

ANTIMICROBIAL PEPTIDES AGAINST ENTEROCOCCUS SPP. BIOFILMS: A SYSTEMATIC REVIEW

PEPTÍDEOS ANTIMICROBIANOS CONTRA BIOFILMES DE ENTEROCOCCUS
SPP.: UMA REVISÃO SISTEMÁTICA

PÉPTIDOS ANTIMICROBIANOS FRENTE A BIOPELÍCULAS DE
ENTEROCOCCUS SPP.: UNA REVISIÓN SISTEMÁTICA

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ABSTRACT

Introduction: Infections caused by *Enterococcus* spp. remain a clinical challenge due to intrinsic antimicrobial resistance and strong biofilm-forming capacity. The increasing prevalence of vancomycin-resistant enterococci further restricts therapeutic options, reinforcing the need for alternative strategies such as antimicrobial peptides. **Methods:** This systematic review followed PRISMA guidelines, with its protocol registered on the OSF platform, and included studies published between 2015 and 2025 in PubMed, Web of Science, and the Virtual Health Library. Eligible studies evaluated antimicrobial peptides activity against *Enterococcus* biofilms through inhibition, eradication, or mechanistic analyses. Data were extracted on peptide characteristics, cytotoxicity, mechanisms of action, and therapeutic applications, and risk of bias was assessed using a ten-item checklist for in vitro studies. **Results:** Most studies focused on *E. faecalis* using in vitro models. Antimicrobial peptides demonstrated consistent ability to inhibit biofilm formation and reduce biomass of mature structures. Reported mechanisms included membrane permeabilization, extracellular matrix disruption, interference with adhesion pathways, and modulation of metabolic and intracellular processes. Cationic amphipathic peptides showed the strongest preventive effects, while engineered analogs, lipopeptides, and enzyme-linked peptides enhanced antibiofilm potency. Synergy with antibiotics or adjunctive therapies was frequently observed. However, methodological variability and limited physiologically relevant models

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restrict translational potential. Conclusion: Overall, antimicrobial peptides represent a promising therapeutic platform, but standardized methodologies and robust in vivo studies are essential for clinical application.

Keywords: Gram-Positive Pathogens. Biofilm Inhibition. Therapeutic Peptides. Peptide Optimization. Antibiofilm Therapy.

RESUMO

Introdução: Infecções causadas por *Enterococcus* spp. permanecem um desafio clínico devido à resistência intrínseca a antimicrobianos e à forte capacidade de formação de biofilme. A crescente prevalência de enterococos resistentes à vancomicina restringe ainda mais as opções terapêuticas, reforçando a necessidade de estratégias alternativas, como os peptídeos antimicrobianos. **Métodos:** Esta revisão sistemática seguiu as diretrizes PRISMA, com protocolo registrado na plataforma OSF, e incluiu estudos publicados entre 2015 e 2025 nas bases PubMed, Web of Science e Biblioteca Virtual em Saúde. Os estudos elegíveis avaliaram a atividade de peptídeos antimicrobianos contra biofilmes de *Enterococcus* por meio de análises de inibição, erradicação ou mecanismos de ação. Dados sobre características dos peptídeos, citotoxicidade, mecanismos de ação e aplicações terapêuticas foram extraídos, e o risco de viés foi avaliado utilizando uma lista de verificação de dez itens para estudos *in vitro*. **Resultados:** A maioria dos estudos concentrou-se em *E. faecalis* utilizando modelos *in vitro*. Os peptídeos antimicrobianos demonstraram capacidade consistente de inibir a formação de biofilme e reduzir a biomassa de estruturas maduras. Os mecanismos relatados incluíram permeabilização da membrana, ruptura da matriz extracelular, interferência nas vias de adesão e modulação de processos metabólicos e intracelulares. Peptídeos anfipáticos catiônicos apresentaram os efeitos preventivos mais acentuados, enquanto análogos modificados, lipopeptídeos e peptídeos conjugados a enzimas potencializaram a atividade antibiofilme. A sinergia com antibióticos ou terapias adjuvantes foi frequentemente observada. No entanto, a variabilidade metodológica e a escassez de modelos fisiologicamente relevantes limitam o potencial de translação clínica. **Conclusão:** De modo geral, os peptídeos antimicrobianos representam uma plataforma terapêutica promissora, mas a padronização de metodologias e a realização de estudos *in vivo* robustos são essenciais para a aplicação clínica.

Palavras-chave: Patógenos Gram-Positivos. Inibição de Biofilme. Peptídeos Terapêuticos. Otimização de Peptídeos. Terapia Antibiofilme.

RESUMEN

Introducción: Las infecciones causadas por *Enterococcus* spp. siguen representando un desafío clínico debido a su resistencia intrínseca a los antimicrobianos y a su gran capacidad para formar biopelículas. La creciente prevalencia de enterococos resistentes a la vancomicina limita aún más las opciones terapéuticas, lo que refuerza la necesidad de estrategias alternativas, como el uso de péptidos antimicrobianos. **Métodos:** Esta revisión sistemática siguió las directrices PRISMA, con su protocolo registrado en la plataforma OSF, e incluyó estudios publicados entre 2015 y 2025 en PubMed, Web of Science y la Biblioteca Virtual en Salud. Los estudios seleccionados evaluaron la actividad de péptidos antimicrobianos frente a biopelículas de *Enterococcus* mediante análisis de inhibición, erradicación o mecanismos de acción. Se extrajeron datos sobre las características de los péptidos, su citotoxicidad, mecanismos de acción y aplicaciones

terapéuticas, y se evaluó el riesgo de sesgo utilizando una lista de verificación de diez puntos para estudios *in vitro*. Resultados: La mayoría de los estudios se centraron en *E. faecalis* utilizando modelos *in vitro*. Los péptidos antimicrobianos demostraron una capacidad constante para inhibir la formación de biopelículas y reducir la biomasa de estructuras maduras. Los mecanismos descritos incluyeron la permeabilización de la membrana, la alteración de la matriz extracelular, la interferencia con las vías de adhesión y la modulación de procesos metabólicos e intracelulares. Los péptidos anfipáticos catiónicos mostraron los efectos preventivos más potentes, mientras que los análogos diseñados, los lipopéptidos y los péptidos conjugados con enzimas mejoraron la eficacia contra las biopelículas. Se observó frecuentemente sinergia con antibióticos o terapias adyuvantes. Sin embargo, la variabilidad metodológica y la escasez de modelos fisiológicamente relevantes limitan el potencial de traslación clínica. Conclusión: En conjunto, los péptidos antimicrobianos constituyen una plataforma terapéutica prometedora; no obstante, resulta esencial contar con metodologías estandarizadas y estudios *in vivo* sólidos para su aplicación clínica.

Palabras clave: Patógenos Gram-Positivos. Inhibición de Biopelículas. Péptidos Terapéuticos. Optimización de Péptidos. Terapia Antibiopelícula.



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INTRODUCTION

The genus *Enterococcus* comprises opportunistic pathogens of major clinical importance because of their ability to persist in healthcare environments, acquire antimicrobial resistance, and form biofilms that promote chronic colonization and recurrent infections (Alamer *et al.*, 2022; Blot *et al.*, 2022; Trinchera *et al.*, 2025). *Enterococcus faecalis* and *Enterococcus faecium* are the species most frequently associated with human infections, including urinary tract infections, bloodstream infections, endocarditis, intra-abdominal infections, wound infections, and infections related to indwelling medical devices (Pishkar *et al.*, 2024; Şchiopu *et al.*, 2023; Shridhar & Dhanashree, 2019). The dissemination of vancomycin-resistant enterococci has further reduced therapeutic options, making these microorganisms an important public health concern (Antochevis *et al.*, 2025; H. Zhang *et al.*, 2023).

The clinical relevance of *Enterococcus* spp. is associated with both intrinsic resistance and the acquisition of additional resistance determinants through mutation and horizontal gene transfer. These determinants may be carried by plasmids, transposons, integrons, and insertion sequences, facilitating their dissemination in healthcare environments mortality (Ho *et al.*, 2025; G. Liu *et al.*, 2022; Maddamsetti *et al.*, 2024). In addition, enterococci express virulence factors

involved in adhesion, aggregation, tissue invasion, and biofilm formation, including the enterococcal surface protein encoded by the *esp* gene, which may promote persistence on host tissues and abiotic surfaces (Shridhar & Dhanashree, 2019).

Biofilms are structured communities of sessile microorganisms attached to biotic or abiotic surfaces and embedded in a self-produced extracellular matrix (Asfaw, 2019; Michaelis & Grohmann, 2023). This matrix restricts antimicrobial penetration and protects embedded cells from environmental stress and host immune responses (Almatroudi, 2025; Zafer *et al.*, 2024). Physiological heterogeneity within biofilms also favors slow-growing and persister cells, which are less susceptible to antimicrobials whose activity depends on active metabolism (Almatroudi, 2025; Azeem *et al.*, 2025; Trinchera *et al.*, 2025). Consequently, biofilm-associated bacteria may tolerate antimicrobial concentrations substantially higher than those required against their planktonic counterparts (Mishra *et al.*, 2022).

Enterococcal biofilms are particularly relevant in chronic and device-associated infections involving intravascular catheters, urinary devices, ureteral stents, prosthetic materials, wounds, periodontal tissues, and the endodontic environment (Nesuta *et al.*, 2017; Şchiopu *et al.*, 2023). Their spatial organization and close cell-to-cell contact may also favor horizontal gene transfer and the dissemination of antimicrobial resistance determinants. Thus, the combination of multidrug resistance and biofilm-associated tolerance substantially limits the effectiveness of conventional therapies (Michaelis & Grohmann, 2023; Trinchera *et al.*, 2025).

The increasing prevalence of multidrug-resistant enterococci and the reduced effectiveness of conventional antimicrobial treatments have intensified the search for alternative strategies capable of targeting both planktonic and biofilm-associated populations (Antochevis *et al.*, 2025; García-Solache & Rice, 2019). Among these approaches, antimicrobial peptides (AMPs) have gained attention because of their broad antimicrobial activity and multiple mechanisms of action. These biologically active molecules are involved in the innate defense of organisms from different biological kingdoms and may be synthesized through ribosomal or non-ribosomal pathways (J. Li *et al.*, 2021; L. Zhang & Gallo, 2016).

AMPs exhibit considerable structural diversity, but many possess cationic and amphipathic properties that favor interactions with negatively charged microbial surfaces and lipid membranes (Kim *et al.*, 2025; S. Liu *et al.*, 2025). In Gram-positive bacteria, these interactions may involve teichoic and lipoteichoic acids in the cell envelope (Ma *et al.*, 2024; Maria-Neto *et al.*, 2015). Their antimicrobial activity is frequently associated with membrane

disruption, increased permeability, ionic imbalance, and loss of intracellular components. However, some AMPs may also interfere with DNA replication, RNA and protein synthesis, enzymatic activity, energy metabolism, cell-wall synthesis, and bacterial division (Fleitas *et al.*, 2025; S. Liu *et al.*, 2025).

Beyond their activity against planktonic cells, AMPs may prevent initial adhesion, reduce extracellular-matrix production and biofilm biomass, and, depending on their structural properties and the developmental stage of the biofilm, act against established communities (Oh *et al.*, 2022). Some peptides may also affect persister cells and stress-response pathways associated with biofilm tolerance to conventional antimicrobials (Mishra *et al.*, 2022). Nevertheless, inhibition of biofilm formation and eradication of mature biofilms represent distinct outcomes because the extracellular matrix may impair peptide diffusion and limit access to cells in deeper biofilm layers (Ma *et al.*, 2024).

AMPs may also be combined with conventional antimicrobial agents, as peptide-induced membrane permeabilization may facilitate antibiotic entry and increase its availability at intracellular targets, potentially producing additive or synergistic effects. However, synergistic effects should be demonstrated using standardized quantitative methods, and the resulting combinations should be evaluated for cytotoxicity, stability, and potential antagonistic interactions (Zhu *et al.*, 2022).

Despite their therapeutic potential, the clinical translation of AMPs remains limited by proteolytic degradation, instability in biological fluids, possible cytotoxicity, reduced activity in the presence of salts and serum proteins, and the predominance of evidence derived from in vitro studies using reference strains and simplified biofilm models (Dad *et al.*, 2025; Kim *et al.*, 2025). Furthermore, the available evidence regarding AMPs against *Enterococcus* biofilms remains heterogeneous and has not been fully consolidated with respect to peptide origin, mechanisms of action, antibiofilm efficacy, combination strategies, biocompatibility, and translational potential (Gao *et al.*, 2024; Mishra *et al.*, 2022).

Therefore, this systematic review aimed to identify and critically analyze antimicrobial peptides evaluated against biofilms formed by *Enterococcus* spp., with emphasis on their origin, mechanisms of action, antibiofilm activity, combinations with conventional antimicrobial agents, biocompatibility, and potential clinical applications.

METHODOLOGY

This systematic review was conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines, and its protocol was prospectively registered in the Open Science Framework (OSF; <https://osf.io/q74ek/overview>). The review question was structured using the PICO framework to investigate the antibiofilm activity of antimicrobial peptides (AMPs) against *Enterococcus* spp. biofilms.

A literature search was performed in April 2025 in PubMed, Web of Science, and the Virtual Health Library (VHL) using the following search strategy: ("Antimicrobial cationic Peptides" OR "Antimicrobial peptides") AND ("Biofilm" OR "bacterial biofilm*") AND ("Enterococcaceae" OR "Enterococcus"). The search was restricted to studies published between 2015 and 2025.

Original peer-reviewed studies evaluating the antibiofilm activity of AMPs against *Enterococcus* spp., their mechanisms of action, or their use alone or in combination with conventional antimicrobial agents were included. Reviews, conference abstracts, case reports, theses, dissertations, studies unrelated to human health, and studies that did not investigate AMPs as the primary intervention were excluded.

Records were exported to Rayyan for duplicate removal and screening. Two independent reviewers screened titles, abstracts, and full texts according to the eligibility criteria, with disagreements resolved by consensus or consultation with a third reviewer. Data extracted from the included studies comprised publication characteristics, AMP origin, *Enterococcus* species and strains, experimental models, antibiofilm assays, cytotoxicity, combination therapies, in vivo validation, and reported mechanisms of action.

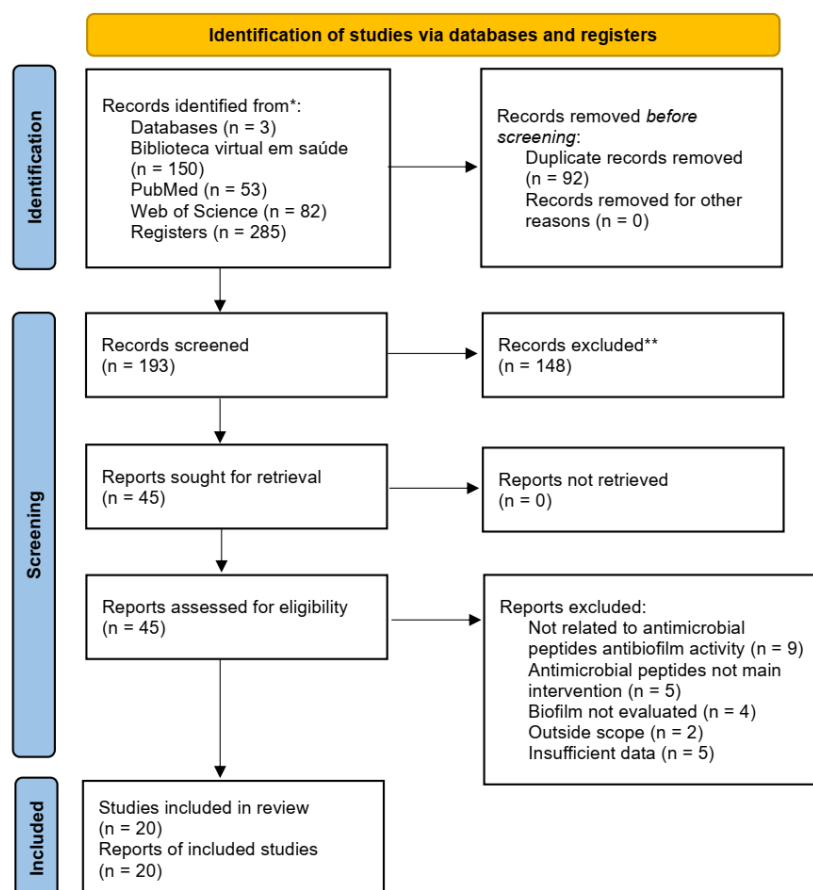
Methodological quality was independently assessed by two reviewers using ten predefined criteria adapted from Silva *et al.* (2025), including experimental design, sample-size calculation, peptide characterization, methodological reporting, use of reference strains, statistical analysis, control measures, and observer blinding. Each fulfilled criterion received one point, and studies were classified as having a low, moderate, or high risk of bias according to the total score.

RESULTS AND DISCUSSIONS

A total of 285 records were identified in the Virtual Health Library (n = 150), PubMed (n = 53), and Web of Science (n = 82). After the removal of 92 duplicates, 193 records were screened by title and abstract, of which 148 were excluded. Forty-five articles underwent full-text assessment, and 25 were subsequently excluded because they did not evaluate the antibiofilm activity of AMPs against *Enterococcus* spp. (n = 9), investigated other interventions (n = 5), examined biofilms formed by other microorganisms (n = 4), addressed food or environmental applications (n = 2), or provided insufficient methodological information (n = 5). Thus, 20 studies were included in the final analysis, as shown in Figure 1.

Figure 1

Screening, eligibility, and inclusion of articles according to the PRISMA flow diagram.



Source: The authors.

The included studies consisted predominantly of in vitro investigations, with fewer ex

vivo, in situ, and in vivo models. Although the search included publications up to April 2025, no eligible studies published in 2025 met the inclusion criteria. This finding suggests that research specifically investigating the antibiofilm activity of antimicrobial peptides against *Enterococcus* spp. remains limited despite the growing interest in alternative strategies for combating multidrug-resistant pathogens. Table 1 summarizes the characteristics of the included studies, including the antimicrobial peptides evaluated, their origins, the *Enterococcus* species investigated, and the main findings reported.

Table 1

Characteristics of the studies evaluating antimicrobial peptides against Enterococcus spp. biofilms.

Study (author/year)	Peptide/composition	Origin	Enterococcus strains	Main outcome
(Sivakamavalli <i>et al.</i> , 2015)	Crustin	Animal	<i>E. faecalis</i>	Inhibited planktonic growth and early biofilm formation; non-hemolytic up to 20 µg/mL. Antibiofilm activity with low hemolysis
(Berditsch <i>et al.</i> , 2016)	Gramicidin S	Microbial	<i>E. faecalis</i>	Showed activity against planktonic cells and biofilms formed by reference, clinical, and tetracycline-resistant isolates. KR-12-a5 showed the best antimicrobial and antibiofilm activity with low cytotoxicity; LL-37 was inactive and hBD-3-1CV showed limited activity and greater cytotoxicity.
(Caiaffa <i>et al.</i> , 2017)	LL-37, KR-12-a5, hBD-3-1CV	Animal-derived and synthetic	<i>E. faecalis</i>	HYL-20K and HYL-21 were active against planktonic cells and biofilms, whereas HYL-20 and HYL-20-OH were inactive.
(Nesuta <i>et al.</i> , 2017)	HYL-20, HYL-21, HYL-20K, HYL-20-OH	Synthetic	<i>E. faecalis</i>	The dimer (AU) ₂ K was more
(Freitas <i>et al.</i> , 2019)	AU, (AU) ₂ K	Synthetic	<i>E. faecalis</i> , VRE <i>E. faecium</i>	

				active than AU and enhanced photodynamic antibiofilm activity against both species. Antimicrobial and antibiofilm activity was structure-dependent; selected lipopeptides inhibited biofilm formation and eradicated mature biofilms. Both peptides showed concentration-dependent activity, with DJK-5 demonstrating greater efficacy against mature biofilms. Gramicidin S showed the strongest activity, Temporin L had intermediate or partial effects, and IDR-1018 was inactive; all peptides showed toxicity in the zebrafish embryo model. DJK-5 was more effective than IDR-1018 against biofilms formed in an ex vivo human dentin model. Both peptides were active against planktonic cells and biofilms without significant cytotoxicity in L929 cells. Demonstrated potent antimicrobial and antibiofilm activity, although the most active
(Paduszynska <i>et al.</i> , 2019)	Gemini lipopeptide series	Synthetic	<i>E. faecalis</i>	
(Ye <i>et al.</i> , 2019)	IDR-1018, DJK-5	Synthetic	<i>E. faecalis</i>	
(Berditsch <i>et al.</i> , 2019)	Gramicidin S, Temporin L, IDR-1018	Mixed: microbial, animal-derived, and synthetic	<i>E. faecalis</i> ; <i>E. faecium</i> VRE	<i>E.</i>
(Huang <i>et al.</i> , 2019)	IDR-1018, DJK-5	Synthetic	<i>E. faecalis</i> clinical isolate.	
(Mergoni <i>et al.</i> , 2020)	KP, L18R	Synthetic	<i>E. faecalis</i>	
(Neubauer <i>et al.</i> , 2021)	Monomeric USCLs and gemini lipopeptide series	Synthetic	<i>E. faecium</i> VRE	

(D. Li <i>et al.</i> , 2021)	L163, L163-Ac, L163FΔL, L163(S-S)	Synthetic	<i>E. faecalis</i> and resistant <i>Enterococcus clinical isolates</i>	compounds showed cytotoxicity and hemolysis near effective concentrations. L163 derivatives were active against planktonic cells and biofilms and exhibited low hemolysis. Demonstrated ex vivo antibiofilm activity in dentinal tubules without detectable cytotoxicity. Both peptides reduced biofilm biomass and were non-cytotoxic under the evaluated conditions. Lysozyme-derived peptides showed bactericidal and antibiofilm activity against susceptible, multidrug-resistant, and vancomycin-resistant isolates. The derivatives were active against planktonic cells and biofilms and showed low toxicity in an ex vivo infected pig-skin model.
(Caiaffa <i>et al.</i> , 2021)	LL-37, KR-12-a5	Synthetic	<i>E. faecalis</i>	LL-37 alone was ineffective, whereas the Ply2660-LL-37 combination showed synergistic activity against planktonic cells and biofilms and was effective in a murine infection model. Demonstrated
(Mergoni <i>et al.</i> , 2021)	KP, L18R	Synthetic	<i>E. faecalis</i>	
(Rouchon <i>et al.</i> , 2022)	Lysozyme, Lysozyme HI, LP9	Animal-derived and synthetic	<i>E. faecalis</i> reference, mutant, and clinical isolates	
(Mishra <i>et al.</i> , 2022)	LfcinB6 derivative series	Synthetic	Resistant <i>E. faecalis</i> and <i>E. faecium</i> isolates	
(H. Zhang <i>et al.</i> , 2023)	Ply2660, LL-37	Microbial and animal-derived	<i>E. faecalis</i> ; <i>E. faecium</i> , including VRE	
(Hu <i>et al.</i> , 2024)	buCaTHL4B, Im-4	Synthetic	<i>E. faecalis</i>	

				antibiofilm activity despite the absence of relevant planktonic growth inhibition at concentrations up to 80 µg/mL. Showed antimicrobial and antibiofilm activity in an ex vivo endodontic model without causing dentin erosion. Demonstrated antibiofilm activity against vancomycin-resistant <i>E. faecium</i> and showed no relevant cytotoxicity toward eukaryotic cells.
(Sinha, 2024)	GH12	Synthetic	<i>E. faecalis</i>	
(Kranjec <i>et al.</i> , 2024)	EJs, MP1	Synthetic microbial	and <i>E. faecium</i> , including VRE	

Caption: VRE = vancomycin-resistant enterococci; USCLs = ultrashort cationic lipopeptides.

Source: The authors.

Across the 20 included studies, a wide diversity of antimicrobial peptides was investigated against *Enterococcus* spp. biofilms, with recurrent interest in compounds such as Gramicidin S, DJK-5, L18R, KR-12-a5, KP, IDR-1018, and LL-37, which were evaluated in more than one study. This recurrence suggests that these peptides are considered promising candidates for structural optimization and further translational investigation.

Synthetic peptides and structurally modified analogs predominated, reflecting the increasing use of rational peptide engineering to improve antimicrobial potency, stability, selectivity, and resistance to degradation. Natural peptides of animal or microbial origin were less frequent but remained important as templates for the development of optimized derivatives.

Most studies evaluated *E. faecalis*, including reference strains, clinical isolates, multidrug-resistant isolates, and genetically modified strains, whereas studies involving *E. faecium* were less frequent but commonly focused on vancomycin-resistant isolates, highlighting their clinical relevance. Nevertheless, the predominance of in vitro models and reference strains limits the generalizability of the findings to the diversity of enterococcal infections encountered in clinical practice.

Most included studies reported activity against both planktonic cells and biofilms. However, antibiofilm efficacy varied according to peptide structure, concentration, bacterial strain, biofilm maturity, and experimental conditions. Preventive activity against initial adhesion and biofilm development was more frequently demonstrated than complete eradication of mature biofilms.

Crustin reduced *E. faecalis* biofilm biomass by interfering with the early stages of adhesion and extracellular matrix production. Similarly, KR-12-a5 demonstrated activity against both planktonic cells and biofilms while exhibiting lower cytotoxicity than related peptides, highlighting the importance of cationic charge and amphipathic structure for membrane interaction (Caiaffa *et al.*, 2017; Sivakamavalli *et al.*, 2015).

Aurein-derived peptides (AU and (AU)₂K), lipopeptides, GH12, KP, L18R, DJK-5, and several L163 derivatives also demonstrated relevant antibiofilm activity. Although most peptides inhibited biofilm formation, eradication of mature biofilms was generally less effective. DJK-5 consistently outperformed IDR-1018, including in ex vivo endodontic models, whereas some lipopeptides achieved high activity only at concentrations associated with increased cytotoxicity (Freitas *et al.*, 2019; Huang *et al.*, 2019; Neubauer *et al.*, 2021; Ye *et al.*, 2019).

These findings confirm that inhibition of biofilm formation and eradication of mature biofilms should not be interpreted as equivalent outcomes. Early-stage biofilms are more accessible to peptides, whereas mature structures present a dense extracellular matrix, metabolic heterogeneity, and persister cells that may restrict peptide penetration and reduce efficacy (Huang *et al.*, 2019; Mishra *et al.*, 2022; Oh *et al.*, 2022; Ye *et al.*, 2019). Consequently, future studies should report preventive and eradication effects separately and employ standardized definitions of minimum biofilm inhibitory and eradication concentrations (Mishra *et al.*, 2022; Oh *et al.*, 2022).

Cytotoxicity was evaluated in only 11 of the 20 included studies. Several peptides, including KR-12-a5, KP, L18R, L163 derivatives, LfcinB6 derivatives, and the Ply2660–LL-37 combination, showed low toxicity or acceptable compatibility with mammalian cells (Caiaffa *et al.*, 2017, 2021; J. Li *et al.*, 2021; Mergoni *et al.*, 2020, 2021; Mishra *et al.*, 2022; H. Zhang *et al.*, 2023). In contrast, gramicidin S and some gemini lipopeptides exhibited cytotoxicity at concentrations close to those required for antibiofilm activity (Berditsch *et al.*, 2019; Neubauer *et al.*, 2021). These findings indicate that antimicrobial potency alone is insufficient for selecting candidates for clinical development, emphasizing the importance of systematic safety assessment

using clinically relevant models (Dad *et al.*, 2025; Kim *et al.*, 2025).

In addition to their antimicrobial efficacy and safety profile, the included studies investigated a wide range of mechanisms underlying the antibiofilm activity of AMPs. Although membrane disruption was the predominant mechanism, several peptides also targeted intracellular pathways, stress-response signaling, bacterial adhesion, and extracellular matrix production. The main mechanisms reported in the included studies are summarized in Table 2.

Table 2

Proposed mechanisms of action of antimicrobial peptides against Enterococcus spp. biofilms..

Study (author/year)	Main mechanism(s)
(Sivakamavalli <i>et al.</i> , 2015)	Membrane permeabilization; reduced bacterial adhesion and biofilm formation.
(Berditsch <i>et al.</i> , 2016)	Membrane disruption; intracellular interaction with ppGpp and nucleotide metabolism.
(Caiaffa <i>et al.</i> , 2017)	Membrane permeabilization and pore formation.
(Nesuta <i>et al.</i> , 2017)	Membrane disruption; protease resistance (HYL-21/HYL-20K).
(Freitas <i>et al.</i> , 2019)	Lipoteichoic acid binding; inhibition of bacterial adhesion.
(Paduszynska <i>et al.</i> , 2019)	Membrane permeabilization and inhibition of adhesion.
(Ye <i>et al.</i> , 2019)	ppGpp degradation; metabolic inhibition; biofilm destabilization.
(Berditsch <i>et al.</i> , 2019)	Membrane disruption and intracellular metabolic targets.
(Huang <i>et al.</i> , 2019)	ppGpp inhibition and biofilm penetration.
(Mergoni <i>et al.</i> , 2020)	Cell wall interaction and membrane penetration.
(Neubauer <i>et al.</i> , 2021)	Membrane permeabilization (dose-dependent).
(D. Li <i>et al.</i> , 2021)	Enhanced peptide stability and membrane interaction.
(Caiaffa <i>et al.</i> , 2021)	Membrane permeabilization (proposed mechanism).
(Mergoni <i>et al.</i> , 2021)	Cell wall interaction and membrane destabilization
(Rouchon <i>et al.</i> , 2022)	Membrane disruption and bacterial lysis.
(Mishra <i>et al.</i> , 2022)	Membrane disruption; modulation of adhesion genes and metabolism.
(H. Zhang <i>et al.</i> , 2023)	Synergistic peptidoglycan degradation and membrane disruption.
(Hu <i>et al.</i> , 2024)	Membrane disruption and cytoplasmic leakage.
(Sinha, 2024)	Membrane disruption; ATPase and EPS inhibition.
(Kranjec <i>et al.</i> , 2024)	Membrane receptor interaction; inhibition of protein synthesis.

Source: The authors.

Membrane disruption was the predominant mechanism reported among the included studies. Most cationic and amphipathic peptides interacted with negatively charged components of the enterococcal envelope, including lipoteichoic acids and membrane phospholipids, leading to membrane permeabilization, depolarization, cytoplasmic leakage, and ultimately cell death. This mechanism was consistently described for several peptide families, including gramicidin S, KR-12-a5, aurein derivatives, lipopeptides, GH12, and buCaTHL4B, supporting membrane targeting as the principal mode of antibiofilm activity (Berditsch *et al.*, 2016, 2019; Caiaffa *et al.*, 2017; Freitas *et al.*, 2019; Hu *et al.*, 2024; Paduszynska *et al.*, 2019; Sinha, 2024).

However, membrane disruption alone does not explain the differences in activity observed among peptides, particularly against mature biofilms. Several AMPs also targeted intracellular and regulatory pathways. DJK-5 and IDR-1018 interfered with the bacterial alarmone (p)ppGpp, impairing stress adaptation and biofilm maintenance, whereas gramicidin S interacted with phosphate-containing nucleotides and GH12 inhibited F₁F₀-ATPase activity and extracellular polysaccharide synthesis (Berditsch *et al.*, 2016; Huang *et al.*, 2019; Sinha, 2024; Ye *et al.*, 2019). Other reported mechanisms included modulation of adhesion-related genes, disruption of the extracellular matrix, and interference with bacterial energy metabolism, indicating that AMP activity is frequently multimodal rather than exclusively membranolytic (Mishra *et al.*, 2022).

Nevertheless, mechanistic evidence remained heterogeneous across studies. In several investigations, the proposed mechanisms were inferred from peptide structure or previous publications rather than experimentally demonstrated. Moreover, only a limited number of studies explored structure–activity relationships, making it difficult to identify the physicochemical properties most strongly associated with antibiofilm efficacy (Kim *et al.*, 2025; Neubauer *et al.*, 2021).

Several studies investigated AMPs in combination with conventional antimicrobials or complementary therapies, including chlorhexidine, sodium hypochlorite, calcium hydroxide, photodynamic therapy, antibiotics, and the phage endolysin Ply2660. Among these, the combination of Ply2660 and LL-37 demonstrated complementary mechanisms, in which enzymatic degradation of peptidoglycan facilitated membrane disruption by LL-37, resulting in enhanced activity against planktonic cells and biofilms and efficacy in a murine infection model (H. Zhang *et al.*, 2023). Likewise, aurein-derived peptides combined with photodynamic therapy enhanced activity against *E. faecalis* and vancomycin-resistant *E. faecium* (Freitas *et al.*, 2019).

These findings suggest that AMPs may be particularly valuable as adjunctive therapies rather than direct replacements for conventional antimicrobials. By increasing membrane permeability, disrupting the biofilm matrix, or interfering with bacterial stress responses, AMPs may enhance bacterial susceptibility to other agents while potentially allowing lower concentrations of each component (Freitas *et al.*, 2019; H. Zhang *et al.*, 2023; Zhu *et al.*, 2022).

However, evidence supporting synergistic activity remained limited. In most studies, synergism was inferred from reductions in biofilm biomass or viable-cell counts rather than demonstrated using standardized interaction assays. Future investigations should therefore confirm synergistic effects using validated methods, such as checkerboard assays, fractional

inhibitory concentration indices, and time-kill analyses, while also evaluating antagonism, cytotoxicity, formulation stability, and performance under clinically relevant conditions (Zhu *et al.*, 2022).

The methodological quality of the included studies was subsequently assessed to identify potential sources of bias and limitations in the available evidence. The results of this assessment are summarized in Table 3.

Table 3

Assessment of the risk of bias of the studies included in the review.

Study (author/year)	Item evaluated										Score	Risk of bias
	1	2	3	4	5	6	7	8	9	10		
(Sivakamavalli <i>et al.</i> , 2015)	✓	✗	✓	✓	✓	✓	✓	✗	✗	✗	6	medium risk
(Berditsch <i>et al.</i> , 2016)	✓	✗	✓	✓	✓	✓	✓	✗	✓	✗	7	low risk
(Caiaffa <i>et al.</i> , 2017)	✓	✗	✓	✓	✓	✓	✓	✓	✓	✗	8	low risk
(Nesuta <i>et al.</i> , 2017)	✓	✗	✓	✓	✓	✓	✓	✓	✓	✗	8	low risk
(Freitas <i>et al.</i> , 2019)	✓	✗	✓	✓	✓	✓	✓	✓	✓	✗	8	low risk
(Paduszynska <i>et al.</i> , 2019)	✓	✗	✓	✓	✓	✓	✓	✗	✗	✗	6	medium risk
(Ye <i>et al.</i> , 2019)	✓	✗	✓	✓	✓	✓	✓	✓	✓	✓	9	low risk
(Berditsch <i>et al.</i> , 2019)	✓	✗	✓	✓	✓	✓	✓	✓	✓	✗	8	low risk
(Huang <i>et al.</i> , 2019)	✓	✓	✓	✓	✗	✓	✓	✓	✓	✗	8	low risk
(Mergoni <i>et al.</i> , 2020)	✓	✗	✓	✓	✓	✓	✓	✓	✓	✗	8	low risk
(Neubauer <i>et al.</i> , 2021)	✓	✗	✓	✓	✓	✓	✓	✓	✓	✗	8	low risk
(D. Li <i>et al.</i> , 2021)	✓	✗	✓	✓	✗	✓	✓	✓	✓	✗	7	low risk
(Caiaffa <i>et al.</i> , 2021)	✓	✗	✗	✗	✓	✓	✓	✓	✓	✗	6	medium risk
(Mergoni <i>et al.</i> , 2021)	✓	✗	✗	✗	✓	✓	✓	✓	✗	✗	5	medium risk
(Rouchon <i>et al.</i> , 2022)	✓	✗	✗	✗	✗	✓	✓	✓	✓	✗	5	medium risk
(Mishra <i>et al.</i> , 2022)	✓	✗	✗	✗	✗	✓	✓	✓	✓	✗	5	medium risk
(H. Zhang <i>et al.</i> , 2023)	✓	✗	✗	✗	✓	✓	✓	✓	✗	✗	5	medium risk
(Hu <i>et al.</i> , 2024)	✓	✗	✓	✗	✓	✓	✓	✓	✓	✗	7	low risk
(Sinha, 2024)	✓	✓	✗	✗	✓	✓	✓	✓	✗	✗	6	medium risk
(Kranjec <i>et al.</i> , 2024)	✓	✗	✗	✗	✗	✓	✓	✓	✗	✗	4	medium risk

Legend: ✓ Item included in the study; ✗ Item not included in the study; Item 1: Presence of a control group; Item 2: Description of the sample size calculation; Item 3: Method of synthesis and characterization of antimicrobial peptides; Item 4: Description of the composition and properties of antimicrobial peptides; Item 5: Use of ATCC standard strains in the experimental design; Item 6: Specification of the microorganism growth stage (planktonic and biofilm); Item 7: Detailed methodology or type of study employed; Item 8: Validation of the significance of the results with statistical tests; Item 9: Comparison with conventional antimicrobials or use of viability and technique controls; Item 10: Blinding of observers.

Eleven studies (55%) were classified as having a low risk of bias, whereas nine (45%) were considered to have a moderate risk. No study was classified as having a high risk of bias. All studies included appropriate control groups and reported microbial growth conditions or

biofilm stage, supporting an acceptable baseline methodological quality.

Despite this overall classification, important methodological limitations were identified. Only two studies reported sample-size calculations, seven did not clearly describe peptide synthesis or characterization, and eight provided incomplete information regarding peptide composition or physicochemical properties. In addition, statistical analyses were absent in three studies, conventional antimicrobial or viability controls were lacking in six, and observer blinding was reported in only one study. These shortcomings reduce reproducibility and increase the risk of experimental bias.

The representativeness of the experimental models also remains limited. Five studies did not include reference strains, whereas many others relied predominantly on ATCC strains. Future investigations should combine standardized reference strains with genetically diverse clinical isolates, particularly multidrug-resistant and vancomycin-resistant strains, to improve the clinical applicability of the findings.

This review also has limitations. The exclusion of grey literature may have favored studies reporting positive findings, and the absence of Embase and Scopus from the search strategy may have resulted in the omission of eligible studies. Furthermore, incomplete reporting of peptide purity, synthesis, structural confirmation, and stability limited comparisons among studies and restricted mechanistic interpretation.

Future studies should standardize MBIC and MBEC definitions, distinguish biofilm prevention from mature-biofilm eradication, and incorporate clinically relevant models, including multidrug-resistant isolates, multispecies biofilms, host-derived matrices, and device-associated infections. Greater emphasis should also be placed on peptide stability under physiological conditions, structural optimization, advanced delivery systems, quantitative synergy analyses, and comprehensive toxicological evaluation before clinical translation.

CONCLUSION

This systematic review demonstrated that antimicrobial peptides exhibit consistent antibiofilm activity against *Enterococcus* spp., particularly by preventing bacterial adhesion and early biofilm formation, although some peptides and combination strategies also showed activity against mature biofilms. Their efficacy is supported by multiple mechanisms, including

membrane disruption, intracellular targeting, modulation of stress-response pathways, and interference with the extracellular matrix.

Synthetic and structurally modified peptides predominated among the investigated compounds, reflecting current efforts to improve stability, selectivity, and therapeutic performance. Combination approaches involving antibiotics, antiseptics, photodynamic therapy, and phage endolysins further suggest that AMPs may be particularly valuable as adjunctive therapies.

Despite these promising findings, clinical translation remains limited by the predominance of in vitro studies, methodological heterogeneity, scarce in vivo validation, and incomplete toxicological evaluation. Future research should prioritize standardized antibiofilm methods, clinically relevant infection models, multidrug-resistant and vancomycin-resistant isolates, and robust safety assessments to support the development of effective AMP-based therapies against *Enterococcus* biofilms.

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