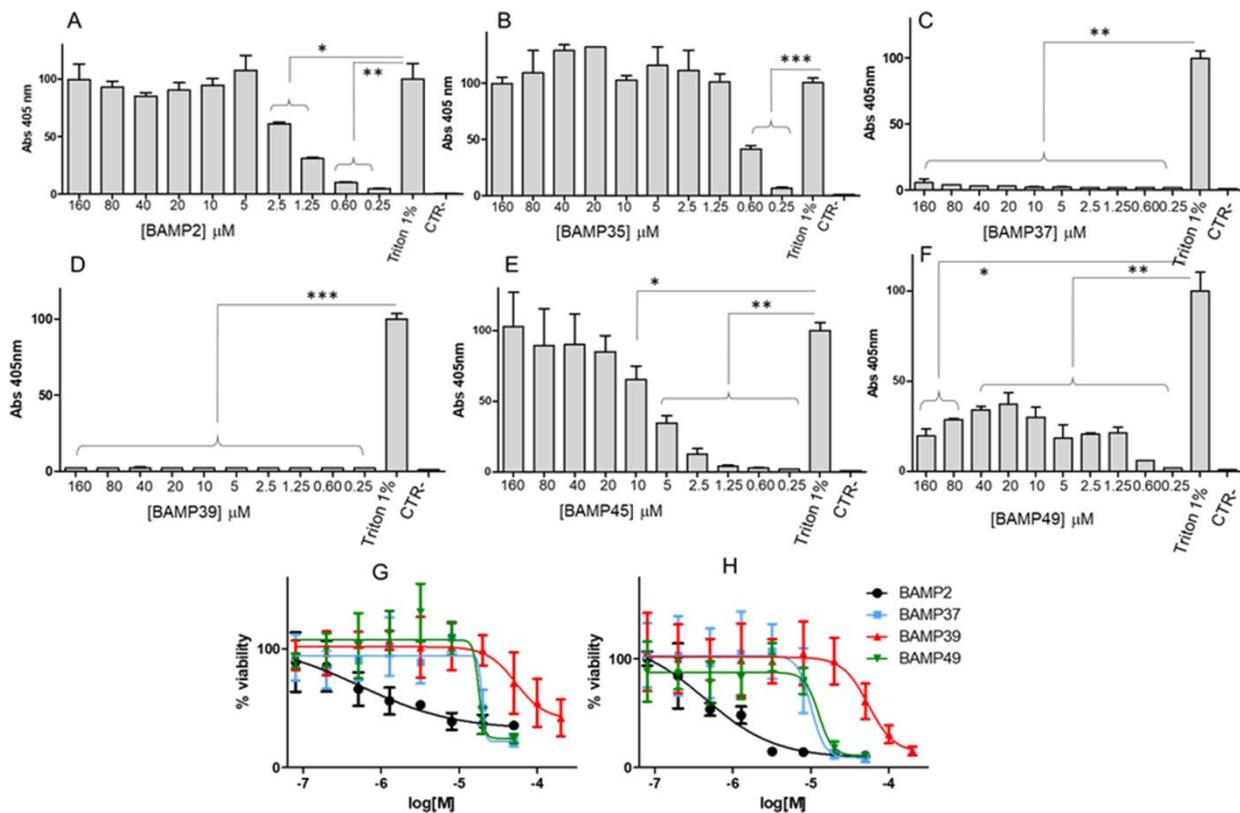


Fig.23 Hemolytic activity of BAMP peptides. All experiments were repeated at least three times ( $n = 3$ ), statistical analysis was performed using the paired two-tailed  $t$  test. The asterisk indicates significant differences between columns (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ). (G) 4 h and (H) 24 h cytotoxicity of the peptides against RAW264.7 murine macrophages.

## 2.9 SKIN INFECTION MODEL

For the infection model, *E. coli* (luxCDABE) was injected subcutaneously into the shaved dorsum of the mice. Two groups were established: the control group, which received 50  $\mu$ L of sterile saline, and the treatment group, which was administered 0.4 mg of BAMP2 (16 mg/kg) in 50  $\mu$ L saline. Treatments were delivered subcutaneously at the infection site 2 hours post-infection and repeated every 24 hours for three doses.

Daily images of the mice's backs were captured and analyzed as radiance to assess bacterial burden. By day 2, the group treated with BAMP2 showed a 75% reduction in bacterial load compared to the untreated group, with significant differences observed on days 2 and 3 (Figure 22A, B). Over time, luminescence decreased in all groups due to the mice's immune response and the natural decay of luciferase expression.

BAMP2's anti-inflammatory properties were evaluated by measuring inflammatory monocyte recruitment at the infection site using anti-Ly6-G/Ly6-C antibodies on harvested tissue slices. Infected skin had over 20% more monocytes than healthy skin, but treatment with BAMP2 reduced monocyte levels to nearly those of healthy tissue, indicating its ability to mitigate inflammation (Figure 22C, D). This aligns with AMPs' known immunomodulatory effects, which help resolve inflammation and prevent excessive immune responses, such as those seen in sepsis.

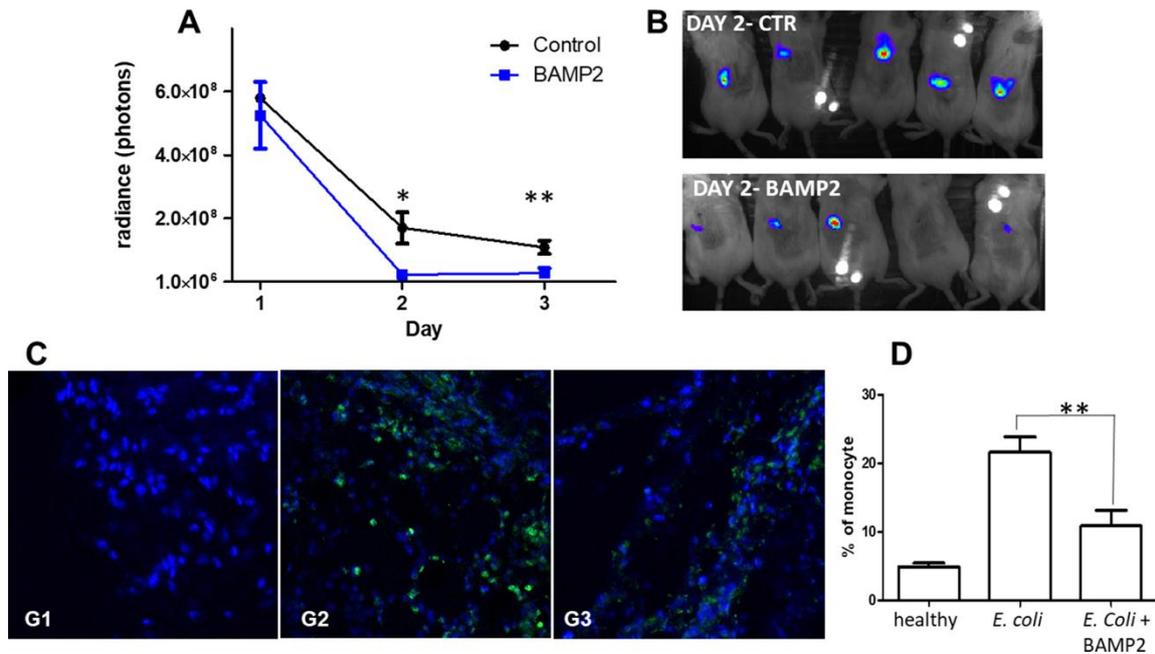


Fig.22 The number of *E. coli* cells was measured in radiance with an IVIS Lumina X5 imager. The two groups, treated with BAMP2 and untreated, were compared. The unpaired *t* test was run with  $n = 5$ , 95% confidence interval,  $p < 0.05$ . (B) Images taken 48 h after infection of the group treated with BAMP2 and of the untreated control group (white dots are an artifact of the visible light beam used to take the picture, not calculated in radiance). (C) Immunohistochemistry of fixed cryosections of skin. The blue signal (DAPI) was used to evaluate the total number of cells in the tissue slice sample, and the green signal (anti-Ly6-G/Ly6-C-488) indicates monocytes. (D) Percentage of monocytes on the total number of cells. Confocal microscopy images were analyzed with ImageJ ( $n = 3-6$ ), followed by the two-tailed *t* test (\*\* $p < 0.05$ ).

## DISCUSSION

AMR poses a significant global health threat by reducing the effectiveness of treatments for infectious diseases, leading to prolonged illnesses, higher mortality rates, and increased healthcare costs. Misuse and overuse of antibiotics in healthcare and agriculture are key drivers of AMR, accelerating its spread. Addressing AMR is crucial to maintain the efficacy of antimicrobial therapies and preventing a public health crisis.<sup>4,5</sup>

In the oral cavity, the microbiome forms biofilms on various surfaces, enabling bacterial communication and, in some cases, pathogenic behavior. This can result in dysbiosis and conditions such as periodontitis and endodontitis.<sup>18</sup> These biofilms are particularly resistant to antibiotics due to their structure, which limits antibiotic penetration and promotes the emergence of resistant bacterial phenotypes. *E. faecalis*, a primary cause of secondary endodontic infections, colonizes treated root canals and forms robust biofilms that withstand standard irrigation techniques and conventional antibiotics.<sup>33,34,36,38</sup>

The inability to completely eliminate oral infections often results in chronic conditions, leading to both local and systemic inflammation. Bacteria and inflammatory mediators can enter the bloodstream, exacerbating systemic diseases such as cardiovascular conditions.<sup>40-42</sup>

M33D and its analogue M33i/l have been studied for their potential as antibacterial agents in intracanal irrigation, targeting biofilm reduction and bacterial load elimination. Additionally, their immunomodulatory properties have been explored for treating oral diseases and preventing systemic complications like cardiovascular disease.

Computational analysis using the APPTTEST protocol indicated that both M33D and M33i/l possess nearly identical amphipathic tertiary structures, characterized by a random, linear configuration instead of a helical one. This amphipathic characteristic is crucial to their mechanism of action: the peptides embed themselves into bacterial membranes, disrupting their stability, which leads to membrane damage and ultimately results in bacterial death.

Protease stability tests indicated that both peptides, thanks to their tetra-branched structures and D-configurations, are highly resistant to proteolytic degradation (Figure 12).

As illustrated in Table 1, the peptides showed strong antibacterial activity against oral pathogens, with MIC values ranging from 0.3  $\mu\text{M}$  to 1.5  $\mu\text{M}$ . Notably, M33i/l exhibited improved effectiveness against a clinical *E. coli* LC711 strain that is resistant to colistin, a peptide antibiotic with a similar mechanism of action to M33D. This enhanced efficacy may be attributed to the i/l substitution, which likely increases bacterial susceptibility and limits resistance mechanisms. Aside from this distinction, the antibacterial performance of M33D and M33i/l was similar.

Mechanistic studies demonstrated that both peptides function by forming pores in bacterial membranes, with initial pore formation occurring within 10 minutes (Figure 13). This rapid action disrupts membrane integrity, resulting in effective bacterial permeabilization in both *E. faecalis* PE and colistin-resistant *E. coli* LC711 isolates.

To assess the potential use of M33D and M33i/l as antibacterial agents in intracanal irrigation procedures, biofilm prevention concentration (BPC) and minimal biofilm inhibition concentration (MBIC) assays were developed, along with a dentin-based root canal irrigation model. Both peptides exhibited comparable performance in BPC and MBIC tests against *E. faecalis* and *E. coli* reference strains, as well as clinical isolates (Table 2). These results are particularly significant when contrasted with the MBIC values of standard antibiotics like ampicillin and linezolid against *E. faecalis*, which are 2048 and 1024 times higher than their MICs, respectively.<sup>100</sup>

Moreover, in the dentin slice model infected with *E. faecalis* PE, which simulates an irrigation process, treatment with either peptide led to a reduction in bacterial load by about 95% compared to a simple wash without peptide exposure, highlighting their effectiveness in preventing bacterial regrowth (Figure 15).

The localized application of these peptides in dentistry allows for the use of concentrations significantly higher than the MIC, well above their cytotoxic EC50. Their robust antibiofilm activity, combined with their amphipathic nature, is likely attributed to their detergent-like properties due to the presence of both charged and lipophilic residues, which enhance biofilm disruption.

Given the established connection between oral infections and cardiovascular diseases (CVDs), the immunomodulatory effects of M33D and M33i/l were also examined. Both peptides showed the ability to neutralize LPS in a dose-dependent manner (Fig. 14) and significantly decreased the release of inflammatory cytokines, including IL-1 $\beta$  and TNF- $\alpha$  in LPS-stimulated murine macrophages, as well as IL-6 in human cardiac fibroblasts (Fig. 16). This dual action indicates that these peptides not only kill bacteria by disrupting their membranes but also bind to LPS released during bacterial death, thus limiting the systemic circulation of pro-inflammatory cytokines and reducing atherosclerotic damage. Furthermore, both peptides exhibited minimal cytotoxicity and no hemolytic activity (Fig. 17). These results position M33D and M33i/l as promising candidates for antiseptic agents in intracanal irrigation procedures, providing an effective approach to tackle dental infections and potentially reducing severe systemic complications, such as CVDs.

AMPs are naturally occurring molecules found in various organisms, known for their strong antibacterial properties. By making chemical modifications, these peptides can be optimized to

improve their effectiveness against a wide range of bacterial strains, including those resistant to antibiotics, while reducing toxicity.<sup>92</sup>

To explore their therapeutic potential, a small library of 18 peptides was created by synthesizing sequences of 9-14 residues derived from natural AMPs into tetra-branched structures. Initial screening against *Escherichia coli* TG1 revealed six promising candidates: BAMP2, BAMP35, BAMP37, BAMP39, BAMP45, and BAMP49.

The branched structure of these peptides offered several benefits, particularly increased resistance to protease degradation, which prolonged their serum half-life. The selected peptides showed activity against planktonic forms of Gram-negative bacteria, including *E. coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*. Notably, BAMP2, BAMP35, BAMP39, and BAMP45 were also effective against colistin-resistant strains.

The branched structure of these peptides effectively increased their resistance to proteases, thereby prolonging their serum half-life.

The peptides showed activity against planktonic forms of Gram-negative *E. coli*, *P. aeruginosa*, and *K. pneumoniae*. BAMP2, BAMP35, BAMP39, and BAMP45 were also effective against colistin-resistant strains.

Further analysis concentrated on their capacity to inhibit and disrupt biofilms, which are complex bacterial communities often resistant to treatment. As reported in Table 4, BAMP2 was identified as the most effective peptide against *E. coli* biofilms, successfully preventing biofilm formation and breaking down existing layers at both the MIC and double the MIC. In contrast, BAMP37, BAMP39, and BAMP49 were more effective at preventing biofilm formation but demonstrated less ability to disrupt established *E. coli* biofilms. When tested against *K. pneumoniae* biofilms, all peptides showed significant disruption, achieving up to 80% biofilm eradication at elevated concentrations (50  $\mu$ M).

The mechanism of action for these peptides involved membrane disruption, leading to structural damage to bacterial cells, such as wrinkling, enlargement, and loss of surface smoothness. These effects resulted in rapid dysregulation of cellular homeostasis within 10 minutes. Additionally, the cationic structure of the branched AMPs enhanced their binding to LPS at MIC and sub-MIC concentrations (10 and 5  $\mu$ g/mL), which contributed to their anti-inflammatory properties.

Among the peptides, BAMP2 exhibited the most potent antibacterial and antibiofilm properties. However, it also had a higher hemolytic effect, which limits its use for intravenous applications. Nevertheless, BAMP2 was chosen for testing in a mouse model of skin infection. In this model, BAMP2 significantly lowered the bacterial load within 48 hours and reduced the recruitment of immune cells to the infection site. This result was likely attributed to its capacity to decrease the bacterial burden and bind to LPS, thereby masking bacterial toxins and diminishing chemotaxis. The

rising threat of AMR underscores the critical need for new antibiotics. Natural AMPs, particularly those enhanced through branching, show great potential due to their resistance to proteases, high effectiveness, and minimal environmental impact. Utilizing these peptides for treating non-life-threatening infections could help maintain the effectiveness of current antibiotics for more severe cases. By offering effective alternatives for prevention and minor infections, these peptides could also encourage further investment in the creation of new antibiotics.

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